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

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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_2 is relatively inert and cannot be directly utilized by most plants (Burt, 2013). Biological nitrogen fixation is the conversion of atmospheric N_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_2) into ammonium (NH_4^+), a form that plants can assimilate (Zehr *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_2 to NH_4^+ (Peters *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 *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 (*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 *et al.*, 2018; Amicucci *et al.*, 2019; Bennett *et al.*, 2020; Pankievicz *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 (*Sorghum bicolor* (L.) Moench), sugarcane (*Saccharum* spp.), foxtail millet (*Setaria italica* L.*)*, and pearl millet (*Pennisetum glaucum*). They serve multiple functions, including anchorage, water, and nutrient uptake (Hostetler *et al.*, 2021; Sparks, 2023). Sorghum (*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

81 drought-prone regions, makes it an attractive option for sustainable energy production (Regassa &
82 Wortmann, 2014; Rao *et al.*, 2019). Sorghum's high biomass yield and rapid growth rate contribute
83 to its potential as a bioenergetic feedstock (Rooney *et al.*, 2007; Mullet *et al.*, 2014). Efficient
84 water usage and lower input requirements than other energy crops add to its potential as a
85 sustainable crop (Moore *et al.*, 2021). In addition, sorghum increases its biomass when inoculated
86 with specific plant growth-promoting bacteria (PGPB), predominantly those belonging to the
87 genera *Azospirillum*, *Paraburkholderia,* and *Herbaspirillum* (Smith *et al.*, 1984; Pereira *et al.*,
88 1989; Pacovsky, 1990; dos Santos *et al.*, 2017; dos Reis Antunes *et al.*, 2019; Kuramae *et al.*,
89 2020).

90 Despite the similarity of sorghum's mucilage-producing aerial roots to those in maize
91 known to host nitrogen-fixing diazotrophs, evidence of N-fixation in sorghum has not previously
92 been reported. Images of aerial roots of sorghum covered with mucilage, as well as a micrograph
93 depicting *Azospirillum brasilense,* were featured in the abstract book of a conference on N-fixation
94 in cereals hosted by the International Crops Research Institute for the Semi-Arid Tropics, India
95 (ICRISAT) in 1984 but no data were published. The similarity between these sorghum aerial roots
96 and those from the Sierra Mixe maize prompted us to explore N-fixation in sorghum aerial roots.
97 To this end, we explore the diversity of aerial roots in accessions sorghum and the production of
98 mucilage and their microbiome. We demonstrated that, like the maize landraces from the Sierra
99 Mixe, specific sorghum accessions also acquired nitrogen from their aerial roots by the
100 microbiome established in their mucilage.
101

102 **Materials and methods**

103 *Plant material and phenotyping*

104 Sorghum accessions were selected from the sorghum minicore (Upadhyaya et al., 2009) and
105 augmented with accessions kindly provided by Dr. Vetriventhan Mani and Dr. Vania C. R.
106 Azevedo from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT,
107 Hyderabad, Telangana, India) (**Supplementary Table 1**). The plants were cultivated in pots
108 containing Sungrow media in the Walnut Street Greenhouses at the University of Wisconsin-
109 Madison with temperatures at 25 ˚C during the day and 17 ˚C at night. The plants were started on
110 a long daylight regimen (14/10), and once the plants were 1-1.5 m tall, then were switched to short
111 days (12/12) due to their photoperiod sensitivity. Sorghum plants were watered with municipal

112 water or NPK fertilizer. At the flowering stage, when aerial roots were visible on many nodes, the
113 root diameter of 3 roots at the top node was measured with a digital caliper, as well as the number
114 of nodes forming aerial roots and the number of aerial roots formed on the top node. Overhead
115 water was applied, and 30 minutes after the water application, the mucilage volume of three young
116 and old aerial roots was quantified. The same accessions were grown in West Madison Agricultural
117 Research Station (43.0610329663, -89.5325872441) and University of Florida North Florida
118 Research and Education Center - Suwannee Valley near Live Oak, FL (30.313277, 82.902158) in
119 1.5 m rows, with 10 cm between plants, and 76 cm between rows. Only the number of nodes and
120 root diameter were phenotyped in the field experiments.
121
122 *Bacterial isolation from sorghum*
123 Mucilage samples were collected from a West Madison Agricultural Research Station field trial in
124 September 2022. The mucilage samples were inoculated in BMGM semisolid medium (Estrada de
125 Los Santos, 2001) and incubated at room temperature for one week. After that, the microaerophilic
126 pellicles formed were transferred to a new medium and incubated for the same period. This
127 procedure was repeated seven times. After the last incubation period, the bacteria were inoculated
128 in a solid BMGM medium supplemented with 0.5 g of yeast extract per liter. The bacteria were
129 purified in the same medium and stored in glycerol at -80 C. The total collection had 164 isolates
130 screened by clonal strains using the Box-PCR approach. Box-PCR was performed using the Box-
131 A1 primer (5'-CTACGGCAAGGCGACGCTGACG-3') (Versalovic *et al.*, 1994), and the PCR
132 products were subjected to horizontal electrophoresis with agarose gel (1.2% w v^{-1}). The identical
133 profiles were excluded, and 103 non-clonal strains. The bacterial strains were identified by 16S
134 rRNA sequencing. The 16S rRNA was amplified by PCR using the primers 27F (5'-
135 AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') and
136 sequenced in an Applied Biosystems 3500 genetic analyzer at the Functional Biosciences facilities.
137 The bad-quality sequences were trimmed using Sequence Scanner v. 2.0, and the contigs of almost
138 complete 16S rRNA sequences were assembled in BioEdit v. 7.7 (Hall, 1999). The sequences were
139 compared against those deposited within the 16S rRNA reference database of GeneBank using the
140 BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The maximum likelihood phylogenetic
141 tree was constructed using MEGA 11 software (Kumar *et al.*, 2018).
142

143 **Bacterial isolates and growth conditions**

144 Ten strains were used for different experiments (**Supplementary Table 5**). *Azospirillum*
145 *brasilense* FP2 and FP10 (*nifA*$^-$) were kindly provided by Dr. Fábio Pedrosa and Emanuel M. de
146 Souza from UFPR-Brazil (Pedrosa & Yates, 1984). The *Klebsiella variicola* Δ*nifH* mutant was
147 developed by Dr. Maya Venkataraman. *Azospirillum baldaniorum* Sp 245^T, *Klebsiella variicola*
148 A3, *Klebsiella michiganensis* A2, *Azorhizobium caulinodans* ORS571^T, *Stutzerimonas stutzeri*
149 A1501, *Paraburkholderia silvatlantica* SRMrh-20^T, *Azotobacter vinelandii* DJ, *Herbaspirillum*
150 *seropedicae* SmR1. The strains were grown in LB medium (Vervliet *et al.*, 1975), except for *A.*
151 *vinelandii*, which was grown in Burk's sucrose medium (Toukdarian and Kennedy 1986). The
152 cells were grown overnight at 30 ˚C while shaking at 130 rpm.

153

154 **Mucilage glycosyl composition**

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

160

161 **Free sugar quantification**

162 Mucilage was collected at two time points from the sorghum accession IS23992 grown under
163 greenhouse conditions. Benedict's reaction was used to quantify free sugars, as described in
164 Hernandez-Lopez *et al*. (2020). Briefly, Benedict reagent (700 µl) was incubated with sorghum
165 mucilage (30 µl) for 10 mins at 95 ºC. Subsequently, the reaction was centrifuged at 10,000 g for
166 5 mins. The supernatant was read in a spectrophotometer λ = 740 nm (Bio-Rad SmartSpect^{TM}
167 Plus), and quantification was determined using a glucose standard curve.

168

169 **Metabolomics**

170 Mucilage samples for metabolome analysis were analyzed at West Coast Metabolomics Center -
171 UC Davis for analysis. Data were normalized and processed with MetaboAnalyst
172 (https://www.metaboanalyst.ca/). This algorithm offers several different methods. Sparse Partial

173 Least Squares - Discriminant Analysis and Significance Analysis of Microarray were used to
174 analyze the data.
175
176 **16S rRNA amplicon sequencing**
177 DNA was isolated from the mucilage samples from sorghum and maize using the FastDNA™
178 SPIN Kit for Soil (MP Biomedicals) following the manufacturer's instructions. DNA was
179 quantified using a Nanodrop One (ThemoScientific). The 16S rRNA region was amplified with
180 the standard bacterial primers 335F and 769R with GoTaq Mastermix (Promega) according to the
181 manufacturer's instructions. Amplification reaction volumes were 50 µl containing 1 µl of
182 10 pmol μl^{-1} primer and 4 ng of template DNA. Thermocycler conditions were as follows: 95°C
183 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
184 72°C for 5 min. PCR products were separated on an agarose gel and extracted using the GeneJET
185 Gel Extraction Kit (Thermofisher Scientific). Novogene (Santa Clara, CA, USA) conducted library
186 preparation and sequencing. Amplicons were sequenced with an Illumina paired-end platform
187 generating 250 bp paired-end raw reads. OTUs abundance information was normalized using a
188 standard sequence number corresponding to the sample with the fewest sequences. Subsequent
189 PCoA and beta diversity analyses were performed based on these normalized data. These data
190 were processed with QIIME2 (Version 1.7.0) and displayed with R software (Version 2.15.3).
191
192 **Acetylene reduction assay (ARA)**
193 Nitrogenase activity was evaluated in ten bacterial strains (**Supplementary Table 6**) grown in
194 sorghum mucilage collected from the greenhouse. The bacteria were grown in three ml of LB
195 media overnight at 30 ºC and shaken at 180 rpm. Then 30 µl (OD_{600nm} = 0.1) were inoculated in
196 sterile vials containing three ml of mucilage. Vials were capped and incubated for 24 hours at 30
197 ºC. The cap was replaced with a crimp cap, and 1 ml of air was replaced with acetylene, followed
198 by a 3-days incubation period at 30 ºC. Gas chromatography was performed in a GC-2010
199 (Shimazu) with an HS20 autosampler by injecting one ml of air sample into the equipment.
200 Controls included a vial with acetylene to check for traces of ethylene and *A. brasilense* FP2
201 without fixing activity. The indirect measure of the nitrogenase activity was expressed in nmol of
202 ethylene produced per hour per mL of mucilage.
203

204 **^{15}N gas enrichment**

205 Aerial roots from sorghum accession IS23992 were collected in sterile flasks. These roots were
206 obtained from a greenhouse with copious amounts of mucilage. *K. variicola A3* and *K. variicola*
207 Δ*nifH* mutant were grown in LB medium. Roots with mucilage were inoculated with bacteria (100
208 µl, OD_{600nm} = 0.1) in roots with mucilage. Flasks were injected with $^{15}N_2$ gas to reach a
209 concentration of 15% (v/v), and flasks were incubated for three days at room temperature (20 ºC).
210 To determine the ^{15}N enrichment, pheophytin was extracted from aerial roots as described by
211 (Kahn *et al.*, 2002; Van Deynze *et al.*, 2018). The samples were analyzed by isotope ratio mass
212 spectrometry (IRMS) at the soil science department at the University of Wisconsin-Madison.
213

214 **Humidity experiments Greenhouse experiments**

215 Sorghum accessions IS23992 and IS24453 were grown in the Walnut Street Greenhouses at the
216 University of Wisconsin-Madison in Pro-Mix LP15 medium with temperatures at 28 ˚C in the day
217 and 25 ˚C at night. Plants were grown under a light regime of 12-hour photoperiod from 6 am to
218 6 pm. Greenhouses were under high humidity (relative humidity 75%) and low humidity (relative
219 humidity 30%) controlled with a humidifier system (Smart Fog, Reno, Nevada). The phenotypes
220 evaluated included number of nodes with roots, the number of roots at the top node, and the root
221 diameter. The measurements followed the previously described procedure.
222

223 **Statistical analyses**

224 All the statistical analyses were conducted in R version 4.2.1 and 2.15.3 (Foundation for Statistical
225 Computing). ANOVA and Tukey's HSD were conducted with the package ggpubr (Version 0.6.0)
226 and agricolae (Version 1.3-5). Microbiome studies were performed with QUIIME2 (Version 1.7.0)
227 (Caporaso *et al.*, 2010).
228
229

230 **Results**

231 **Specific sorghum accessions developed aerial roots.**

232 A collection of specific accessions selected from the ICRISAT minicore (Upadhyaya *et al.*, 2009)
233 collection and the more extensive ICRISAT collection exhibited the development of aerial roots
234 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.

297 **Carbohydrates are the primary molecules of sorghum mucilage.**
298 The mucilage produced by the Sierra Mixe landraces consists of an intricate polysaccharide
299 composed of several monomeric units, including arabinose, fucose, galactose, glucuronic acid,
300 mannose, and xylose (Van Deynze *et al.*, 2018; Amicucci *et al.*, 2019). In the maize landraces,
301 water triggered the expression of plant genes related to synthesizing carbohydrates (Pankievicz *et*
302 *al.*, 2022). To determine the sorghum mucilage (**Figure 3A**) composition, gas
303 chromatography/mass spectrometry (GC/MS) analysis was conducted. The chemical composition
304 analysis revealed the same monosaccharides were present in sorghum mucilage as in maize
305 mucilage (Van Deynze *et al.*, 2018). However, differences in the proportion of specific
306 monosaccharides were observed (**Figure 3B**). Specifically, the monosaccharides arabinose,
307 galactose, glucuronic acid, and mannose had higher ratios in sorghum mucilage than maize
308 mucilage. Galactose accounts for more than 50 % of the total sugars in sorghum mucilage. The
309 amount of fucose in sorghum mucilage was approximately half that found in maize mucilage.
310 These results indicate that the mucilage in the two species consists of the same monosaccharides
311 but that the structure likely differs based on the observation that the proportions in which the
312 monosaccharides are present differ.
313
314 As shown in Pankievicz *et al.* (2022), the exposure of aerial root to water induced the expression
315 of genes associated with carbohydrate synthesis/degradation. To investigate this degradation
316 process, we assessed the levels of free sugars in sorghum mucilage at two time points: one and
317 five hours after its production from accession IS23992, cultivated in the Walnut Street greenhouse.
318 Using Benedict's reaction (Hernández-López et al. 2020), we observed that the free sugar
319 concentration increased over time, rising from an average of 86 mM at one hour to 109 mM after
320 five hours (Figure 3C). This finding suggests that sorghum aerial roots on these roots produce
321 enzymes breaking the mucilage polysaccharide into simple sugars. We cannot exclude the
322 contribution of microbes on these roots to this mucilage degradation process as the aerial roots
323 were not germ-free, but very few microbes are isolated from sorghum mucilage when grown in
324 this greenhouse.
325
326 To explore the differences in the soluble metabolites in the mucilage between maize and sorghum
327 (**Supplementary Table 2**), we performed a metabolomics analysis using gas chromatography

328 coupled with time-of-flight mass spectrometry (GC/TOF-MS). We analyzed our data with the
329 sparse Partial Least Squares - Discriminant Analysis (sPLS-DA) algorithm to reduce the number
330 of variables (metabolites) and produce robust and easy-to-interpret results. The principal
331 component analysis revealed two clusters where samples were grouped based on the genotype
332 (**Figure 3C**). We use a Significance Analysis of Microarray (SAM) to identify differentially
333 expressed proteins. The top ten most abundant metabolites include six unknown metabolites as
334 well as octadecanoic, montanic acid (octacosanoic acid), pinitol (1D-*chiro*-inositol), and *N*-acetyl
335 aspartate diethyl ester (**Supplementary Table 3**). These metabolomic differences may contribute
336 to the functional differences observed in sorghum and maize mucilage.
337
338 **Aerial root mucilage from sorghum contains a distinctive type of border cell.**
339 Plant mucilage is also produced at the root cap of underground roots (Hawes & Lin, 1990; Brigham
340 *et al.*, 1995; Knee *et al.*, 2001). Within the mucilage, a specific type of cell called border cells
341 (BCs) are responsible for mucilage production (Hawes & Pueppke, 1986; Pankievicz *et al.*, 2022).
342 To investigate the presence of border cells in sorghum mucilage, we examined mucilage derived
343 from both underground and aerial sorghum roots (**Supplementary Figures 3A and C**). BCs
344 derived from underground roots exhibited a distinct and elongated morphology similar to those
345 observed in BCs from pea (*Pisum sativum* L.), maize, and alfalfa (*Medicago sativa* L.) (Hawes &
346 Pueppke, 1987; Woo *et al.*, 2004; Zhang *et al.*, 2014). In contrast, border cells from aerial roots
347 appeared much larger and elongated, with irregular shapes and sizes (**Supplementary Figure 3C**).
348 The BCs in sorghum aerial roots resembled those found in mucilage from maize aerial roots
349 (Pankievicz *et al.*, 2022). These larger BC may contribute to the abundant mucilage production by
350 maize and sorghum aerial roots.
351
352 Border cells detached from the root cap can remain active for several weeks (Hawes & Pueppke,
353 1986; Vicré *et al.*, 2005). To determine the viability of sorghum border cells, we used a live-dead
354 staining protocol on both types of sorghum mucilage collected after 72 hours. We used a
355 combination of SYTO^{TM} 13 and propidium iodine, which are permeant and non-permeant cell
356 dyes, respectively (Boulos *et al.*, 1999; Ullal *et al.*, 2010). Most cells were viable, as indicated by
357 only SYTO^{TM} 13 in the nuclei. Additionally, BCs from underground roots displayed regular and
358 well-defined nuclei, while BCs from aerial roots exhibited irregular nuclei (**Supplementary**

359 **Figures 3B and D**). To quantitatively assess the morphological differences between the two types
360 of BCs, we measured their length from one cellular pole to another and their cell area. Significant
361 differences were observed in both measurements. BCs from aerial roots generally are twice the
362 size compared to BCs from underground roots. In maize, BC length in aerial roots varied
363 depending on the growth stage, with younger aerial roots having longer BCs and older aerial roots
364 having smaller BCs (Pankievicz *et al.*, 2022). The BCs from sorghum aerial roots in our study
365 were collected at a young growth stage, which explains the twofold size difference compared to
366 underground BCs. Although both types of BCs maintain the function of mucilage production, the
367 observed morphological differences could account for the variation in the amount of mucilage
368 secreted by these cells.
369
370 **Sorghum mucilage hosts a wide range of diazotrophs.**
371 A diverse community of diazotrophs can become established in the mucilage of the Sierra Mixe
372 maize landraces (Van Deynze *et al.*, 2018). To explore the microbial diversity within sorghum
373 mucilage, we conducted a study where we isolated and sequenced bacteria using metabarcoding
374 bacterial 16S rRNA. We focused on mucilage samples from two experimental environments: the
375 West Madison Agricultural Research Station University of Wisconsin and the University of
376 Florida North Research & Education Center - Suwannee Valley. Additionally, we included
377 mucilage samples from Sierra Mixe landraces as references (**Supplementary Table 4**). To analyze
378 the bacterial composition, we processed the variable region of the 16S rRNA and organized it into
379 Operational Taxonomic Units (OTUs). The OTUs provided information on the samples' relative
380 abundance of different taxa. This approach allowed us to identify any outliers that significantly
381 differed from the rest of the samples and visually compare the differences among them (He *et al.*,
382 2015). We performed principal coordinate analysis to examine the beta diversity, representing the
383 samples' diversity. Our findings indicated a distinct separation among samples based on the
384 location and plant origin of the mucilage. The most significant difference was observed between
385 the sorghum and maize samples (**Figure 4A**). Furthermore, we investigated the bacterial phylum
386 in sorghum and maize mucilage. Our analysis revealed that the phylum *Pseudomonadota* was
387 dominant in the mucilage, followed by *Cyanobacteriota* and *Bacillota* (**Figure 4B**). The profiles
388 of samples from Wisconsin were relatively similar regardless of the mucilage's origin, while
389 samples from Florida exhibited enrichment in *Cyanobacteria*, with a similar abundance as

390 *Pseudomonadota* (**Figure 4B**). The isolation of bacterial diazotrophs using the N-free semisolid
391 BMGM medium revealed a high diversity of potential diazotrophs. We obtained 103 non-clonal
392 strains that were identified within 34 different species (**Supplementary Table 5)** with the
393 prevalence of *Pseudomonadota* (*Gammaproteobacteria* - 32, *Alphaproteobacteria* - 30,
394 *Betaproteobacteria* - 5), *Actinomycetota* (28), *Bacteroidota* (3), and *Bacillota* (1) (**Figure 4C**).
395 The nitrogenase activity of one representant strain of each one of the 34 species was evaluated by
396 the acetylene reduction assay and the results indicated the *Agrobacterium pusense* BM_20.4,
397 *Bacillus amyloliquenfaciens* BM_7.6, *Pseudomonas turukhanskensis* BM_14.7, *Microbacterium*
398 *aerolatum* BM_14.4*, Agrobacterium fabacearum BM_6.8, Microbacterium neimengense*
399 BM_10.2*, Enterobacter cancerogenus* BM_14.3*, Epilithonimonas hungarica* BM_19.2*,*
400 *Pseudomonas lutea* BM_5.2*, and Pseudacidovorax intermedius* BM_2.1 showed higher
401 nitrogenase activity than the reference diazotrophs *Klebsiella variicola* A3 and *Azospirillum*
402 *brasilense* FP2 (**Figure 4D**). Only eight bacterial strains showed no nitrogenase activity at all.
403
404 **Mucilage enables biological nitrogen fixation in sorghum.**
405 In the Sierra Mixe maize landraces, the mucilage creates a suitable environment for diazotrophs,
406 contributing to nitrogen acquisition for the plant. This environment provides low oxygen levels
407 and carbohydrates (Van Deynze *et al.*, 2018). We tested whether the mucilage could serve as a
408 proper medium for nitrogen fixation by an acetylene reduction assay (ARA). Nine diazotrophs
409 were isolated from sorghum mucilage collected from Wisconsin and Florida fields
410 (**Supplementary Table 6**). As a negative control, we included *A. brasiliense* FP10, a strain that
411 lacks nitrogenase activity but does not impede growth compared to the wild type (Pedrosa & Yates,
412 1984). To prevent endogenous diazotrophic bacteria in the sorghum mucilage, we collected
413 mucilage from sorghum plants grown in the greenhouse. We specifically selected the sorghum
414 accession IS23992 because it produced numerous nodes with aerial roots in the greenhouse and
415 the field (**Supplementary Figure 2**). Additionally, this accession consistently had an average of
416 250 µl of mucilage (**Figure 1**). The acetylene reduction assay (ARA) results indicated significant
417 nitrogenase activity in mucilage inoculated with the strains *Azotobacter vinelandii* and *Klebsiella*
418 *variicola* (**Figure 5A**). However, the remaining seven strains exhibited almost no nitrogenase
419 activity, with only *Klebsiella michiganensis* showing some ARA activity. The negative control
420 also displayed low activity, possibly due to a small fraction of diazotrophs in the mucilage. This

421 result supports the notion that mucilage is a medium to facilitate the nitrogen fixation activity of
422 specific diazotrophs in the aerial roots of sorghum.
423
424 To validate the plant's ability to take up and assimilate nitrogen, we conducted a $^{15}N_2$ gas
425 enrichment experiment. Aerial roots with mucilage were collected from the greenhouse-grown
426 plants of the IS23992 accession. Based on the acetylene reduction assay results, we inoculated the
427 roots with *Klebsiella variicola* A3 (wild type) and used *Klebsiella variicola* A3 Δ*nifH* as a negative
428 control. Isotope-ratio mass spectrometry (IRMS) was used to measure the ^{15}N content in
429 pheophytin derived from chlorophyll isolated from the aerial roots of sorghum. Roots inoculated
430 with *Klebsiella variicola* exhibited significant incorporation of $^{15}N_2$ gas compared to the negative
431 control (**Figure 5B**). These results confirmed that mucilage from the aerial roots of sorghum
432 supports diazotrophs capable of providing nitrogen to the plant.
433

434 **Discussion**

435 Nitrogen availability limits the performance of economically important cereal crops, including
436 sorghum and maize. Both species belong to the Andropogoneae tribe and share a common ancestor
437 dating 12-16 million years ago (Swigonová *et al.*, 2004). Among them, sorghum is a more versatile
438 and drought-resistant crop, yielding higher biomass, making it a promising candidate for biofuel
439 production (Mathur *et al.*, 2017). Biological nitrogen fixation is a suitable method of overcoming
440 nitrogen deficiencies to minimize the requirement for synthetic fertilizer applications for
441 production. While these symbiotic relationships are well studied in legumes, cereal crops lacked a
442 suitable model until the recent discovery of biological nitrogen fixation in aerial root mucilage in
443 maize landraces, specifically Sierra Mixe (Van Deynze *et al.*, 2018). Here, we determined that
444 specific sorghum accessions harbor the ability to host diazotrophs enabling the fixation of
445 atmospheric nitrogen via their mucilage-producing aerial roots. We also elucidated the similarities
446 and differences in aerial root development, mucilage production, composition, and microbial
447 diversity between sorghum landraces.
448

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

450 Variations in the number of nodes with aerial roots among different genotypes of sorghum was
451 studied earlier. For example, the Chinese landrace Sansui produces 6-8 aerial roots, while the elite

452 cultivar Jiliand only has a single node (Li *et al.*, 2014). Our study further demonstrated that specific
453 sorghum accessions displayed varying numbers of nodes with aerial roots (**Figure 1A**).
454 Additionally, we observed differences in diameter and mucilage production in these accessions,
455 and a positive correlation among these three traits confirmed a similar development as the Sierra
456 Mixe (Van Deynze *et al.*, 2018). Recently, this finding was reinforced by a study involving 59
457 sorghum accessions that also produce mucilage from aerial roots (Xu *et al.*, 2023). The presence
458 of aerial roots in other members of the Poaceae family, such as sugarcane and foxtail millet,
459 suggests a common evolutionary origin (Hostetler *et al.*, 2021).
460
461 High relative humidity levels significantly impact plant growth and the production of various
462 phytohormones, including abscisic acid and ethylene (Arve & Torre, 2015). In the case of rice,
463 elevated humidity has been observed to stimulate the formation of lateral roots (Chhun et al. 2007).
464 This effect is attributed to the increased presence of ethylene, a plant hormone that promotes lateral
465 root development (McDonald & Visser, 2003; Visser & Voesenek, 2005). By providing high
466 humidity, we observed more nodes with aerial roots independently of the sorghum genotype
467 (**Figure 2**). We speculate that this response is due to an ethylene-driven mechanism triggered by
468 the elevated humidity, leading to an increased number of nodes with aerial roots. Although the
469 exact mechanism of how water influences hormone regulation remains unclear, it is evident that
470 humidity plays a crucial role in determining the number of nodes with aerial roots, possibly through
471 a water-sensing mechanism that regulates phytohormone production.
472
473 *Some sorghum accessions produce abundant mucilage from aerial roots.*
474 Plants secrete mucilage from various tissues to adapt to abiotic and biotic stresses. Mucilage is
475 commonly found in diverse plant parts, including roots, seeds, leaves, and stems. Notably, in
476 underground roots, the task of producing and releasing mucilage and other components is carried
477 out by specialized cells known as border cells, which utilize large vesicles. In the case of the maize
478 landraces, the mucilage from their aerial roots resembles the mucilage commonly found at the tips
479 of underground roots in maize. Packievicz *et al*. (2022) demonstrated the presence of border cells
480 in the mucilage from the aerial roots of Sierra Mixe, and they noted differences in size compared
481 to border cells derived from underground roots. Our data corroborated these findings, showing a

482 significant distinction between border cells in underground and aerial roots (**Supplementary**
483 **Figure 3**).
484
485 Carbohydrates constitute the main components of both underground and aerial root mucilage
486 (Osborn *et al.*, 1999; Van Deynze *et al.*, 2018; Amicucci *et al.*, 2019; Nazari *et al.*, 2020). In our
487 research, we investigated the carbohydrate composition of sorghum mucilage, and the results show
488 the presence of similar monosaccharides, albeit in slightly different proportions (**Figure 3**). These
489 findings are consistent with a prior study on maize genotypes, which revealed changes in the
490 carbohydrate profile based on their geographic locations and agroecological systems (Nazari *et*
491 *al.*, 2020). Interestingly, our analysis did not identify the presence of glucose or fructose as
492 reported in the sorghum mucilage from the aerial root in accessions 59 and SL36 (Xu *et al.*, 2023).
493 Instead, the monosaccharide profile closely resembled the Sierra Mixe landraces (Van Deynze *et*
494 *al.*, 2018). This discrepancy suggests that environmental factors significantly influence maize and
495 sorghum mucilage's carbohydrate and metabolite composition. However, the genetic aspect cannot
496 be disregarded entirely, as evidenced by our principal component analysis, which displayed a clear
497 separation based on species (**Figure 3**).
498
499 *Sorghum aerial roots mucilage hosts specific microbial communities containing diazotrophs*
500 Numerous studies have documented the predictable variation in plant microbiome composition
501 based on host and environmental factors (Lundberg *et al.*, 2012; Edwards *et al.*, 2015; Hartman *et*
502 *al.*, 2017; Crosbie *et al.*, 2022). Our microbiome investigation revealed that location and host
503 influence microbial composition in the aerial root mucilage (**Figure 4**). Certain plant enzymes
504 capable of breaking down mucilage into simple sugars are expressed in the Sierra Mixe landraces
505 and may play a role in nurturing their microbiomes. Simultaneously, the microbiome contributes
506 to mucilage degradation through specialized enzymes (Pankievicz *et al.*, 2022). *Pseudomonadota*,
507 *Acidobacteriota*, *Cyanobacteriota*, and *Bacillota* were the most prevalent phyla in sorghum,
508 aligning with findings from other sorghum microbiome studies (Xu *et al.*, 2018). Moreover,
509 specific phyla have been proposed as potential biomarkers for certain biological processes (Bhagat
510 *et al.*, 2021). Our study sheds light on the microbial profiles of sorghum and maize for nitrogen
511 fixation and offers valuable resources for engineering these microbes to enhance nitrogen fixation.
512 These resources will serve as a foundation for investigating microbial community interactions and

513 their roles in facilitating nitrogen fixation. Such insights could pave the way for possible
514 inoculation strategies in the future.
515
516 Sorghum benefits triggered by the inoculation of diazotrophic bacteria were reported several years
517 ago (Wong & Stenberg, 1979; Pal & Malik, 1981; Morgenstern & Okon, 1987), and the delivering
518 of nitrogen from the air to the host plants by biological fixation was confirmed in the last few years
519 in inoculated plants (dos Santos *et al.*, 2017) and in plants associated with the native communities
520 (Barros *et al.*, 2020). However, those reports have no information about the plants' aerial roots or
521 mucilage production. Our results indicated production of aerial roots and mucilage is a crucial
522 aspect of nitrogen fixation in sorghum, indicating the ability of the plants reported by Santos *et al.*
523 (2017) and Barros *et al.* (2020) to produce aerial roots and mucilage.
524
525 Microbial communities can be well established in plant mucilages due to the abundance of carbon,
526 and the diazotrophs could benefit from the microaerophilic conditions. The mucilage viscosity is
527 very close to that observed in nitrogen-free semisolid media, which is a classical approach to
528 isolating diazotrophs from non-legumes (Baldani *et al.*, 2014), indicating the abundance of
529 diazotrophs in sorghum mucilage. The existence of efficient diazotrophs colonizing sorghum aerial
530 root mucilage was confirmed in the present study with the application of a nitrogen-free semisolid
531 medium approach, indicating a wide diversity of diazotrophs and corroborating the 16S rRNA
532 amplicon sequence data. The highlights of unusual bacteria with the highest nitrogenase activity,
533 such as *Agrobacterium* spp. and *Epilithonimonas hungarica*, for example, indicate that, under the
534 conditions assessed, the diazotrophic community of sorghum mucilage is different from those
535 communities from sorghum underground roots since other studies using the same medium and
536 approach could not identify these diazotrophs (Silva *et al.*, 2018; dos Reis Antunes *et al.*, 2019).
537
538 *Nitrogen fixation occurs in sorghum aerial root mucilage*
539 Nitrogen fixation in cereals can reduce part of our dependence on synthetic nitrogen-based
540 fertilizers. Our research has shown that mucilage is crucial in creating a favorable environment for
541 diazotrophs to thrive (**Figure 5A**), enabling plants to acquire nitrogen (**Figure 5B**). To achieve
542 nitrogen fixation in cereals, synthetic biology offers promising approaches through transferring
543 nitrogenase genes to plants, engineering a root nodule symbiosis, or remodeling associative

544 diazotrophs. Some progress has been made in expressing *nif* genes responsible for encoding
545 nitrogenase components in plant cells (Allen *et al.*, 2017; Xiang *et al.*, 2020). However, challenges
546 remain, particularly related to the stability of this enzyme in plants, which is sensitive to oxygen
547 and requires high energy levels (Rutten & Poole, 2019; Lindström & Mousavi, 2020). Another
548 approach involves the formation of nodule-like structures to host bacteria, necessitating the
549 transfer of several genes involved in nodule organogenesis and infection mechanisms, but this is a
550 very long-term perspective (Oldroyd & Dixon, 2014; Pankievicz *et al.*, 2019). The genetic
551 remodeling of associative diazotrophs to generate ammonium excreeting bacteria is a short-term
552 promising approach, but using genetically engineered bacteria in the field is currently allowed in
553 only a handful of countries. While exploring these approaches is valuable, we should recognize
554 the huge potential within plant natural diversity to find and breed for better hosts for diazotrophs
555 using non-transgenic approaches. Some maize landraces of Sierra Mixe have demonstrated their
556 ability to acquire nitrogen via aerial roots. Similar traits in sorghum accessions indicate that
557 exploring natural diversity in other cereals for nitrogen fixation is a promising avenue. Doing so
558 can expedite our efforts to introduce nitrogen fixation into cereals for developed and developing
559 countries worldwide. Established sorghum breeding programs could breed for aerial development
560 and mucilage secretion. These natural traits hold considerable value to reduce our dependence on
561 nitrogen fertilizers for cereal crops, reducing costs for growers, the contamination of streams and
562 groundwater, the degradation of coastal zones, and greenhouse gas emissions.
563

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572

573 **Competing interests**

574 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.

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Data Availability

Not applicable

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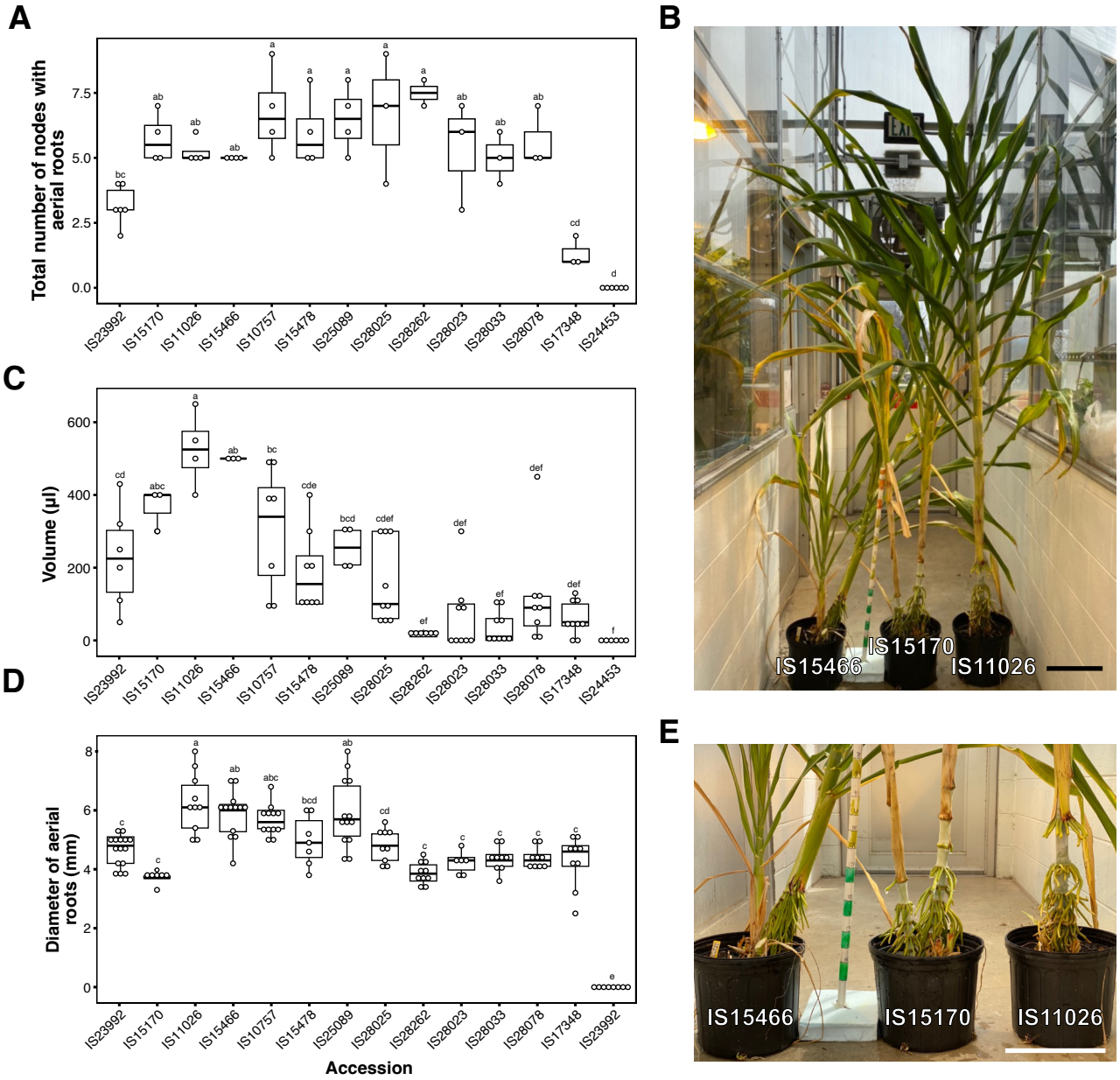


Figure 1. Sorghum screening of selected accessions. Different sorghum accessions display a diversity in traits associated with aerial roots and mucilage production. A) Number of nodes in different sorghum accessions. B) Representative image of selected sorghum accessions. Scale bar = 28 cm. C) Volume 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

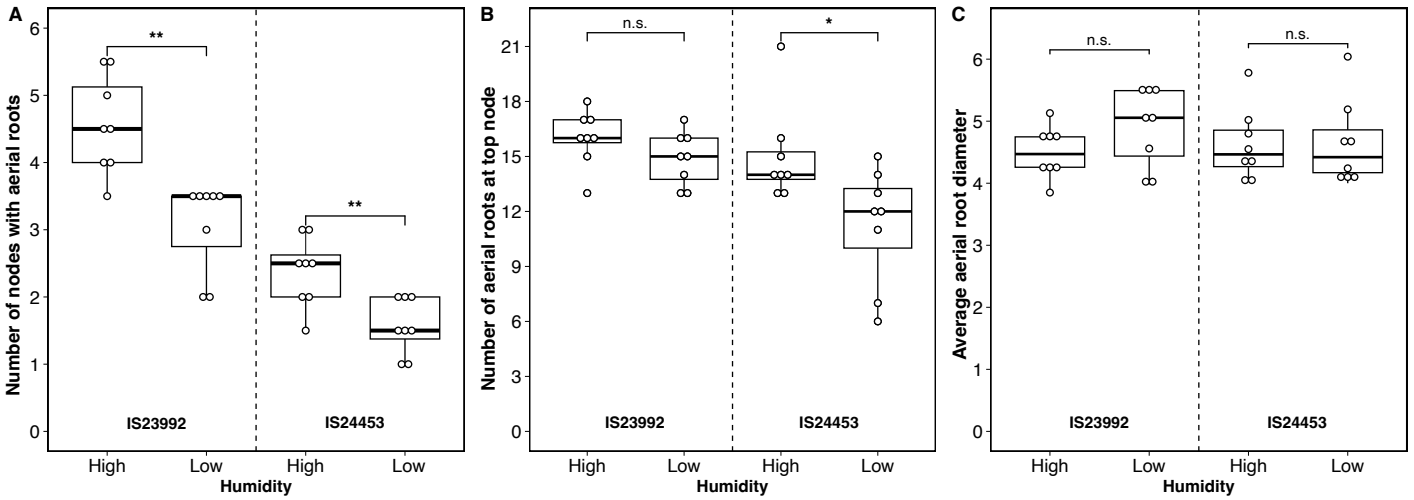


Figure 2. Effect of humidity in sorghum accession IS23992 and IS24453. Boxplots of different phenotypes measured in the sorghum accessions. A) Number of nodes with aerial roots. B) Number of roots on the top node C) Average root diameter on the top node. A Wilcoxon test was performed in R (Ver 4.2.1) 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).

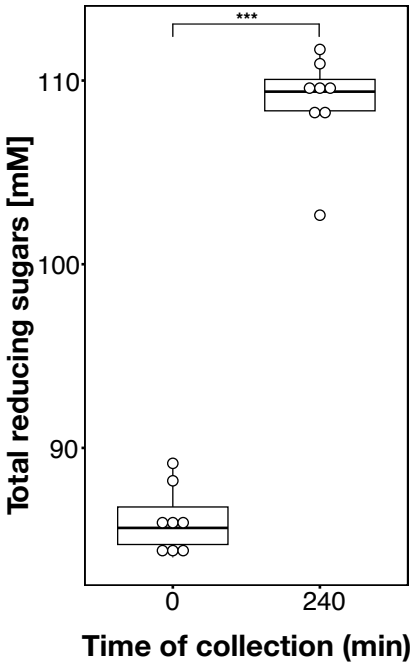
A

B

Monosaccharide	Amount *S. bicolor* (%)[1]	Amount *Z. mays* (%)[2]
Arabinose (Ara)	17.47	12.27
Fucose (Fuc)	13.31	40.68
Xylose (Xyl)	2.26	2.92
Glucuronic acid (GlcA)	8.06	3.09
Mannose (Man)	7.23	2.92
Galactose (Gal)	51.68	38.12
Total	100	100

[1] Data obtained from 1.64 mg of sample
[2] Data from Van Deynze *et al.* 2018

C



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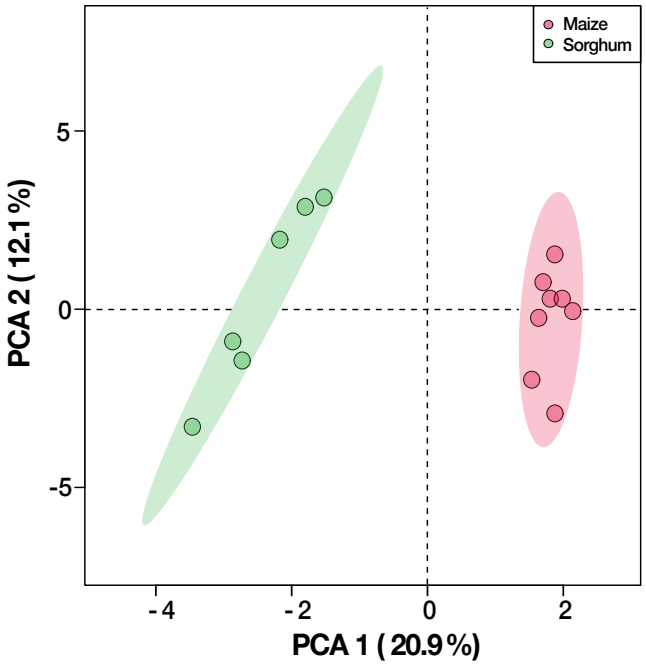


Figure 3. Sorghum aerial root mucilage composition. Sorghum release mucilage from their aerial roots A) Production of mucilage is triggered by water and under high humidity environments. Scale bar (5 cm) B) Monosaccharides composition of sorghum and maize mucilage. Both mucilages have the same monosaccharides 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.

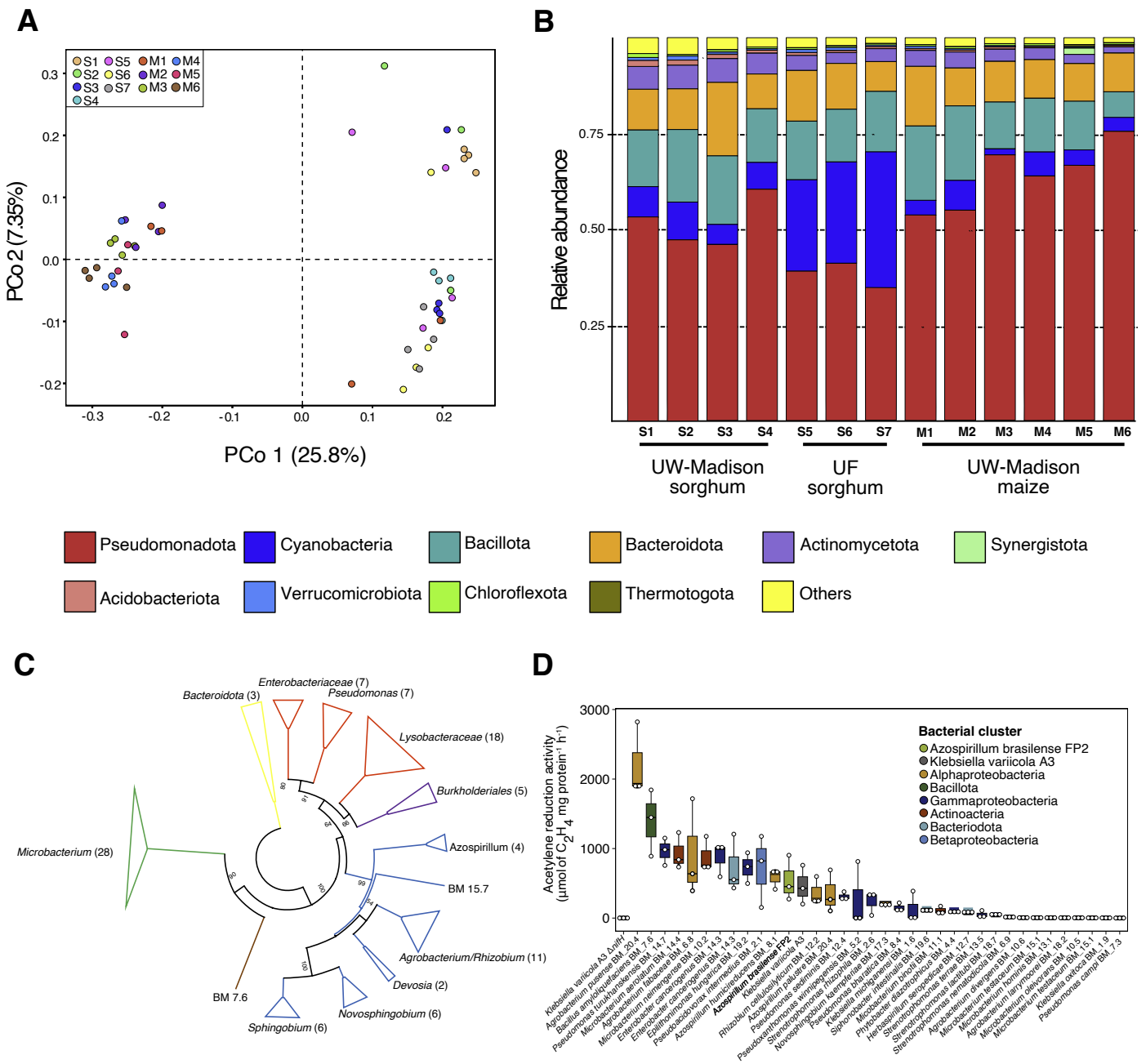


Figure 4. Microbiome profile of sorghum mucilage. Community profile showing the relative abundance present in sorghum and maize. Samples S1 to S4 and M1 to M6 were collected from the field experiments at the University of Wisconsin (UW-Madison) and samples S5 to S7 were collected at University of Florida (UF) A) Principal coordinate analysis plot of sorghum and maize samples based on 16S amplicons. B) Relative abundance 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 variicola* A3 ΔnifH. Species codes (alphabetic order): *Agrobacterium divergens, Agrobacterium fabaceae, Agrobacterium larrymoorei, Agrobacterium pusense, Azospirillum brasilense, Azospirillum humicireducens, Azospirillum palustre, Bacillus amyloliquefaciens, Enterobacter cancerogenus, Epilithonimonas hungarica, Herbaspirillum seropedicae, Klebsiella michiganensis, Klebsiella oxitoca, Klebsiella variicola, Microbacterium aerolatum, Microbacterium binotii, Microbacterium hominis, Microbacterium neimengense, Microbacterium oleivorans, Microbacterium testaceum, Novosphingobium kaempferiae, Phytobacter diazotrophicus, Pseudoacidovorax intermedius, Pseudomonas bharatica, Pseudomonas campi, Pseudomonas lutea, Pseudomonas sediminis, Pseudomonas turukhanskensis, Pseudoxanthomonas winnipegensis, Rhizobium cellulosilyticum, Siphonobacter intestinalis, Stenotrophomonas lactitubi, Stenotrophomonas nematodicola, Stenotrophomonas rhizophila, Stenotrophomonas terrae.*

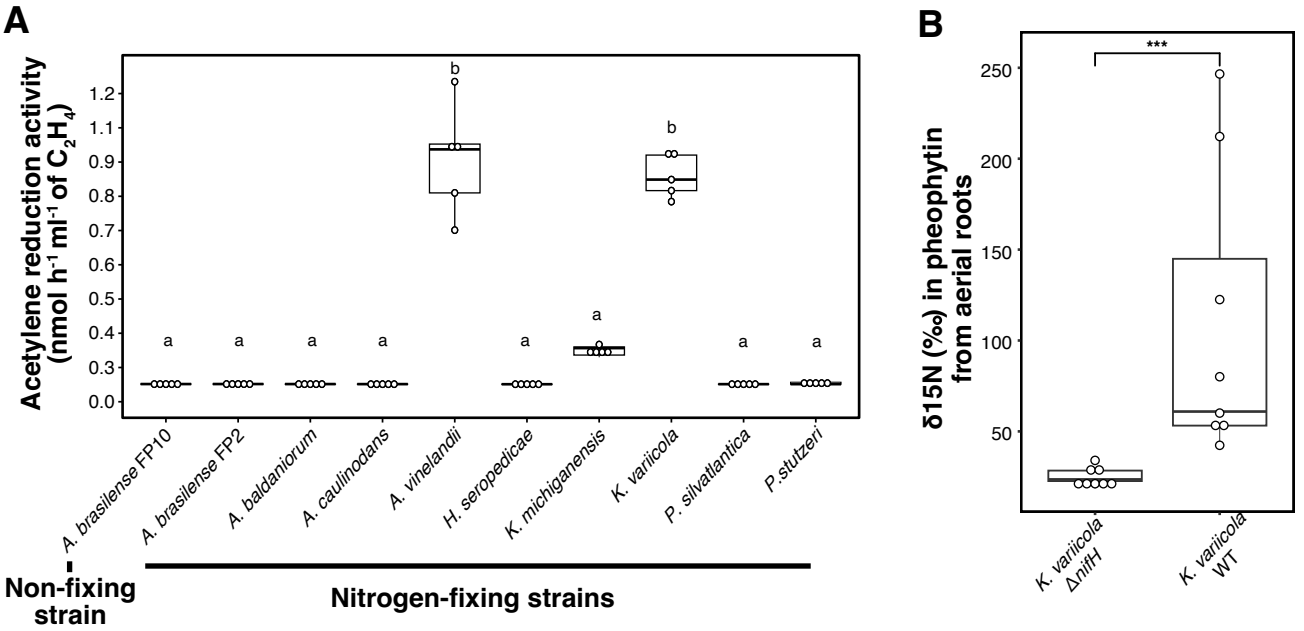
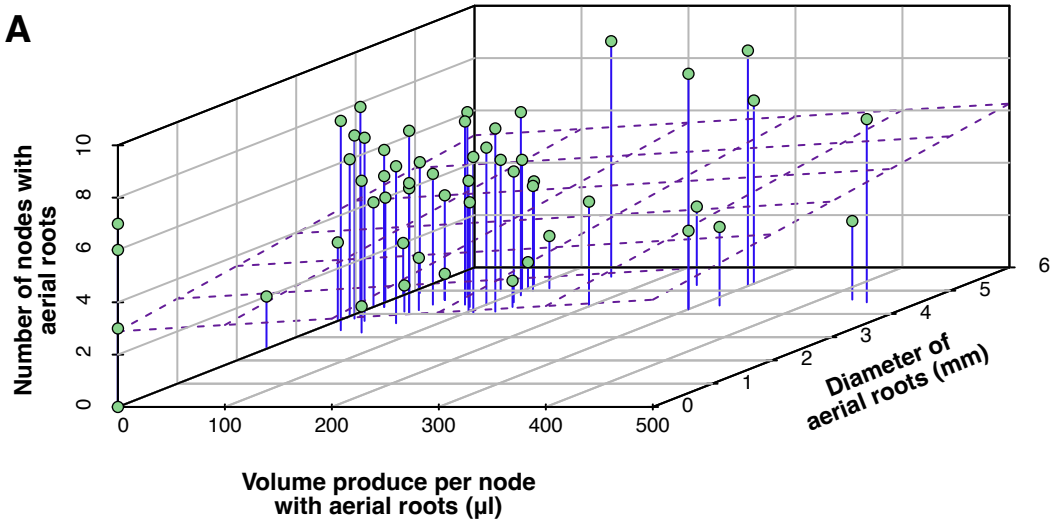
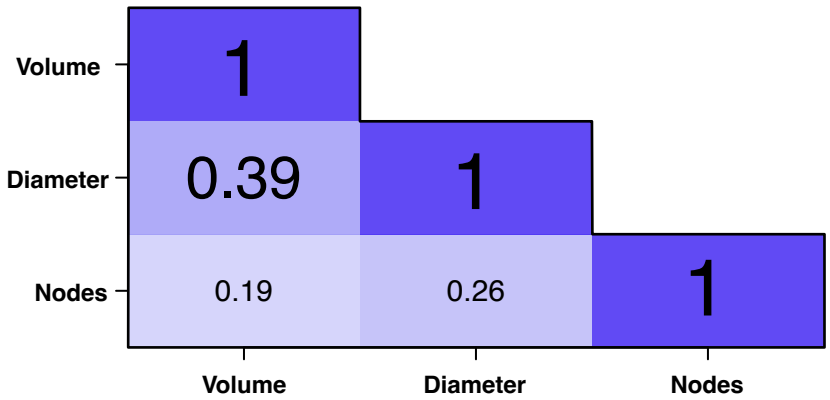
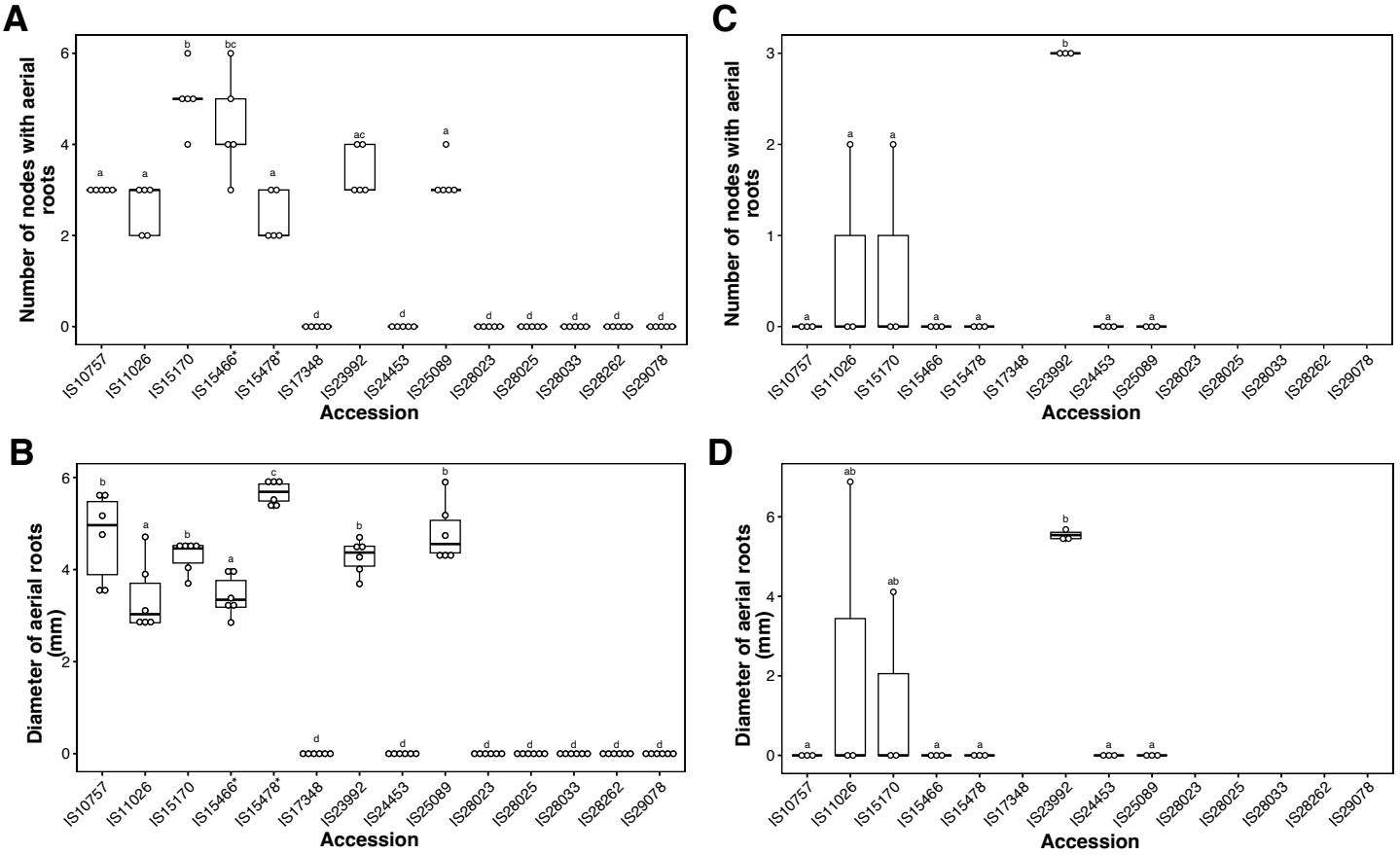


Figure 5. Biological nitrogen fixation in aerial roots from sorghum. A) Acetylene reduction assay in mucilage generated in the greenhouse. Mucilage was used as media to grow ten strains *Azospirillum brasilense* FP2, *Azospirillum baldaniorum*, *Azorhizobium caulinodans*, *Azotobacter vinelandii*, *Herbaspirillum 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 $^{15}N_2$ 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).

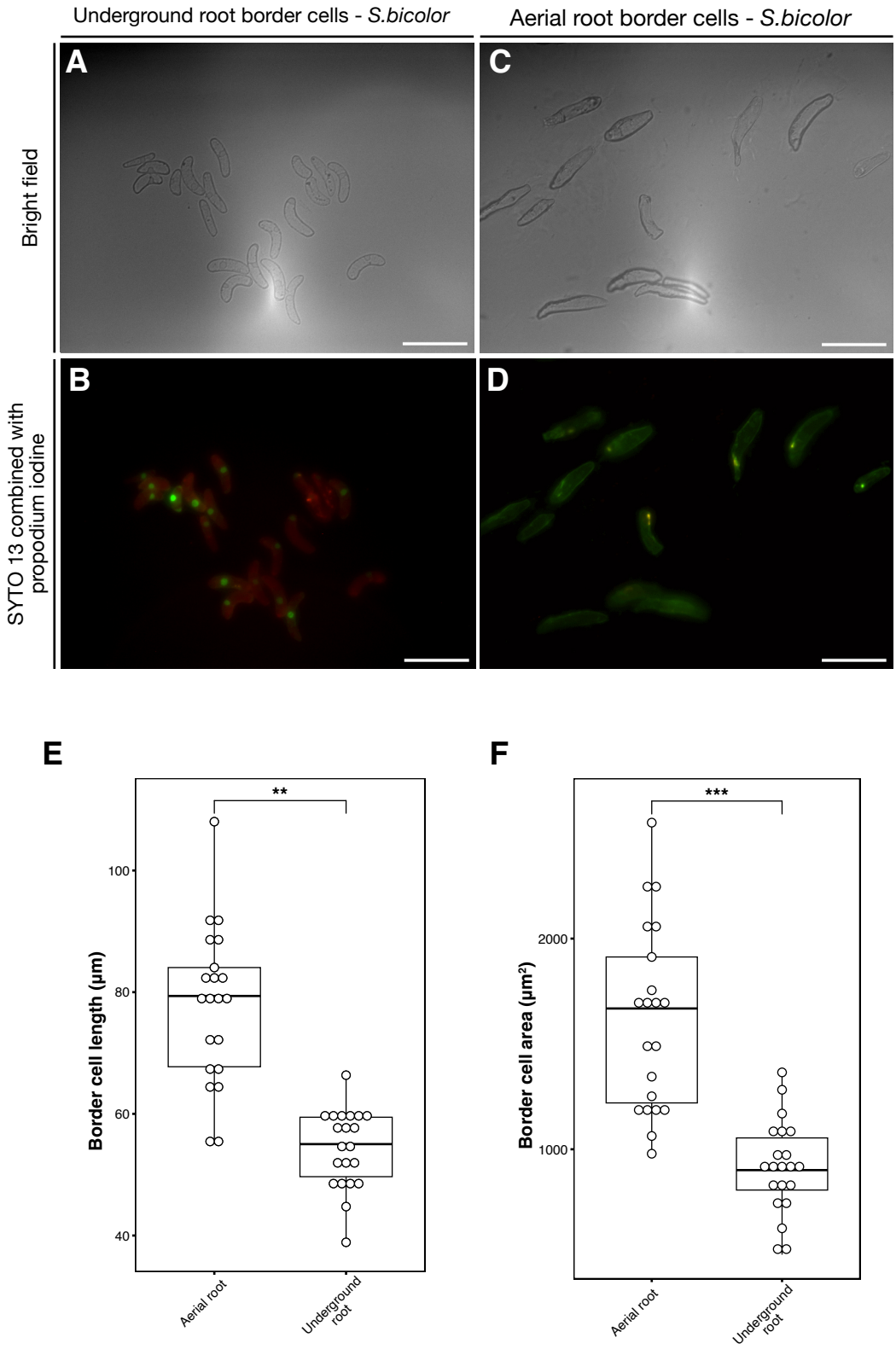




Supplementary Figure 1. Trait correlations. A) Scatter plot shows the correlation between the number of nodes, mucilage volume and diameter. A low positive correlation is observed among all traits. B) Correlation matrix for number of nodes, mucilage volume and diameter.



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 µm^2. AR (aerial roots) and UR (underground roots). The p-values were ** < 0.01 and *** < 0.001 based on a Wilcoxon test (n = 20)