Full title: Mucilage Produced by Sorghum (*Sorghum bicolor*) Aerial Roots Hosts Diazotrophs that Provide Significant Amounts of Nitrogen to the Plant

Short title: Biological Nitrogen Fixation on the Aerial Roots of Sorghum

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Abstract

Sorghum (*Sorghum bicolor*) is a significant crop globally, serving as an important source of food, feed, and fodder, and is increasingly recognized as an energy crop due to its high potential for biomass production. Certain sorghum accessions exhibit prolific aerial root development and produce abundant carbohydrate-rich mucilage after precipitation. This aerial root mucilage bears resemblance to that found in landraces of maize (*Zea mays*) from southern Mexico, which have previously been found to harbor diazotrophs. In this study, we examined the aerial root development of specific sorghum accessions and investigated the influence of humidity on this trait. Our microbiome analysis of the aerial root mucilage of maize and sorghum revealed the presence of numerous diazotrophs in sorghum mucilage, with *Pseudomonadota*, *Bacillota*, and *Bacteriodota* being the predominant phyla observed. However, the community composition varied significantly depending on the host plant and location. Through acetylene reduction, $^{15}N_2$ gas feeding, and ^{15}N isotope dilution assays, we determined that these sorghum accessions can acquire approximately 40% of their nitrogen from the atmosphere through these symbiotic associations on aerial roots. The nitrogen fixation occurring in sorghum aerial root mucilage presents a promising opportunity to reduce reliance on synthetic fertilizers and advance sustainable agricultural practices for food, feed, fodder, and bioenergy production.

Key Words: Biological Nitrogen Fixation, Mucilage, Diazotrophs, Microbiome, Aerial roots.

Introduction

Nitrogen (N) is an essential nutrient for plant growth and development. However, despite its abundance in the atmosphere as dinitrogen (N_2), plants cannot access it directly [1]. Biological nitrogen fixation is the conversion of dinitrogen into ammonium performed by bacteria and archaea known as diazotrophs that express the nitrogenase enzyme [2,3]. This process is essential in agriculture, enabling certain plants, such as legumes, that associate with diazotrophs called rhizobia, to obtain large amounts of nitrogen directly from the atmosphere. Legumes host rhizobia in specialized organs called root nodules, inside which rhizobia fix nitrogen and transfer it to the plant in exchange for carbon from photosynthesis [4]. This symbiosis is critical for the productivity and sustainability of agricultural systems worldwide by reducing the need for synthetic fertilizers and enhancing soil fertility [5–7]. In contrast, cereal crops like maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L.) Moench) generally require substantial nitrogen inputs. While synthetic nitrogen fertilizers, derived from natural gas using the Haber-Bosch process, can address this limitation, their extensive use has led to significant economic and environmental concerns, including nitrogen leaching and the generation of greenhouse gases. For decades, approaches have been proposed to enhance biological nitrogen fixation in cereals, including engineering root nodules in cereals or the expression of the nitrogenase directly in plant cells [8,9]. Another strategy is to manipulate the diazotrophs naturally associated with cereals. Such associative diazotrophs have been documented, for instance, in the rhizosphere, the xylem, or the root mucilage of cereals [10,11]. A synthetic community comprising *Klebsiella variicola* MNAZ1050 and *Citrobacter sp* MNAZ1397 was reported recently to contribute 11% of nitrogen maize through biological nitrogen fixation [10]. Some approaches also rely on altering the genetic circuits controlling nitrogenase expression in associative diazotrophs to induce the release of ammonium on the root surface [12]. While exciting, all these engineering approaches require long development times and are often

based on complex genetic manipulations that limit their development and adoption. To enhance the benefits of biological nitrogen fixation for cereal crops in the shorter term, we explored plant natural diversity to identify genotypes of cereals that could be better hosts for diazotrophs than currently available cultivars.

In 2018, we and colleagues published the characterization of maize landraces cultivated and selected by Indigenous communities in the Sierra Mixe, Oaxaca, Mexico, that secrete viscous mucilage from their aerial roots. Unlike brace roots, these aerial roots are adventitious nodal roots that do not reach the ground. We demonstrated that this mucilage supports communities of diazotrophs that can provide 29-82% of the plant's nitrogen needs [13–16]. These landraces could serve as the foundation for nitrogen fixation in maize through conventional breeding with limited impact on yield [17]. However, the production of aerial roots is not unique to maize. Such roots have been reported throughout the Andropogoneae and Paniceae tribes that include maize, sorghum, sugarcane (*Saccharum* spp.), foxtail millet (*Setaria italica* L.*)*, and pearl millet (*Pennisetum glaucum* (L) R. Br.). These roots serve anchorage functions, especially under flooded conditions, and are important for water and nutrient acquisition [18,19]. Sorghum is a versatile and resilient crop used for food, feed, and fodder production. It has recently gained significant attention as a promising bioenergy crop due to the high biomass productivity of some genotypes [20,21]. Its ability to thrive in diverse climatic conditions, including drought-prone regions, makes it an attractive option for sustainable food and bioenergy production worldwide [22,23].

Despite the similarity between sorghum aerial roots and those in maize known to host diazotrophs, evidence of biological nitrogen fixation in sorghum has yet to be well characterized. Images of aerial roots of sorghum covered with mucilage, as well as a micrograph depicting the bacterium

96 *Azospirillum brasilense,* were featured in the abstract book of a conference on nitrogen fixation in
97 cereals hosted by the International Crops Research Institute for the Semi-Arid Tropics, India
98 (ICRISAT) in 1984. Despite this decades-old report, no data have been published on biological
99 nitrogen fixation in sorghum aerial roots [24]. The similarity between these sorghum aerial roots
100 and those from the Sierra Mixe maize prompted us to explore nitrogen fixation in sorghum. We
101 examined the diversity of aerial roots in sorghum accessions, mucilage production, and their
102 microbiome. We demonstrated that, like the maize landraces from the Sierra Mixe, some sorghum
103 accessions can acquire significant amounts of nitrogen from their aerial roots via the diazotrophs
104 established in their mucilage.
105
106 **Results**
107
108 **Specific sorghum accessions developed aerial roots.**
109 Sorghum accessions selected from the sorghum minicore [25] and augmented with accessions
110 provided by ICRISAT exhibited extensive development of thick aerial roots, similar to those in
111 Sierra Mixe maize landraces [13,25]. Fourteen accessions were grown in a greenhouse at the
112 University of Wisconsin-Madison and assessed for the number of nodes with aerial roots to
113 characterize this trait better (**Supplementary Table 1**). The number of nodes with aerial roots
114 varied from 0 to 8 between accessions. Accessions IS17348 and IS24453 exhibited minimal and
115 no development of aerial roots, respectively. (**Figures 1A**). Upon exposure to water, a number of
116 accessions demonstrated substantial mucilage production from their aerial roots. Notably,
117 accessions IS10757, IS11026, IS15170, IS15466, IS23992, IS25089, and IS28025 consistently
118 produced an average of 200 µl of mucilage per aerial root with comparable lengths. However,
119 accessions IS17348, IS24453, IS28023, IS28033, IS28262, and IS29078 exhibited lower mucilage

120 production, averaging less than 100 µl per root with similar lengths. (**Figure 1B and C**). Sorghum
121 aerial root diameters were measured to determine if the sorghum accessions exhibited thick aerial
122 roots, as shown for the Sierra Mixe maize landraces. The diameter of aerial roots ranged from 0
123 (when absent, only the accession IS24453) to 8 mm (**Figures 1D and 1E**). Through this screening,
124 we identified IS10757, IS11026, IS15170, and IS25089 as the most promising accessions,
125 distinguished by both thick aerial roots and high mucilage production. These results indicate
126 significant variation in the number and diameter of aerial roots and mucilage production among
127 sorghum accessions. A positive correlation was observed between the number of nodes forming
128 aerial roots, root diameter, and mucilage production volume (**Supplementary Figure 1A**). The
129 correlation between the aerial root diameter and mucilage volume was modest, $r^2 = 0.4$ (p-value =
130 0.0024), but low for the number of nodes producing aerial roots and volume, $r^2 = 0.2$ (p-value =
131 0.1517, non-significant) (**Supplementary Figure 1B**). This result indicates that, as we reported
132 for maize, thicker roots are associated with higher mucilage production in these sorghum
133 accessions, too [15].
134
135 **Fig 1. Sorghum screening of selected accessions under greenhouse conditions.**
136 Different sorghum accessions display diverse traits associated with aerial roots and mucilage
137 production. A) Number of nodes in different sorghum accessions (n = 3-6). Most of the accessions
138 evaluated showed more than 5 nodes with aerial roots. B) Representative image of selected
139 sorghum accessions. Scale bar = 28 cm. C) Volume of mucilage per root (µl) produced by different
140 sorghum accessions (n = 4-9). The production of mucilage varied among all the accessions. D)
141 Diameter (mm) of aerial roots in different sorghum accessions (n = 6-15). Thick roots with
142 diameters bigger than 4 mm were noted in nearly all the accessions. E) Aerial roots of the sorghum

accessions in D. Scale bar = 28 cm. An ANOVA (F-test) to calculate significant differences between means was conducted with the package multcompView (0.1-10).

To test these findings under field conditions, the same set of 14 sorghum accessions were grown at the University of Florida North Florida Research and Education Center-Suwannee Valley in 2021 (Live Oak, Florida, USA) and the University of Wisconsin-Madison West Madison Agricultural Research Station in 2022 (Madison, Wisconsin, USA). Florida has a humid subtropical climate, whereas Wisconsin has a continental climate that is affected by the humidity from Lake Superior and Lake Michigan. In these field experiments, the number of nodes with aerial roots, the root diameter, and the number of roots at the top node were recorded (**Supplementary Figure 2**). Environmental conditions, likely a combination of soil properties and humidity, played a significant role, as not all accessions developed aerial roots in both locations. The sorghum accessions generally produced more aerial roots in Florida than in Wisconsin. In Florida, approximately half of the 14 accessions produced nodes with aerial roots; in Wisconsin, fewer than a quarter of the accessions did (**Supplementary Figures 2A and B**). Furthermore, larger aerial root diameters were recorded in Florida compared to Wisconsin (**Supplementary Figures 2C and D**). In Florida, the count of aerial roots at the top node exceeded that in Wisconsin, averaging six roots compared to three roots at the top node, respectively (**Supplementary Figures 2E and F**). Overall, accessions IS11026, IS15170, and IS23992 demonstrated the potential to develop traits related to aerial root formation and mucilage production, highlighting the significant influence of environmental factors on these characteristics.

Humidity influences the number of nodes in sorghum.

166 Environmental factors can affect plant growth and development, including moisture, light
167 intensity, and temperature [26]. We investigated the impact of humidity on our previous findings
168 under greenhouse and field conditions,1 we evaluated two sorghum accessions: IS23992, which
169 produces many aerial roots, and IS24453, which lacks aerial roots under field conditions (**Figure**
170 **1 and Supplementary Figure 2**). We grew these accessions in a greenhouse under either high
171 humidity (75%) or low humidity (30%) conditions. For both sorghum accessions, we observed a
172 significant increase in the number of nodes with aerial roots when grown under high humidity,
173 with a more pronounced effect observed in IS23992 (**Figure 2A**). Interestingly, IS24453 exhibited
174 aerial root development in this experiment, contrasting with the results observed in the field
175 experiments (**Supplementary Figure 2**). Moreover, the number of aerial roots at the top node was
176 only significantly affected by humidity in IS24453 (**Figure 2B**). To determine whether the increase
177 in the number of nodes with aerial roots in humid conditions was due to differences in root
178 diameter, we measured the diameter of aerial roots at the top node under both humidity conditions.
179 The aerial root diameter did not significantly differ between these accessions (**Figure 2C**). These
180 findings highlight the critical role of humidity as a key environmental factor in aerial root
181 development in sorghum.
182
183 **Fig 2. Effect of humidity in sorghum accession IS23992 and IS24453.**
184 Boxplots of different phenotypes measured in the sorghum accessions under high (75%) and low
185 (30%) relative humidity. A) Number of nodes with aerial roots (n = 8). The number of nodes with
186 aerial roots significantly increased in both accessions. B) Number of roots on the top node (n = 8).
187 Only IS24453 increased the number of roots at the top node. C) Average root diameter on the top
188 node (n = 8). The root diameter was not affected by the humidity in both accessions. A Wilcoxon

189 test was performed in R (Ver 4.2.1) with the package ggpubr (Ver 0.6.0). Significance levels * p-
190 value ≤ 0.05, ** p-value ≤ 0.01, and n.s. not significant.
191
192 **Sorghum mucilage is composed of carbohydrates.**
193 The viscous mucilage produced by the Sierra Mixe maize landraces consists of a complex
194 polysaccharide with a galactose backbone and side chains of fucose, arabinose, xylose, mannose,
195 and glucuronic acid [13,14]. Visually the mucilage from different sorghum accessions, such as
196 IS2392 (**Figure 3A**), looked similar. To identify any chemical differences between maize and
197 sorghum mucilage, we conducted a total carbohydrate composition analysis using gas
198 chromatography/mass spectrometry (GC/MS). We collected mucilage from accession IS11026 in
199 the greenhouse, as this accession produced a substantial amount of mucilage under greenhouse
200 conditions (**Figure 1C**). For a clear distinction between sorghum and maize mucilage comparison,
201 we presented the proportions (mole %) of the quantified monosaccharides. (**Figure 3B and**
202 **Supplementary Table 2**). Arabinose, galactose, glucuronic acid, and mannose had higher ratios
203 in sorghum than in maize mucilage. In particular, galactose accounts for nearly 50% of the total
204 sugars in sorghum mucilage, and the proportion of fucose in sorghum mucilage was approximately
205 half of that in maize mucilage. Using this approach, we did not detect the presence of other
206 carbohydrates, such as glucose, rhamnose, ribose, and galacturonic acid. These results indicate
207 that the aerial root mucilage of the two species consists of the same monosaccharides, but that their
208 proportions differ.
209
210 **Fig 3. Sorghum aerial root mucilage composition.**
211 Sorghum aerial roots release mucilage . A) Mucilage production is triggered by water and under
212 high-humidity conditions. The mucilage from different accessions appears similar. Accession

213 IS2392 serves as a representative example of sorghum mucilage. Scale bar (5 cm) B)
214 Monosaccharide composition of sorghum accession IS11026 and Sierra Mixe maize mucilage.
215 Both mucilages consist of the same monosaccharides but in different proportions (mole %). C)
216 Concentration of reducing sugars in sorghum mucilage from the accession IS23992 determined
217 with benedict's reaction. A Wilcoxon test was performed to compare means *** p-value ≤ 0.001
218 (n = 8). D) Principal component analysis using Sparse Partial Least Squares. Two clusters are
219 depicted based on the species studied: sorghum accessions IS29091 and IS2245 and maize
220 landraces CB017456 and GRIN 19897.
221
222 In maize landraces from the Sierra Mixe, water triggered the expression of plant genes associated
223 with carbohydrate synthesis and degradation [15]. To investigate this degradation process further,
224 we assessed the levels of free sugars in sorghum mucilage at 1 and 5 hours after adding water to
225 the aerial roots of the accession IS23992. Using Benedict's reaction to quantify free sugars, we
226 observed that the free sugar concentration increased over time, rising from an average of 86 mM
227 at 1 hour to 109 mM after 5 hours (**Figure 3C**)[27]. This finding suggests that sorghum aerial roots
228 produce enzymes that break down the mucilage polysaccharide into simple sugars. We cannot
229 exclude the contribution of microbes in this degradation process as the aerial roots from plants
230 grown in the greenhouse were not germ-free. However, compared to field conditions, very few
231 microbes could be isolated from sorghum mucilage when grown in this greenhouse.
232
233 To explore the differences in the metabolites between maize and sorghum aerial root mucilage, we
234 performed a metabolomics analysis using gas chromatography coupled with time-of-flight mass
235 spectrometry (GC/TOF-MS) (**Supplementary Table 3**). We utilized the sorghum accessions
236 IS29091 and IS2245 from the Wolf *et al.* (2023) [28] study, which investigated the diversity of

237 aerial roots in sorghum, along with maize landraces CB017456 and GRIN19897, which display
238 aerial root. In total, 252 metabolites were detected in maize and sorghum mucilage (**Dataset S1)**.
239 A principal component analysis (PCA) revealed two clusters where samples were grouped based
240 on plant species (**Figure 3D**). We used a Significance Analysis of Microarray (SAM) to identify
241 metabolites that differed among the mucilage samples of the two species. The top 10 most
242 differentially abundant metabolites between sorghum and maize included 6 unknown metabolites
243 but also octadecanoic acid, montanic acid (octacosanoic acid), pinitol (1D-*chiro*-inositol), and *N*-
244 acetyl aspartate diethyl ester (**Supplementary Table 4**). These metabolomic differences may
245 contribute to functional differences between sorghum and maize mucilage.
246
247 **Aerial root mucilage from sorghum contains abundant and large border cells.**
248 The root cap of underground roots produces small quantities of mucilage [29–31]. In particular,
249 border cells (BCs) that detach from the root cap are responsible for mucilage production [15,32].
250 To investigate sorghum mucilage production, we examined mucilage derived from both
251 underground and aerial roots in sorghum accession IS23992 (**Supplementary Figures 3A and C**).
252 BCs derived from underground roots exhibited a distinct and elongated morphology similar to
253 those observed in BCs from pea (*Pisum sativum* L.), maize, and alfalfa (*Medicago sativa* L.)[33–
254 35]. In contrast, BCs from sorghum aerial roots appeared much larger and elongated, with irregular
255 shapes and sizes (**Supplementary Figure 3C**). Interestingly, these BCs from sorghum aerial roots
256 resembled those recently reported in mucilage from maize aerial roots [15]. These larger BCs may
257 contribute to the abundant mucilage production by maize and sorghum aerial roots.
258
259 Border cells detached from the underground root cap can remain active for several weeks [32,36].
260 To determine the viability of sorghum border cells, we used a live-dead staining protocol on both

261 types of sorghum mucilage collected after 72 hours. We used a combination of SYTO™ 13 and
262 propidium iodine, which are permeating and non-permeating cell dyes, respectively [37,38]. Most
263 cells were viable, as indicated by only SYTO™ 13 in the nuclei. Additionally, BCs from
264 underground roots displayed regular and well-defined nuclei, while BCs from aerial roots
265 exhibited irregular nuclei (**Supplementary Figures 3B and D**). To quantify the morphological
266 differences between the two types of BCs, we measured their length between the two cellular poles
267 and their cell area. Significant differences were observed in both measurements. BCs from aerial
268 roots are generally twice the size of BCs from underground roots. Although both types of BCs
269 maintain the function of mucilage production, the observed morphological differences may
270 account for the variation in the amount of mucilage secreted by these cells.
271
272 **Sorghum mucilage hosts a wide range of diazotrophs.**
273 To explore the microbial diversity within sorghum mucilage, we isolated and identified bacteria
274 using 16S amplicon sequencing. The sorghum association panel [39] and sorghum minicore [25]
275 were used to explore the genetic control of aerial root development in sorghum [28], obtain large
276 amounts of mucilage, and characterize aerial root microbiomes [28,39]. We used mucilage
277 samples from accessions IS2245, IS23992, IS29092 and IS2902 from the fields at the University
278 of Wisconsin West Madison Agricultural Research Station and the UF North Florida Research &
279 Education Center - Suwannee Valley. We also included mucilage samples from Sierra Mixe
280 landraces at a neighboring West Madison Agricultural Research Station field as reference
281 (**Supplementary Table 5**). To analyze the bacterial composition, we amplified and sequenced the
282 16S V3-V4 region and grouped the reads into Operational Taxonomic Units (OTUs) to provide
283 information on the relative abundance of different taxa [40]. We performed principal coordinate
284 analysis to examine the similarity between samples. As expected, the plant origin of the mucilage

285 was the main factor influencing beta diversity, although location also plays a role in this diversity
286 (**Figure 4A**). However, Analysis of Molecular Variance (AMOVA) uncovered instances where no
287 statistically significant differences were detected between the microbiomes of maize and sorghum
288 (**Supplementary Table 6**). We also investigated the taxonomy of bacteria in sorghum and maize
289 mucilage. The phylum Pseudomonadota (former Proteobacteria) was dominant in the mucilage of
290 both maize and sorghum, followed by Bacillota and Bacteroidota (**Figure 4B**). The profiles of
291 samples from Wisconsin were relatively similar regardless of the plant of origin, while samples
292 from Florida exhibited enrichment in Cyanobacteria (**Figure 4B**). In both mucilage samples, 34
293 distinct bacterial genera were identified, showing, as expected, differences in their abundance
294 across the samples (**Supplementary Figure 4)**.
295
296 **Fig 4. Microbiome profile of sorghum mucilage.**
297 The community profile shows the relative abundance of bacteria present in sorghum (S) and maize
298 (M) mucilages. Samples S1 to S4 (squares) and M1 to M6 (triangles) were collected from the field
299 at the University of Wisconsin-Madison (UW-Madison), and samples S5 to S7 (circles) were
300 collected at the University of Florida (UF). A) Principal coordinates analysis plot of sorghum and
301 maize samples based on 16S amplicon sequences. B) Relative abundance of sorghum and maize
302 samples based on 16S amplicons and OTUs. C) Unrooted maximum-likelihood phylogenetic tree
303 of 103 bacteria isolated from sorghum aerial roots mucilage in BMGM nitrogen-free semisolid
304 medium. The evolutionary distances were computed using the Jukes-Cantor method and are in the
305 units of the number of base substitutions per site. This analysis involved 103 nucleotide sequences.
306 All positions containing gaps and missing data were eliminated (complete deletion). There were
307 908 positions in the final dataset. Numbers at nodes are bootstrap values (1000 replications, values
308 < 50% are not shown). Colors: Red: Gammaproteobacteria, Purple: Betaproteobacteria, Blue:

309 Alphaproteobacteria, Brown: Bacillota, Green: Actinomycetota, Yellow: Bacteroidota. D)
310 Nitrogenase activity of 34 isolated from sorghum aerial roots mucilage in BMGM nitrogen-free
311 semisolid medium (n=3). The red arrows show the reference strains for comparison. Bacteria:
312 *Klebsiella variicola* A3 Δ*nifH*. Species codes (alphabetic order): *Agrobacterium divergens*,
313 *Agrobacterium fabaceae*, *Agrobacterium larrymoorei*, *Agrobacterium pusense*, *Azospirillum*
314 *brasilense*, *Azospirillum humicireducens*, *Azospirillum palustre*, *Bacillus amyloliquefaciens*,
315 *Enterobacter cancerogenus*, *Epilithonimonas hungarica*, *Herbaspirillum seropedicae*, *Klebsiella*
316 *michiganensis*, *Klebsiella oxytoca*, *Klebsiella variicola*, *Microbacterium aerolatum*,
317 *Microbacterium binotii*, *Microbacterium hominis*, *Microbacterium neimengense*, *Microbacterium*
318 *oleivorans*, *Microbacterium testaceum*, *Novosphingobium kaempferiae*, *Phytobacter*
319 *diazotrophicus*, *Pseudoacidovorax intermedius*, *Pseudomonas bharatica*, *Pseudomonas campi*,
320 *Pseudomonas lutea*, *Pseudomonas sediminis*, *Pseudomonas turukhanskensis*,
321 *Pseudoxanthomonas winnipegensis*, *Rhizobium cellulosilyticum*, *Siphonobacter intestinalis*,
322 *Stenotrophomonas lactitubi*, *Stenotrophomonas nematodicola*, *Stenotrophomonas rhizophila*, and
323 *Stenotrophomonas terrae*.
324
325 In a second experiment, we isolated bacteria from the same sorghum association panel but
326 expanded the number of accessions (**Supplementary Table 7**) [28,39]. The isolation of
327 diazotrophs was performed using the nitrogen-free semi-solid BMGM medium, which revealed a
328 high diversity of potential diazotrophs. We isolated 103 non-clonal strains from 34 different
329 bacterial species with the prevalence of Pseudomonadota (Gammaproteobacteria - 31,
330 Alphaproteobacteria - 33, Betaproteobacteria - 5), Actinomycetota (28), Bacteroidota (5), and
331 Bacillota (1) (**Figure 4C and Supplementary Table 7**). Subsequently, we assessed the
332 nitrogenase activity via the acetylene reduction assay (ARA) in one representative strain selected

333 from our prior isolates. Additionally, we introduced the reference strains *Azospirillum brasilense*
334 FP2[41], a bacterium known for promoting plant growth and commonly associated with crops, and
335 *Klebsiella variicola* A3, isolated from sorghum mucilage and established as a standard for nitrogen
336 fixation in our laboratory. As a negative control, we included the *Klebsiella variicola* A3 Δ*nifH*
337 mutant, unable to fix nitrogen. The strains *Agrobacterium pusense* 20.4, *Bacillus*
338 *amyloliquefaciens* 7.6, *Pseudomonas turukhanskensis* 14.7, *Microbacterium aerolatum* 14.4*,*
339 *Agrobacterium fabacearum* 6.8*, Microbacterium neimengense* 10.2*, Enterobacter cancerogenus*
340 14.3*, Epilithonimonas hungarica* 19.2*, Shinella oryzae* 21.1*, and Pseudacidovorax intermedius*
341 2.1 showed higher nitrogenase activity than the reference diazotrophs *K. variicola* A3 and *A.*
342 *brasilense* FP2 (**Figure 4D**). These findings validate that sorghum mucilage hosts a variety of
343 diazotrophs capable of potentially aiding in nitrogen fixation[42].
344
345 **Mucilage enables efficient biological nitrogen fixation in sorghum.**
346 In Sierra Mixe maize landraces, the mucilage fosters a nitrogen-acquiring habitat for diazotrophs
347 with low oxygen levels and an abundance of carbohydrates [13]. Using ARA, we tested whether
348 sorghum mucilage could serve as a proper medium for nitrogen fixation. A panel of nine
349 diazotrophs isolated from sorghum and maize mucilage along with other isolates from cereals (e.g.,
350 maize, rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and sugarcane) were used for this
351 experiment (**Supplementary Table 8**). As a negative control, we included *A. brasiliense* FP10, a
352 strain lacking nitrogenase activity yet exhibiting a growth rate equivalent to the wild-type FP2
353 strain [41]. To limit the abundance of environmental diazotrophic bacteria in the sorghum
354 mucilage, we collected mucilage from sorghum plants grown in the greenhouse. We selected
355 sorghum accession IS23992 because it produced numerous nodes with aerial roots in greenhouse
356 and field conditions (**Figure 1 and Supplementary Figure 2**). ARA indicated significant

357 nitrogenase activity in mucilage inoculated with the strains *Azotobacter vinelandii* DJ, a reference
358 diazotroph for decades, and *Klebsiella variicola* A3 that we isolated sorghum mucilage (**Figure**
359 **5A**). However, all seven other strains tested exhibited limited nitrogenase activity, with only a
360 *Klebsiella michiganensis* strain isolated from maize mucilage showing modest ARA activity. This
361 experiment indicated that the sorghum aerial root mucilage provides a proper environment for
362 some diazotrophs to fix nitrogen, particularly those isolated from mucilage.
363
364 **Fig 5. Biological nitrogen fixation on aerial roots from sorghum.**
365 A) Acetylene reduction assay in mucilage generated in the greenhouse. Mucilage was used as
366 media to grow 9 nitrogen-fixing strains (*Azospirillum brasilense* FP2, *Azospirillum baldaniorum*,
367 *Azorhizobium caulinodans*, *Azotobacter vinelandii* DJ, *Herbaspirillum seropedicae*, *Klebsiella*
368 *michiganensis*, *Klebsiella variicola* A3, *Paraburkholderia silvatlantica*, and *Pseudomonas*
369 *stutzeri*) and *Azospirillum brasilense* FP10 as a non-fixing control. Tukey's honestly significant
370 difference (HSD) test was performed in R, and significant group means are displayed as lower
371 case letters (n = 6). B) Analysis of ^{15}N gas feeding experiment on aerial roots of sorghum accession
372 IS23992. Aerial roots were inoculated with *K. variicola* Δ*nifH* (negative control) or *K. variicola*
373 A3 (positive control). *** indicates p-value ≤ 0.001 based on Wilcoxon signed-rank test (n = 8).
374 C) ^{15}N isotope dilution experiment on aerial roots of sorghum accessions IS23992 and IS24453.
375 Accessions IS23992 and IS24453 were inoculated with the nine strains from the ARA experiment
376 (diazotrophs), and three non-fixing mutant strains *Azospirillum brasilense* FP10, *Azotobacter*
377 *vinelandii* DJ100 Δ*nifH*, *Azorhizobium caulinodans* ORS571 Δ*nifA*. Whole plants at the flowering
378 stage were ground, and the average of three replicates was plotted. Percent Ndfa was calculated
379 for both accessions using the plants inoculated with diazotrophs and using as reference the plants
380 inoculated with mutant strains. An ANOVA was performed to compare means between treatments

381 and genotypes using the package multcompView. D) Scatterplot of the percentage of nitrogen
382 derived from the atmosphere (%Ndfa) in different sorghum samples from the accessions IS23992
383 and IS24453, indicated with circles and triangles, respectively. Solid and dotted lines are
384 regression lines for IS23992 and IS24453, respectively. Samples were collected every two weeks,
385 starting at stage 3 (Samples S3 to S5) and after flowering (S6). Error bars display standard
386 deviations. Pair-wise comparisons were made with Wilcoxon's test;**** means p-value ≤ 0.0001
387 and n.s. Indicates non-significant.
388
389 To test the plant's ability to assimilate nitrogen fixed by diazotrophs, we conducted a $^{15}N_2$ gas
390 feeding experiment. Aerial roots producing mucilage were collected from the greenhouse-grown
391 sorghum plants of the accession IS23992. Based on the ARA results, we inoculated the roots with
392 wild-type fixing *Klebsiella variicola* A3 or its non-fixing Δ*nifH* mutant strain as a negative
393 control[42]. Isotope-ratio mass spectrometry (IRMS) was used to quantify the $\delta^{15}N$ in chlorophyll
394 from sorghum aerial roots. Roots inoculated with wild-type fixing *K. variicola* exhibited
395 significant incorporation of ^{15}N in plant chlorophyll compared to aerial roots inoculated with the
396 non-fixing strain (**Figure 5B**). To provide additional evidence of fixed nitrogen uptake, we
397 conducted a ^{15}N isotope dilution assay. Two sorghum accessions, IS23992 and IS24453, exhibiting
398 high and low numbers of aerial roots, respectively, were grown in pots in the greenhouse. These
399 accessions were inoculated either with a panel of diazotrophs from the ARA experiment or three
400 non-fixing mutants (**Supplementary Table 8**). Atom%^{15}Nexcess was evaluated by IRMS in
401 leaves collected at four vegetative stages (S3, S4, S5, and S6) and whole plant samples at S6.
402 Significantly lower Atom%^{15}Nexcess values were observed in the IS23992 accession, which
403 produces many aerial roots, when inoculated with nitrogen-fixing bacteria compared to plants
404 inoculated with the non-fixing controls. Atom%^{15}Nexcess values of the IS24453 accession, which

405 makes few aerial roots, inoculated with fixers or non-fixers, were similar to those of the IS23992
406 genotype inoculated with non-fixers (**Figure 5C**). Based on these ^{15}N dilution data, we evaluated
407 the percentage of nitrogen derived from the atmosphere (%Ndfa) at these four stages of sorghum
408 development (**Supplementary Figure 5**). %Ndfa increased steadily over time, reaching 43% on
409 whole plants at S6 (**Figure 5D**). Even though all the techniques to quantify nitrogen fixation (ARA,
410 $^{15}N_2$ gas feeding, and ^{15}N isotope dilution) were used in the greenhouse and have their own biases,
411 our results collectively indicate that sorghum accessions with a large number of aerial roots can
412 support an active community of diazotrophs and obtain a significant fraction of their nitrogen from
413 the biological nitrogen fixation.
414

415 **Discussion**

416 *Some sorghum accessions produce abundant and thick aerial roots under humid conditions.*
417 Previous studies have reported variations in the number of nodes adventitious nodal roots among
418 different sorghum genotypes. For example, the Chinese landrace Sansui produces 6-8 nodes with
419 aerial roots, while the elite cultivar Jiliand only has a single node with roots [43]. Our study based
420 on select sorghum accessions from different sources such as the sorghum mincore panel, the
421 sorghum association panel and ICRISAT confirmed significant variation in the number of nodes
422 with aerial roots (**Figure 1A**). We observed differences in diameter and mucilage production in
423 these accessions, and, as in maize, a positive correlation among these traits was confirmed [13].
424 The presence of aerial roots in other members of the Andropogoneae and Paniceae tribes, such as
425 sugarcane and foxtail millet, suggests a common evolutionary origin of this trait [18]. It will be
426 interesting to explore if similar genes control this trait across these species.
427

428 Water availability, hypoxia, and humidity impact plant growth and development by producing
429 phytohormones, including abscisic acid and ethylene [44]. In the case of rice, high humidity
430 stimulates the development of lateral roots, which was attributed to increased levels of ethylene
431 [45–47]. Abscisic acid also controls plant responses to water availability [48]. Under high
432 humidity, we observed more nodes with aerial roots across sorghum genotypes, possibly by
433 regulating phytohormones like ethylene and abscisic acid. The accession IS24453 failed to develop
434 aerial roots in the initial experiment, where humidity remained at base levels. However, it
435 eventually developed aerial roots upon increasing humidity, likely due to increased phytohormone
436 levels (**Figure 2**). Subsequent experiments should prioritize quantifying these components to
437 mitigate unidentified experimental variables that may influence aerial root development.
438
439 *Some sorghum accessions produce abundant mucilage from aerial roots.*
440 Plants secrete mucilage from various tissues to adapt to abiotic and biotic stresses. In underground
441 roots, mucilage production is carried out by border cells. In the case of the maize landraces, the
442 mucilage from their aerial roots resembles the mucilage commonly found at the tips of
443 underground roots. Pankievicz *et al*. [15] showed the abundance of border cells in the mucilage
444 from the aerial roots of Sierra Mixe maize. They noted differences in border cell sizes compared
445 to border cells from underground roots. This result explained the difference in mucilage production
446 between the two types of roots [15]. Recently, a study involving 146 sorghum accessions described
447 the generation of mucilage from the aerial roots[49]. Our data corroborated these sorghum
448 findings, showing differences between border cell morphologies between underground and aerial
449 roots and mucilage production (**Supplementary Figure 3 and Figure 3A**). Based on these
450 findings, we hypothesize that the border cells in sorghum and maize exhibit greater similarity than
451 those in underground roots. The difference in quantity and size explains the volume generated by

452 each type of root. Additionally, we hypothesize these specialized cells are more abundant and
453 functionally active in younger roots, as observed in maize aerial roots.
454
455 Carbohydrates are the primary components of both underground and aerial root mucilage
456 [13,14,50,51]. In this study, we investigated the carbohydrate composition of sorghum mucilage
457 and showed the presence of similar monosaccharides in maize and sorghum aerial root mucilage,
458 albeit in different proportions (**Figure 3**). These findings are consistent with a prior study on maize
459 genotypes, which revealed changes in the carbohydrate profile based on their geographic locations
460 and agroecological systems [51]. Wolf *et al.* also analyzed the monosaccharide composition in
461 mucilage from sorghum aerial roots, and we found that both studies reported a similar range of
462 values [28]. To fully understand variations in monosaccharide composition in sorghum mucilage,
463 a broader range of environments and genotypes needs to be considered. Interestingly, our analysis
464 did not identify the presence of fructose in the sorghum mucilage, as reported in other studies
465 (**Figure 3B**) [49]. This mucilage composition may suggest that environmental factors significantly
466 influence maize and sorghum mucilage. However, the genetic aspect cannot be disregarded
467 entirely, as evidenced by our principal component analysis, which displayed a clear separation
468 based on species (**Figure 3**). Overall, inter-species differences are more pronounced than inter-
469 accession differences (**Figure 3B and 3D)**, supporting reports that highlight variations in the
470 microbiomes of cereals [52]. Additionally, there must be differences within genotypes, as observed
471 in maize underground mucilage[51].
472
473 *Sorghum aerial roots mucilage hosts specific microbial communities containing diazotrophs.*
474 Several studies have documented variations in plant microbiome composition based on host and
475 environmental factors [53–56]. Our microbiome survey revealed that location and host influence

476 microbial composition in the aerial root mucilage (**Figure 4**). Certain plant enzymes that break
477 down mucilage into simple sugars are expressed in the Sierra Mixe landraces and may play a role
478 in selecting specific microbes. The microbiome may contribute to mucilage degradation through
479 specialized enzymes [15]. Pseudomonadota, Acidobacteriota, Cyanobacteriota, and Bacillota were
480 the most abundant phyla in sorghum, aligning with findings from other sorghum microbiome
481 studies [57]. Our study sheds light on the microbial profiles of sorghum and maize for nitrogen
482 fixation and offers valuable strains for engineering diazotrophs and enhancing nitrogen fixation.
483 These resources will serve as a foundation for investigating microbial community interactions in
484 the mucilage. Such insights could pave the way for inoculation strategies with diazotrophic
485 consortia and/or engineered diazotrophs.
486
487 Sorghum growth promotion enabled by inoculation with diazotrophs has been reported several
488 years ago [58–60]. There was evidence that sorghum could acquire variable amounts of nitrogen
489 from the air after inoculation with diazotrophs or in association with native bacterial communities
490 [61,62]. However, these reports provided no information about the ability of these genotypes to
491 produce aerial roots or mucilage. Our study provides evidence that the production of aerial roots
492 and mucilage is a mechanism in sorghum that facilitates association with diazotrophs, enabling the
493 plant to obtain nitrogen from the atmosphere through biological nitrogen fixation. Microbial
494 communities can thrive in plant mucilages due to the abundance of carbon, and diazotrophs often
495 benefit from the microaerobic conditions. The mucilage viscosity is strikingly similar to nitrogen-
496 free semi-solid media, which is classically used to isolate diazotrophs from non-legumes [63]. The
497 presence of diazotrophs in sorghum aerial root mucilage was confirmed in our study using bacterial
498 isolation in nitrogen-free semisolid media and nitrogenase activity assays.
499

500 *Efficient nitrogen fixation occurs in sorghum aerial root mucilage.*

501 Biological nitrogen fixation in cereals can reduce agriculture's dependence on synthetic nitrogen-
502 based fertilizers. Our research has shown that mucilage is crucial in creating a favorable
503 environment for diazotrophs to thrive, enabling plants to acquire nitrogen (**Figure 5A, B, and C**).
504 Two independent studies have revealed that specific diazotrophs form associations with the
505 underground roots of sorghum plants, delivering nitrogen to the plant. We observed this unique
506 specificity as only the diazotrophs isolated from mucilage exhibited higher nitrogenase activity
507 than those isolated from other grasses, considered the benchmark for studying nitrogen fixation in
508 cereals (**Figure 5A**). The biological nitrogen fixation in sorghum was previously studied through
509 ARA, ^{15}N isotope dilution, and ^{15}N abundance assays [62,64,65]. This study used three
510 complementary approaches (ARA, $^{15}N_2$ gas feeding, and ^{15}N isotope dilution) to provide evidence
511 of efficient nitrogen fixation in sorghum aerial roots (**Figure 5**). While these methods can suffer
512 from inherent biases, they collectively point towards efficient nitrogen fixation in the sorghum
513 mucilage and the acquisition of this nitrogen by the plant. The mucilage offers a favorable
514 environment for diazotrophs; therefore, obtaining mucilage or mucilage-producing plants devoid
515 of diazotrophs can be challenging. Thus, for these experiments, rather than using non-inoculated
516 mucilage or plants as negative controls, we favored inoculation with non-fixing mutants to exclude
517 as much as possible diazotrophs from the environment. ARA revealed that diazotrophs can actively
518 fix nitrogen in this environment (**Figure 5A**). However, ARA cannot prove that the plant can
519 acquire this nitrogen. This was demonstrated in $^{15}N_2$ gas feeding and ^{15}N isotope dilution (**Figures**
520 **5B and 5C**). Previous field studies using ^{15}N isotope dilution in sorghum suggested that
521 diazotrophs can provide between 36 and 78 % of nitrogen depending on the edaphoclimatic
522 conditions [62]. In this study using ^{15}N isotope dilution in greenhouse conditions, we observed that
523 sorghum with aerial roots can acquire up to 43% of the atmospheric nitrogen (**Figure 5C**). ^{15}N

524 dilution can be biased due to the segregation of ^{15}N within the soil, and different plants may acquire
525 nitrogen from different soil layers. In our case, the sorghum root system filled the pots, acquiring
526 nitrogen from all the soil layers and alleviating this concern. However, we should be careful to
527 extrapolate results from greenhouse experiments to field conditions where the environment will
528 undoubtedly positively or negatively affect this plant-microbe relationship.
529
530 *Sorghum,maize and their relatives with abundant and thick aerial roots as promising avenues to*
531 *reduce the use of synthetic nitrogen fertilizers*
532 Farmers in Oaxaca, Mexico, have preserved and cultivated the Sierra Mixe maize landraces
533 for centuries. Field experiments indicated that soils with more than ten years of maize
534 cultivation showed higher %Ndfa values than those with zero to one year of maize history.
535 This consistent cultivation has promoted biological nitrogen fixation in these maize
536 landraces[13]. Consequently, this selection process has significantly enhanced biological
537 nitrogen fixation. To our knowledge, the sorghum accessions we identified have undergone
538 minimal selection for biological nitrogen fixation, which may explain the differences in
539 %Ndfa between sorghum and maize. By adopting modern cultivation practices, we may
540 achieve increased levels of %Ndfa in sorghum.
541
542 Sorghum is renowned for its resilience to drought, which might restrict the areas suitable
543 for exploiting biological nitrogen fixation from aerial roots with mucilage. In maize,
544 drought-adapted cultivars can release mucilage that retains water effectively [66].
545 Therefore, sorghum accessions that produce aerial roots and that originate from drought-

546 prone regions may also produce mucilage under drought conditions. However,
547 (sub)tropical regions or areas with high relative humidity might be ideal for these sorghum
548 accessions.
549
550 This study examined the diversity within the minicore and ICRISAT sorghum collections
551 and identified accessions that developed aerial roots. Sorghum, maize, and sugarcane are
552 all members of the Andropogoneae tribe, and genera from the Paniceae tribe (Setaria and
553 Pennisetum) are known to develop brace roots [18]. In sugarcane, thin roots emerging from
554 nodes have been observed [67]. During a screening of Setaria, we discovered *Setaria italica*
555 and *Setaria magna* accessions with thin aerial roots lacking mucilage. Additionally, we
556 found that Johnsongrass (*Sorghum halepense*) and sudangrass (*Sorghum bicolor* ×
557 *Sorghum arundinaceum*) developed aerial roots with mucilage (**Supplementary Figure**
558 **6**). Notably, these grasses exhibited thicker roots and mucilage. Our research indicates
559 thicker roots are necessary for mucilage production, as seen in maize and sorghum. Two
560 loci associated with aerial root diameter in sorghum were reported by Wolf et al. [39].
561 Based on the observations of aerial root formation in other grasses, additional screenings
562 of germplasm collections can provide more information on the genetic basis of the aerial
563 root formation and aerial root diameter.
564
565 To achieve efficient nitrogen fixation in cereals, synthetic biology offers exciting approaches
566 through transferring nitrogenase genes to plants, engineering root nodule symbiosis, or
567 remodeling associative diazotrophs [68,69]. While exploring these approaches is undeniably

568 valuable, it is important to explore the untapped potential present in the natural diversity of plants
569 to identify and breed superior plant hosts for diazotrophs using non-transgenic methods. Certain
570 maize landraces in Sierra Mixe can obtain significant amounts of nitrogen from the atmosphere
571 through their aerial roots. Our research suggests that a similar trait is present in sorghum, and
572 investigating the natural diversity in cereal crops for nitrogen fixation provides a near-term, non-
573 transgenic pathway to enhance biological nitrogen fixation in these crops. Leveraging our genetic
574 understanding of this trait, we can engage in breeding efforts in sorghum and maize to promote
575 aerial development and mucilage secretion [28]. These natural traits are quite promising to reduce
576 the dependence on synthetic nitrogen fertilizers for cereal crops and improve the sustainability of
577 our agriculture for food, feed, fodder and biofuel production.
578

579 ## Materials and methods

580 ### Plant material and phenotyping

581 Sorghum accessions were selected from the sorghum minicore collection [25] and augmented with
582 accessions kindly provided by Vetriventhan Mani and Vania C. R. Azevedo at the International
583 Crops Research Institute for the Semi-Arid Tropics (ICRISAT, Hyderabad, Telangana, India)
584 (**Supplementary Table 1**). The plants were cultivated in C1200 nursery pots containing Metro-
585 Mix® 360 SUN-COIR™ (Burton, Ohio) medium in the Walnut Street Greenhouses at the
586 University of Wisconsin-Madison with temperatures at 25 ˚C during the day and 17 ˚C at night.
587 The plants were started on a long daylight regimen (14/10), and once they were 1-1.5 m tall, they
588 were switched to short days (12/12) due to their photoperiod sensitivity. Relative humidity was set
589 at 20%. Sorghum plants were watered with municipal water or NPK fertilizer (20-10-20). At the
590 flowering stage, when aerial roots were visible on many nodes, the root diameter of three roots at

591 the top node was measured with a caliper, as well as the number of nodes forming aerial roots and
592 the number of aerial roots formed on the top node. Overhead water was applied, and 30 minutes
593 after the water application, the mucilage volume was quantified from three young and three old
594 aerial roots with similar lengths, of approximately 10 cm. Mucilage was collected into 2 ml tubes
595 by simply removing it from the base to the tip of the root. The same accessions were grown at the
596 University of Wisconsin-Madison West Madison Agricultural Research Station (43.0610329663,
597 -89.5325872441) and the University of Florida North Florida Research and Education Center -
598 Suwannee Valley near Live Oak, FL (30.313277, -82.902158) in 1.5 m rows, with 10 cm between
599 plants, and 76 cm between rows. Only the number of nodes and root diameter were phenotyped in
600 the field experiments.
601
602 **Greenhouse experiments on the influence of humidity levels**
603 Sorghum accessions IS23992 and IS24453 were grown in individual 6-gallon (23-L) pots
604 (Custom-Tainer 2800C-14") with Pro-Mix LP15 medium (Quakertown, Pennsylvania) at the
605 Walnut Street Greenhouses at the University of Wisconsin-Madison. Greenhouse temperature was
606 set at 28 ˚C during the day and 25 ˚C at night. Plants were grown under a light regime of 12-hour
607 photoperiod from 6 am to 6 pm. Greenhouses were kept at high (75%) or low relative humidity
608 30%, controlled with a humidifier system (Smart Fog, Reno, Nevada). The phenotypes evaluated
609 included number of nodes with roots, the number of roots at the top node, and the root diameter.
610 The measurements were obtained with the previously described procedure.
611
612 **Bacterial growth conditions**
613 Twelve strains were used for different experiments (**Supplementary Table 8**). *Azospirillum*
614 *brasilense* FP2 and FP10 (*nifA*$^-$) were kindly provided by Fábio Pedrosa and Emanuel M. de Souza

615 from UFPR-Brazil[41]. The *Klebsiella variicola* Δ*nifH* mutant was developed by Maya
616 Venkataraman (UW-Madison). *Azospirillum baldaniorum* Sp 245^T, *Klebsiella variicola* A3,
617 *Klebsiella michiganensis* A2, *Azorhizobium caulinodans* ORS571^T, *Stutzerimonas stutzeri* A1501,
618 *Paraburkholderia silvatlantica* SRMrh-20^T, *Azotobacter vinelandii* DJ, *Herbaspirillum*
619 *seropedicae* SmR1, *Azorhizobium caulinodans* ORS571 Δ*nifA* were grown in LB medium, except
620 for *A. vinelandii* DJ and *A. vinelandii* DJ100 Δ*nifH*, which were grown in Burk's sucrose
621 medium[70,71]. The cells were grown overnight at 30 ˚C while shaking at 130 rpm.
622
623 **Mucilage sugar composition**
624 Root mucilage from accessions IS11026, was collected from cut aerial roots of different sorghum
625 genotypes from the greenhouse experiment. The roots underwent a 24-hour water exposure in a
626 50 ml tube, and the mucilage from two separate experiments was sent to the Complex
627 Carbohydrate Center Research Center (CCRC) at the University of Georgia, Athens, USA.
628 Glycosyl composition analysis was performed by combined gas chromatography/mass
629 spectrometry (GC/MS) of the per-*O*-trimethylsilyl (TMS) derivatives from the monosaccharide
630 methyl glycosides produced by acidic methanolysis as described previously [13]. The analysis
631 included rabinose, rhamnose, ribose, fucose, xylose, glucuronic acid, galacturonic acid, mannose,
632 galactose, glucose, *N*-acetyl mannosamine, *N*-acetyl galactosamine, *N*-acetyl glucosamine were
633 measured but only monosaccharides that were detected are reported.
634
635 **Total free sugar quantification**
636 Mucilage was collected at two time points from the sorghum accession IS23992 grown under
637 greenhouse conditions. Benedict's reaction was used to quantify free sugars, as described in
638 Hernandez-Lopez *et al*.[27]. Benedict's reagent (700 µl) was incubated with sorghum mucilage

639 (30 µl) for 10 min at 95 ºC. Subsequently, the sample was centrifuged at 10,000 *g* for 5 min. The
640 supernatant was collected, and the absorbance at 740 nm was determined using a BioRad
641 SmartSpect^{TM} Plus spectrophotometer. Sugars were quantified using a calibration based on
642 solutions with different concentrations of D-glucose.
643
644 **Metabolomic analyses**
645 Metabolome analysis of mucilage samples from sorghum accessions IS29091 and IS2241, as well
646 as maize landraces CB017456 and GRIN 19897, was conducted at the West Coast Metabolomics
647 Center at UC Davis following their standard protocols (https://metabolomics.ucdavis.edu/). A
648 minumum of 75 mg of mucilage was sent per sample (Supplementary Table 3). A primary
649 metabole analysis was performed to detect sugar phosphates, amino acids, hydroxyl acids, free
650 fatty acids, purines, pyrimidines, aromatics compounds, and unidentified compounds. Metabolites
651 were quantified by peak heights, normalized, and processed with MetaboAnalyst[72]
652 (https://www.metaboanalyst.ca/). This algorithm offers several different methods. Sparse partial
653 least squares - discriminant analysis and significance analysis of microarray were used to analyze
654 the data.
655
656 **Microscopy**
657 Border cells from underground roots were collected from the root tip of six-day-old seedlings from
658 sorghum accession IS23992 that grew in 1.2% agar at 24 ºC in a growth chamber. Border cells
659 from aerial roots were collected from mucilage produced from the humidity study. The mucilage
660 was triggered by spraying the aerial roots directly with municipal water 5 times every 5 minutes.
661 The mucilage was collected after 1 hour after the final spraying. Approximately 5 µl of mucilage
662 were spread on a slide and combined with 5 µl of distilled water, 10 µl of SYTO green (5 µM),

663 and 10 µl of propidium iodine (10 µg/mL). The cell suspension was observed under a Leica DMi8
664 model S inverted fluorescence microscope. GFP and mCherry filters were used to examine SYTO
665 green and propidium iodide fluorescent signals, respectively. Light microscopy was used to obtain
666 images of border cells from both underground and aerial roots. ImageJ - Fiji (version 2.9.0) [73]
667 was used to measure the area and length of the border cells.
668

669 **Microbiome study and 16S rRNA amplicon sequencing**

670 DNA was isolated from the mucilage samples from sorghum and maize using the FastDNA™
671 SPIN Kit for Soil (MP Biomedicals, Irvine, California) following the manufacturer's instructions.
672 DNA was quantified using a Nanodrop One (Thermofisher Scientific, Waltham, Massachusetts).
673 The V3-V4 region of the *16S rRNA* gene was amplified with the standard bacterial primers 335F
674 and 769R [74] with Go*Taq* Mastermix (Promega, Madison, Wisconsin) according to the
675 manufacturer's instructions. Amplification reaction volumes were 50 µl containing 10 pmol
676 of each primer and 4 ng of template DNA. Thermocycler conditions were as follows: 95°C
677 for 1 minute, then 35 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s, with a final
678 step at 72°C for 5 min. PCR products were separated on an agarose gel and extracted using
679 the GeneJET Gel Extraction Kit (Thermofisher Scientific). Novogene (Santa Clara,
680 California, USA) conducted library preparation and sequencing. Amplicons were sequenced
681 with an Illumina paired-end platform generating 250 bp paired-end raw reads. OTUs
682 abundance was normalized using a standard sequence number corresponding to the sample
683 with the fewest sequences. Sequences exhibiting a similarity of ≥97% were grouped into
684 identical OTUs. A representative sequence was chosen for each OTU to undergo subsequent

685 annotation. Principal component and beta diversity analyses were performed based on these
686 normalized data. These data were processed with QIIME2 (Version 1.7.0) [75] and displayed
687 with R software (Version 2.15.3) package ggplot2 (Version 3.4.3.) [76]. Sequencing data are
688 available at the Short Read Archive of NCBI under BioProject: PRJNA1009066.
689

690 **Isolation of bacteria from sorghum samples**

691 Mucilage samples were collected from a West Madison Agricultural Research Station field trial in
692 2022 from mucilage after rain (**Supplementary Tables 5 and 7**). The sampling was conducted
693 after a continuous rainy weekend with an accumulated rainfall of around 60 mm. In the field, we
694 looked for homogeneous mucilage production in the sorghum association panel [28,39]. A sample
695 was composed of a mix of two replicates from the panel. Mucilage (2-3 mL per plant) was collected
696 with a 1-mL pipette in a 15 mL tube. The samples were kept cold (0 ºC) until analysis. The
697 mucilage samples were added to BMGM semisolid medium and incubated at room temperature
698 for one week [77]. After that, the microaerophilic pellicles that formed were transferred to a fresh
699 medium and incubated for the same period seven more times. After the final incubation period,
700 solid BMGM medium supplemented with 0.5 g/l of yeast extract was inoculated with the bacteria.
701 A total of 164 isolates were screened by clonal strains using the Box-PCR approach with the A1
702 primer (5′-CTACGGCAAGGCGACGCTGACG-3′) and subjected to agarose gel electrophoresis
703 (1.2%) [78]. Identical profiles were excluded, and 103 non-clonal strains were kept. The bacterial
704 strains were identified by sequencing 16S rRNA genes using the primers 27F (5'-
705 AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')[79]. The
706 PCR was initiated with a denaturation cycle at 95°C for 10 minutes, followed by 35 cycles
707 consisting of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension

708 at 72°C for 1 minute and 30 seconds. The process concluded with a final extension at 72°C for 10
709 minutes. PCR product was sequenced in an Applied Biosystems 3500 genetic analyzer at the
710 Functional Biosciences facilities. Sequence Scanner v.2.0 (Informer Technologies, Inc.) was used
711 for quality assessment, and the contigs of almost complete 16S rRNA sequences were assembled
712 in BioEdit v.7.7[80]. The sequences were compared against those deposited within the 16S rRNA
713 reference database of GeneBank using the BLASTn tool [81]. The maximum likelihood
714 phylogenetic tree was constructed using MEGA 11 software [82]. The aligning used 103 different
715 16S rRNA gene sequences from the bacteria isolated from the sorghum mucilage.
716

717 **Acetylene reduction assays (ARA) to quantify nitrogenase activity**

718 Nitrogenase activity was evaluated in ten bacterial strains (**Supplementary Table 8**) grown in
719 sorghum mucilage collected from the greenhouse. The bacteria were grown in 3 ml of LB medium
720 overnight at 30 ºC and shaken at 180 rpm. The culture was centrifuged at 5000 *g* for 5 minutes,
721 and the pellet was resuspended in 1 ml of sterile water. Then 30 µl (OD_{600nm} = 0.1) were inoculated
722 in sterile vials containing 3 ml of mucilage. Vials were capped and incubated for 24 hours at 30
723 ºC. The cap was replaced with a crimp cap, and 1 ml of air was replaced with acetylene, followed
724 by a 3-day incubation period at 30 ºC. Gas Chromatography - Flame Ionization Detection was
725 performed in a GC-2010 (Shimadzu) with an HS20 autosampler by injecting 1 ml of air sample.
726 The injector and the detector were set at 200 ºC with a flow rate of 86.6 ml/min nitrogen carrier
727 gas. The temperature of the column was 100 ºC. Controls included a vial with acetylene to check
728 for traces of ethylene and *A. brasilense* FP2 without fixing activity. The indirect measure of the
729 nitrogenase activity was expressed in nmol of ethylene produced per hour per mL of mucilage.
730 ARA in the 34 isolated diazotrophs was performed similarly but using a semi-solid BMGM
731 medium.

732

733 **$^{15}N_2$ gas feeding experiments**

734 Aerial roots from sorghum accession IS23992 were collected in 150 ml sterile flasks. These roots
735 were obtained from greenhouse-grown plants with copious amounts of mucilage. *K. variicola A3*
736 and the *K. variicola* Δ*nifH* mutant were grown overnight at 30 ºC and shaken at 180 rpm in LB
737 medium. The culture underwent centrifugation at 5000 *g* for 5 minutes, and the resulting pellet
738 was then resuspended in 1 ml of sterile water. Bacteria were directly inoculated onto the surface
739 of mucilage-coated roots (100 µl, OD_{600nm} = 0.1). Flasks were injected with $^{15}N_2$ gas (98%) to
740 reach a concentration of 15% (v/v), and flasks were incubated for three days at room temperature
741 (20ºC). Non-fixing bacteria were included as negative controls to confirm the absence of other
742 sources of ^{15}N in the system. The roots with mucilage were excised from the plant for practicality
743 and proper incubation in $^{15}N_2$ gas. To determine the ^{15}N enrichment specifically in plant tissue,
744 chlorophyll was extracted by peeling the green cell layers from the aerial roots after removal of
745 mucilage. The extraction followed the protocol described by Kahn *et al.* [83]. The samples were
746 analyzed by isotope ratio mass spectrometry (IRMS) at the Department of Soil Science at the
747 University of Wisconsin-Madison to evaluate the $\delta^{15}N$ of the samples.

748

749 **^{15}N isotope dilution experiments**

750 A total of 40 sorghum plants from each of the two accessions, IS23992 and IS24453, were grown
751 in 6-gallon (23-L) pots at the Walnut Street Greenhouses in two adjacent greenhouse rooms to
752 prevent contamination between the two types of bacterial inoculants (fixing and non-fixing). An
753 equal number of plants were placed in the two treatments, and standard humidity conditions were
754 set (relative humidity 50%) with a humidifier system (Smart Fog, Reno, Nevada). After four weeks

755 post-germination, pots were fertilized weekly with 500 ml of Hoagland's No.2 Basal Salt mixture
756 (1.34 g per litter) and 80% ammonium-^{15}N sulfate $(^{15}NH_4)_2SO_4$ as the only source of nitrogen.
757 Five weeks after germination, aerial roots of plants in one room were inoculated with a mixture of
758 nine fixing strains (OD_{600} = 0.1), and the aerial roots on the plants in the other room was inoculated
759 with three non-fixing strains (OD_{600} = 0.1) (**Supplementary Table 8**). Plants were sprayed with
760 250 ml of the inoculum resuspended in a phosphate-buffered saline (PBS) buffer using a backpack
761 sprayer. The third leaf counting from the top was collected before inoculation (stage 3) and then
762 three times every two weeks (stages 4 and 5). At flowering (stage 6), whole plants were collected,
763 ground, and homogenized to a fine powder . Quantification of the samples' ^{15}N content above the
764 atmospheric concentration of N_2 (Atom% ^{15}N excess) was measured by isotope ratio mass
765 spectrometry (IRMS) at the Department of Soil Science at the University of Wisconsin-Madison.
766 The values of whole-plant samples were averaged. The percentage of nitrogen derived from the
767 atmosphere (%Ndfa) was calculated according to the equation described in van Deynze *et al*.[13].
768 The fixers were the accessions inoculated with diazotrophs, whereas the references were those
769 inoculated with the mutant strains.
770
771 ***Setaria* growth conditions**
772 *Setaria* accessions PI43384 and PI689086 were cultivated at the Walnut Street Greenhouses under
773 high relative humidity (80%) using the same system as described for the humidity experiment with
774 sorghum. The plants were grown in 1-gallon (3.8-L) Pro Cal Premium Nursery Pots with Pro-Mix
775 LP15 medium from Quakertown, Pennsylvania. Plants were maintained at 28 ˚C during the day
776 and 25 ˚C at night, with a 12-hour photoperiod from 6 am to 6 pm. The phenotype evaluated
777 included the number of nodes with roots.
778

Raw data

All raw data are available at DOI: 10.6084/m9.figshare.25193696

Statistical analyses

All statistical analyses were conducted in R version 4.2.1 and 2.15.3[84]. ANOVA and Tukey's HSD were conducted with the package ggpubr (Version 0.6.0) and multcompView (Version 0.1-9). Wilcoxon's test was also calculated with package ggpubr (Version 0.6.0). Microbiome studies were performed with QUIIME2 (Version 1.7.0) [85].

Acknowledgments

We thank Sanhita Chakraborty and Kimberly Gibson for their valuable input and engaging discussions, 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 Janet Hedtcke, the field manager at UW-Madison Agricultural Research Station. We thank Maya Venkataraman and Brian F. Pfleger for providing the *K. variicola* Δ*nifH* strain, David Brenner for providing us with seeds from different *Setaria* accessions, and Daniel Carmeli for providing pictures from Johnsongrass and sudangrass. Funding for this project was provided by the United States Department of Energy (DOE), Office of Science (BER), grant no. DE-SC0021052 (JMA, WV).

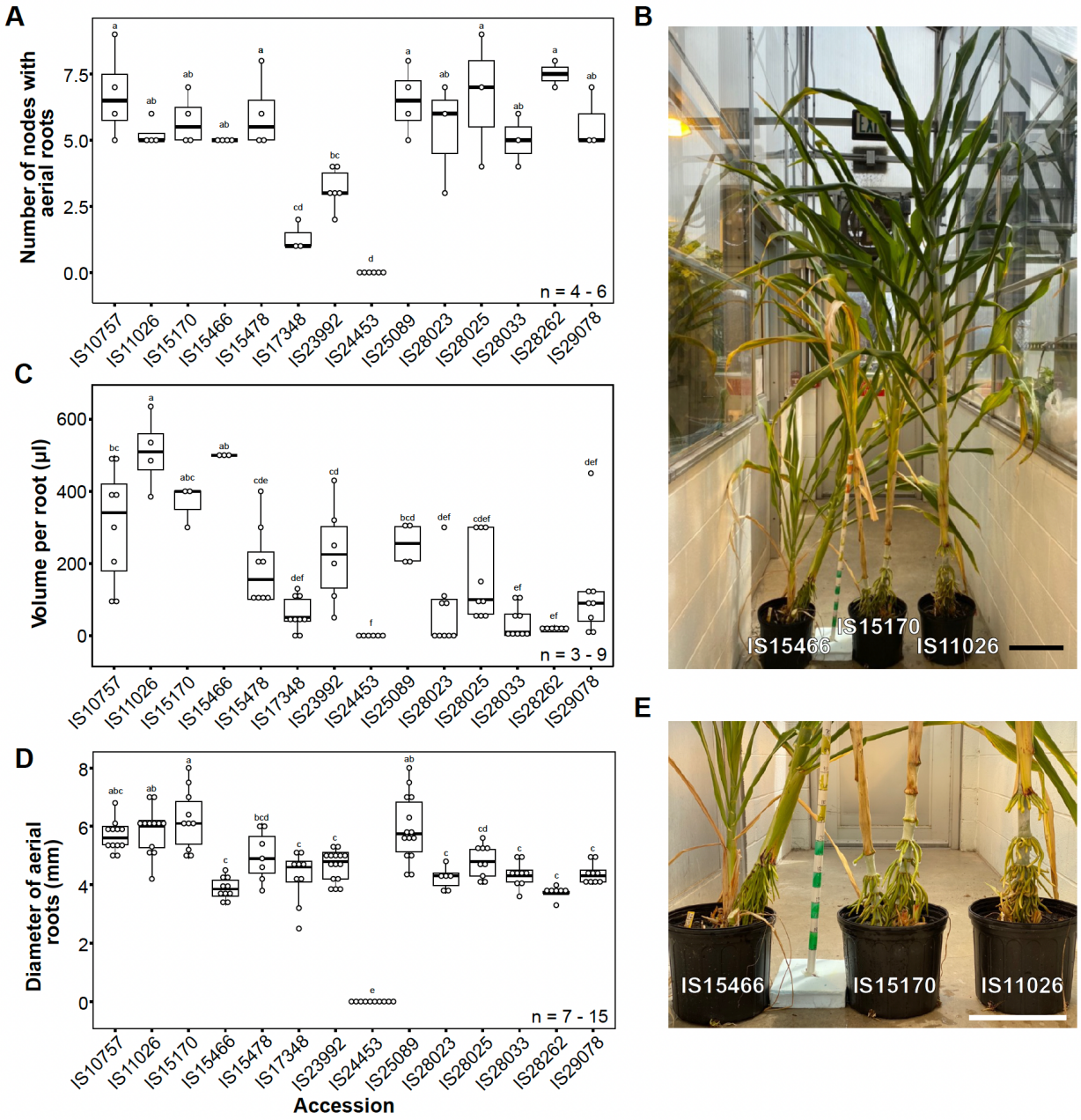


Fig 1. Sorghum screening of selected accessions under greenhouse conditions.

Different sorghum accessions display diverse traits associated with aerial roots and mucilage production. A) Number of nodes in different sorghum accessions (n = 3-6). Most of the accessions evaluated showed more than 5 nodes with aerial roots. B) Representative image of selected sorghum accessions. Scale bar = 28 cm. C) Volume of mucilage per root (µl) produced by different sorghum accessions (n = 4-9). The production of mucilage varied among all the accessions. D)

807 Diameter (mm) of aerial roots in different sorghum accessions (n = 6-15). Thick roots with
808 diameters bigger than 4 mm were noted in nearly all the accessions. E) Aerial roots of the sorghum
809 accessions in D. Scale bar = 28 cm. An ANOVA (F-test) to calculate significant differences
810 between means was conducted with the package multcompView (0.1-10).
811

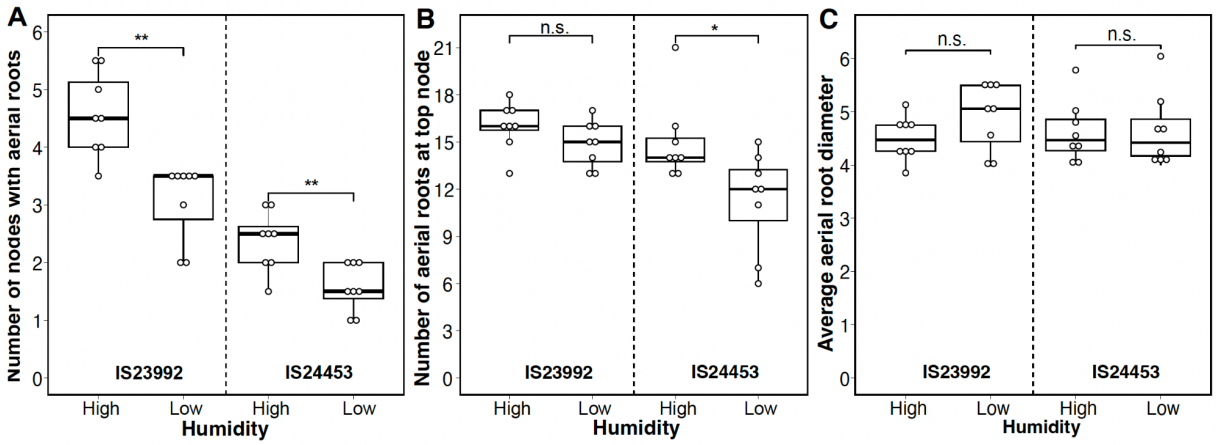


Fig 2. Effect of humidity in sorghum accession IS23992 and IS24453.

Boxplots of different phenotypes measured in the sorghum accessions under high (75%) and low (30%) relative humidity. A) Number of nodes with aerial roots (n = 8). The number of nodes with aerial roots significantly increased in both accessions. B) Number of roots on the top node (n = 8). Only IS24453 increased the number of roots at the top node. C) Average root diameter on the top node (n = 8). The root diameter was not affected by the humidity in both accessions. A Wilcoxon test was performed in R (Ver 4.2.1) with the package ggpubr (Ver 0.6.0). Significance levels * p-value ≤ 0.05, ** p-value ≤ 0.01, and n.s. not significant.

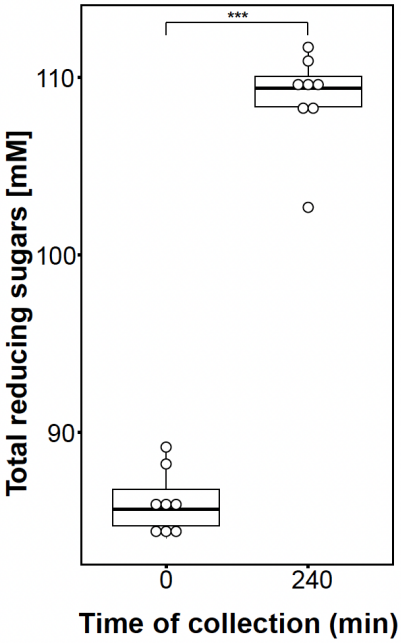
A

B

Monosaccharide	Amount *S. bicolor* (%)[1]	Amount *Z. mays* (%)[2]
Arabinose (Ara)	20.22	13.7
Fucose (Fuc)	15.23	41.8
Xylose (Xyl)	2.66	3.3
Glucuronic acid (GlcA)	7.4	2.7
Mannose (Man)	6.4	2.7
Galactose (Gal)	47.16	35.8
Total	100	100

[1] Data obtained from the average of two biological replicates
[2] Data from Van Deynze *et al.* 2018

C



D

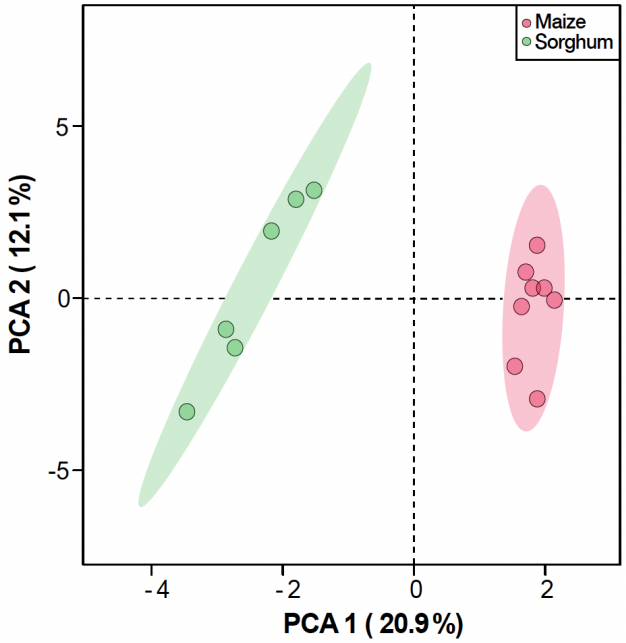


Fig 3. Sorghum aerial root mucilage composition.

Sorghum aerial roots release mucilage . A) Mucilage production is triggered by water and under high-humidity conditions. The mucilage from different accessions appears similar. Accession IS2392 serves as a representative example of sorghum mucilage. Scale bar (5 cm) B) Monosaccharide composition of sorghum accession IS11026 and Sierra Mixe maize mucilage. Both mucilages consist of the same monosaccharides but in different proportions (mole %). C)

829 Concentration of reducing sugars in sorghum mucilage from the accession IS23992 determined
830 with benedict's reaction. A Wilcoxon test was performed to compare means *** p-value ≤ 0.001
831 (n = 8). D) Principal component analysis using Sparse Partial Least Squares. Two clusters are
832 depicted based on the species studied: sorghum accessions IS29091 and IS2245 and maize
833 landraces CB017456 and GRIN 19897.
834

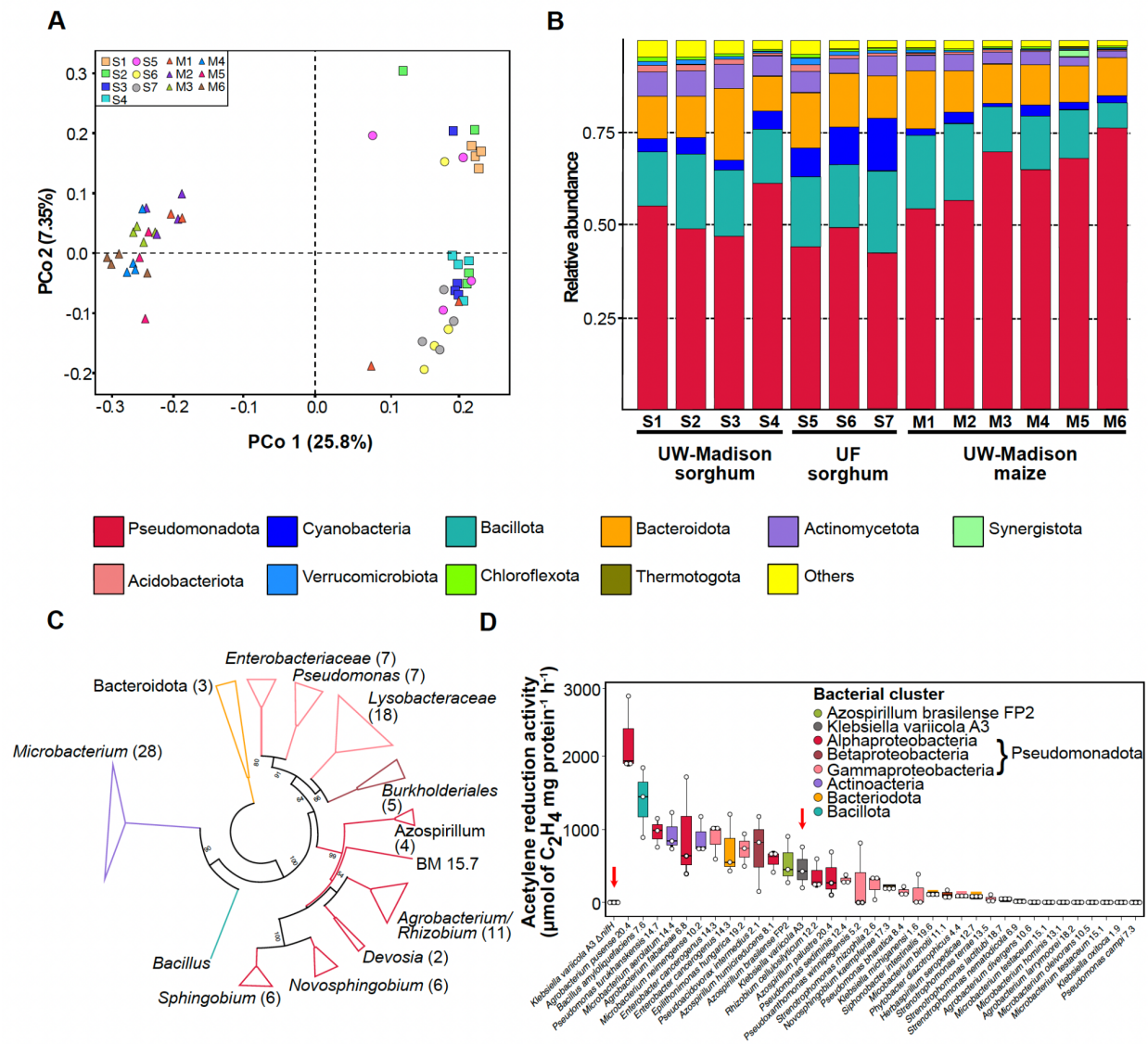


Fig 4. Microbiome profile of sorghum mucilage.

The community profile shows the relative abundance of bacteria present in sorghum (S) and maize (M) mucilages. Samples S1 to S4 (squares) and M1 to M6 (triangles) were collected from the field at the University of Wisconsin-Madison (UW-Madison), and samples S5 to S7 (circles) were collected at the University of Florida (UF). A) Principal coordinates analysis plot of sorghum and maize samples based on 16S amplicon sequences. B) Relative abundance of sorghum and maize samples based on 16S amplicons and OTUs. C) Unrooted maximum-likelihood phylogenetic tree

843 of 103 bacteria isolated from sorghum aerial roots mucilage in BMGM nitrogen-free semisolid
844 medium. The evolutionary distances were computed using the Jukes-Cantor method and are in the
845 units of the number of base substitutions per site. This analysis involved 103 nucleotide sequences.
846 All positions containing gaps and missing data were eliminated (complete deletion). There were
847 908 positions in the final dataset. Numbers at nodes are bootstrap values (1000 replications, values
848 < 50% are not shown). Colors: Red: Gammaproteobacteria, Purple: Betaproteobacteria, Blue:
849 Alphaproteobacteria, Brown: Bacillota, Green: Actinomycetota, Yellow: Bacteroidota. D)
850 Nitrogenase activity of 34 isolated from sorghum aerial roots mucilage in BMGM nitrogen-free
851 semisolid medium (n=3). The red arrows show the reference strains for comparison. Bacteria:
852 *Klebsiella variicola* A3 Δ*nifH*. Species codes (alphabetic order): *Agrobacterium divergens*,
853 *Agrobacterium fabaceae*, *Agrobacterium larrymoorei*, *Agrobacterium pusense*, *Azospirillum*
854 *brasilense*, *Azospirillum humicireducens*, *Azospirillum palustre*, *Bacillus amyloliquefaciens*,
855 *Enterobacter cancerogenus*, *Epilithonimonas hungarica*, *Herbaspirillum seropedicae*, *Klebsiella*
856 *michiganensis*, *Klebsiella oxytoca*, *Klebsiella variicola*, *Microbacterium aerolatum*,
857 *Microbacterium binotii*, *Microbacterium hominis*, *Microbacterium neimengense*, *Microbacterium*
858 *oleivorans*, *Microbacterium testaceum*, *Novosphingobium kaempferiae*, *Phytobacter*
859 *diazotrophicus*, *Pseudoacidovorax intermedius*, *Pseudomonas bharatica*, *Pseudomonas campi*,
860 *Pseudomonas lutea*, *Pseudomonas sediminis*, *Pseudomonas turukhanskensis*,
861 *Pseudoxanthomonas winnipegensis*, *Rhizobium cellulosilyticum*, *Siphonobacter intestinalis*,
862 *Stenotrophomonas lactitubi*, *Stenotrophomonas nematodicola*, *Stenotrophomonas rhizophila*, and
863 *Stenotrophomonas terrae*.
864

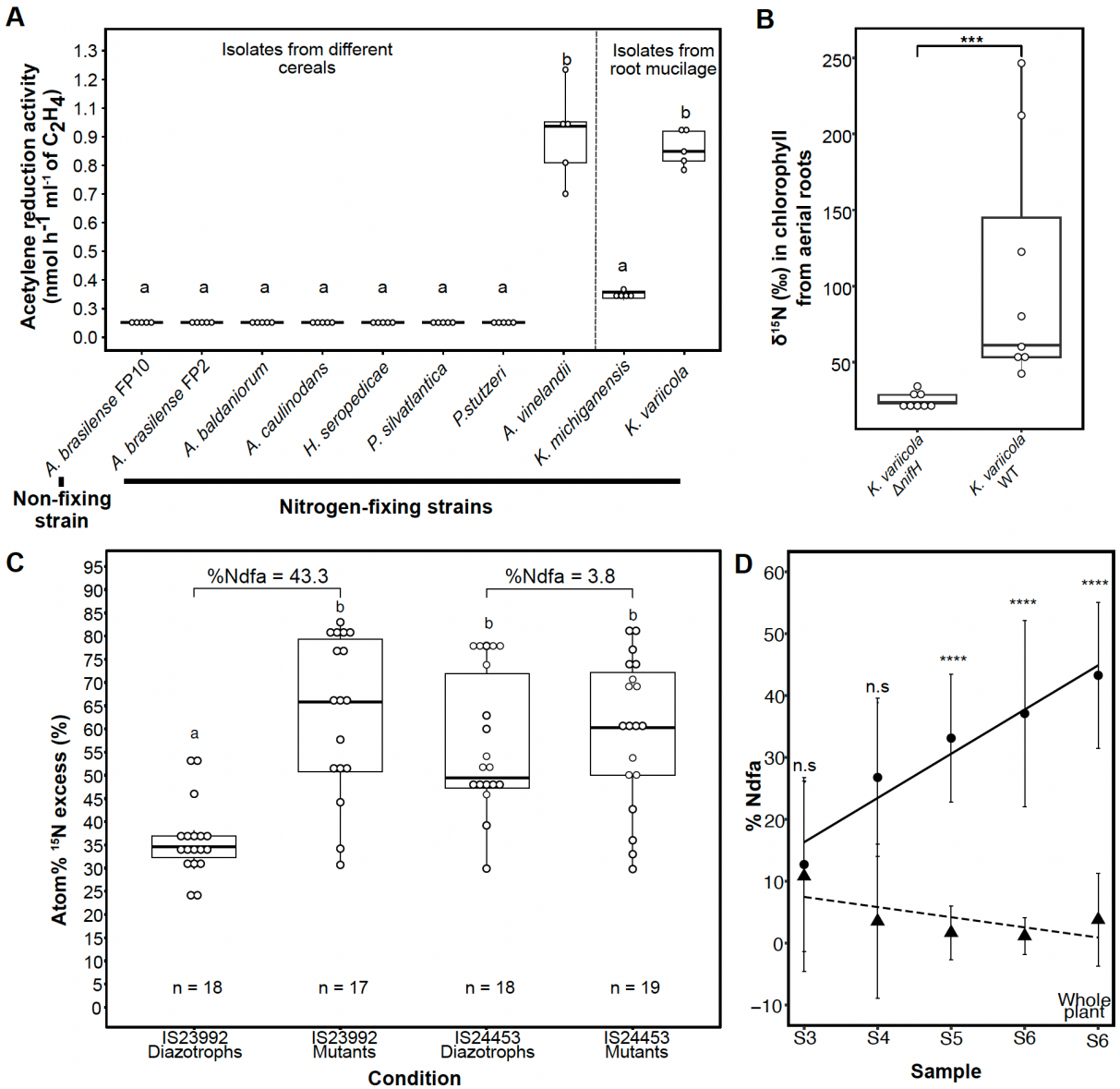
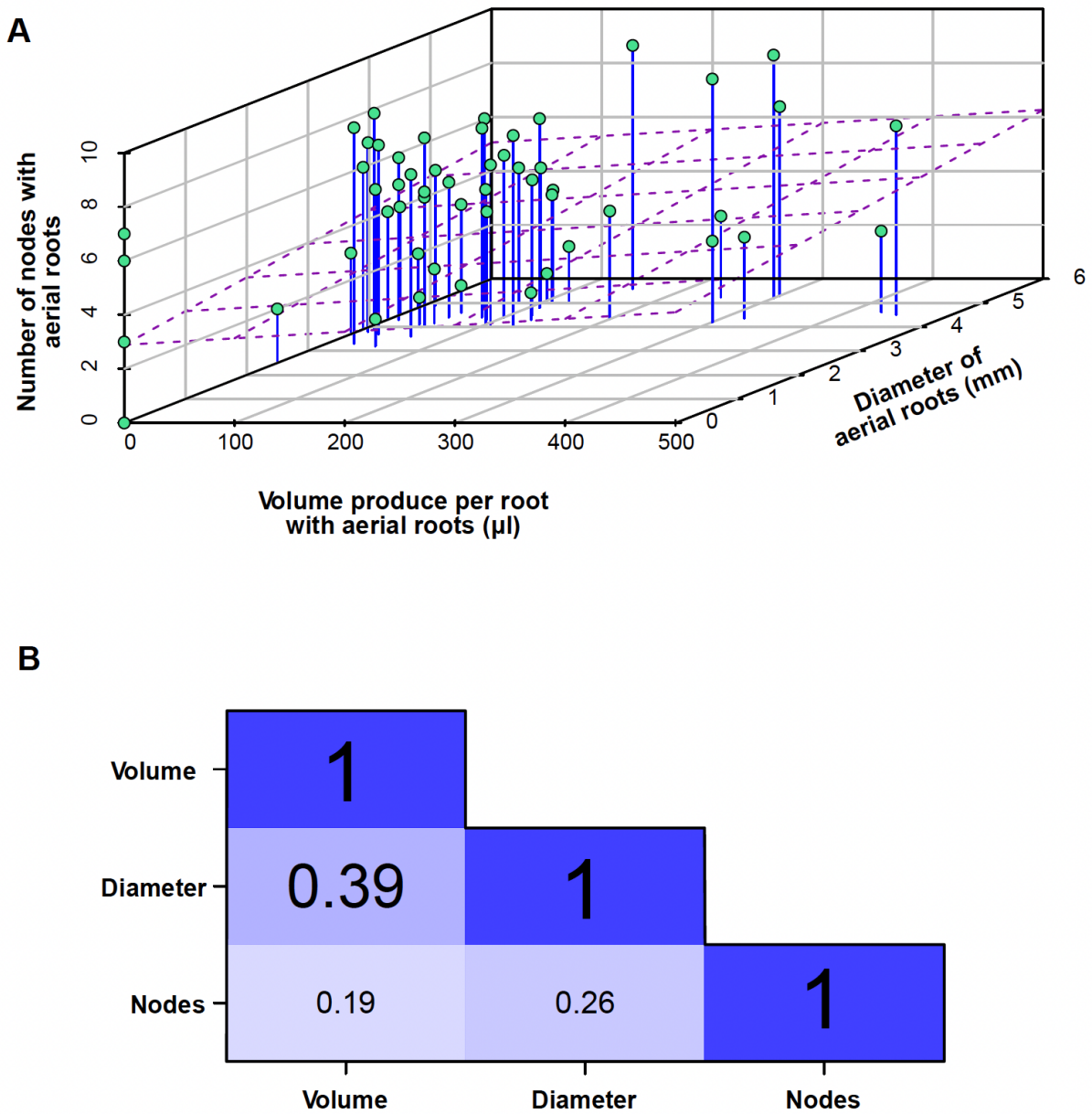


Fig 5. Biological nitrogen fixation on aerial roots from sorghum.

A) Acetylene reduction assay in mucilage generated in the greenhouse. Mucilage was used as media to grow 9 nitrogen-fixing strains (*Azospirillum brasilense* FP2, *Azospirillum baldaniorum*, *Azorhizobium caulinodans*, *Azotobacter vinelandii* DJ, *Herbaspirillum seropedicae*, *Klebsiella michiganensis*, *Klebsiella variicola* A3, *Paraburkholderia silvatlantica*, and *Pseudomonas stutzeri*) and *Azospirillum brasilense* FP10 as a non-fixing control. Tukey's honestly significant difference (HSD) test was performed in R, and significant group means are displayed as lower

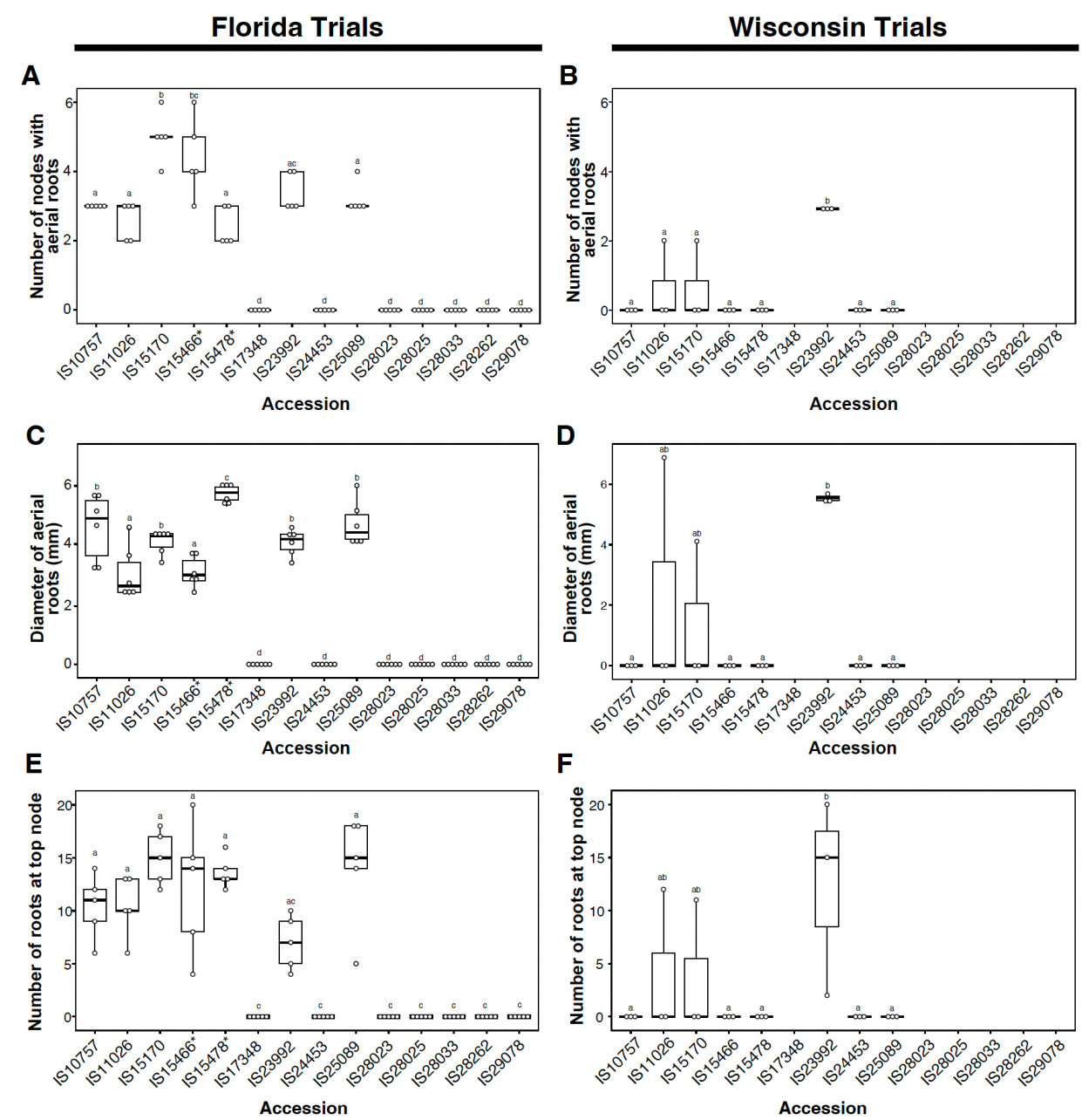
873 case letters (n = 6). B) Analysis of ^{15}N gas feeding experiment on aerial roots of sorghum accession
874 IS23992. Aerial roots were inoculated with *K. variicola* Δ*nifH* (negative control) or *K. variicola*
875 A3 (positive control). *** indicates p-value ≤ 0.001 based on Wilcoxon signed-rank test (n = 8).
876 C) ^{15}N isotope dilution experiment on aerial roots of sorghum accessions IS23992 and IS24453.
877 Accessions IS23992 and IS24453 were inoculated with the nine strains from the ARA experiment
878 (diazotrophs), and three non-fixing mutant strains *Azospirillum brasilense* FP10, *Azotobacter*
879 *vinelandii* DJ100 Δ*nifH*, *Azorhizobium caulinodans* ORS571 Δ*nifA*. Whole plants at the flowering
880 stage were ground, and the average of three replicates was plotted. Percent Ndfa was calculated
881 for both accessions using the plants inoculated with diazotrophs and using as reference the plants
882 inoculated with mutant strains. An ANOVA was performed to compare means between treatments
883 and genotypes using the package multcompView. D) Scatterplot of the percentage of nitrogen
884 derived from the atmosphere (%Ndfa) in different sorghum samples from the accessions IS23992
885 and IS24453, indicated with circles and triangles, respectively. Solid and dotted lines are
886 regression lines for IS23992 and IS24453, respectively. Samples were collected every two weeks,
887 starting at stage 3 (Samples S3 to S5) and after flowering (S6). Error bars display standard
888 deviations. Pair-wise comparisons were made with Wilcoxon's test;**** means p-value ≤ 0.0001
889 and n.s. Indicates non-significant.
890



Suppl. Fig 1. Trait correlations.

A) Scatter plot shows the correlation between the number of nodes, mucilage volume, and diameter. A 3D regression line is dot plotted (purple) to observe the correlation among the traits. B) The correlation matrix for the number of nodes, mucilage volume, and diameter reveals a modest positive correlation across all traits. The correlation coefficient (r) values indicate the

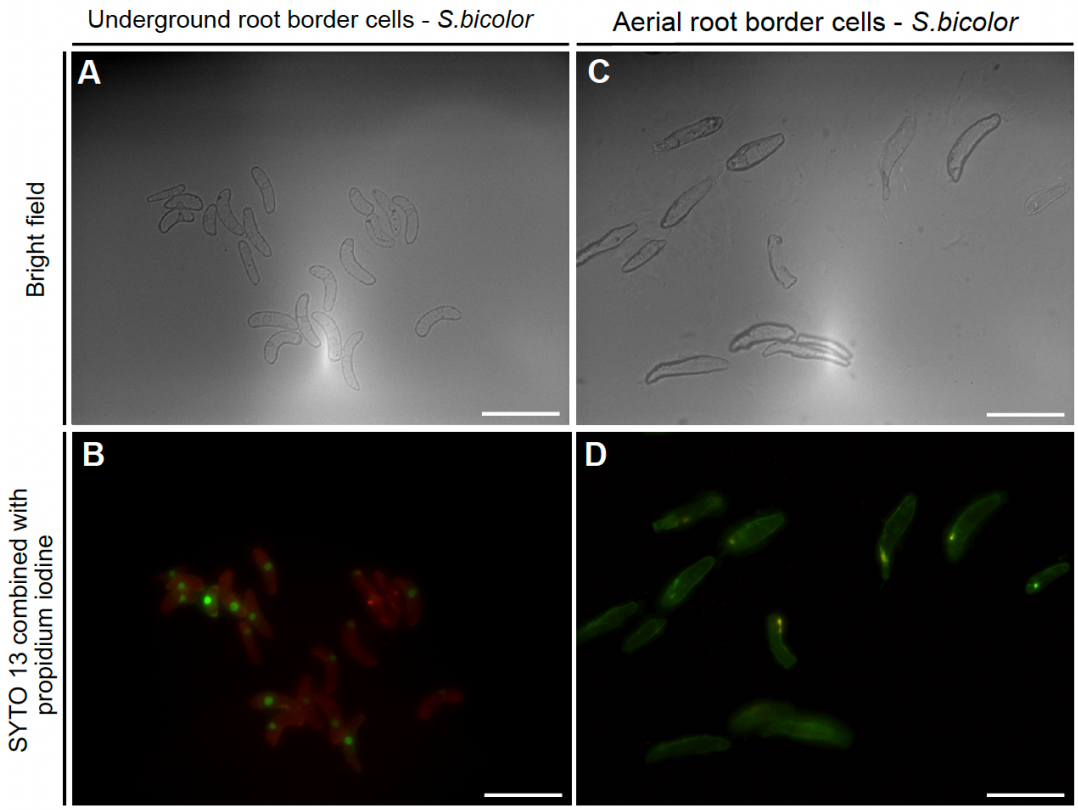
correlation strength between each trait. The correlation plot and values were calculated with the packages “scatterplot3d” [86] and “psych” [87], respectively.

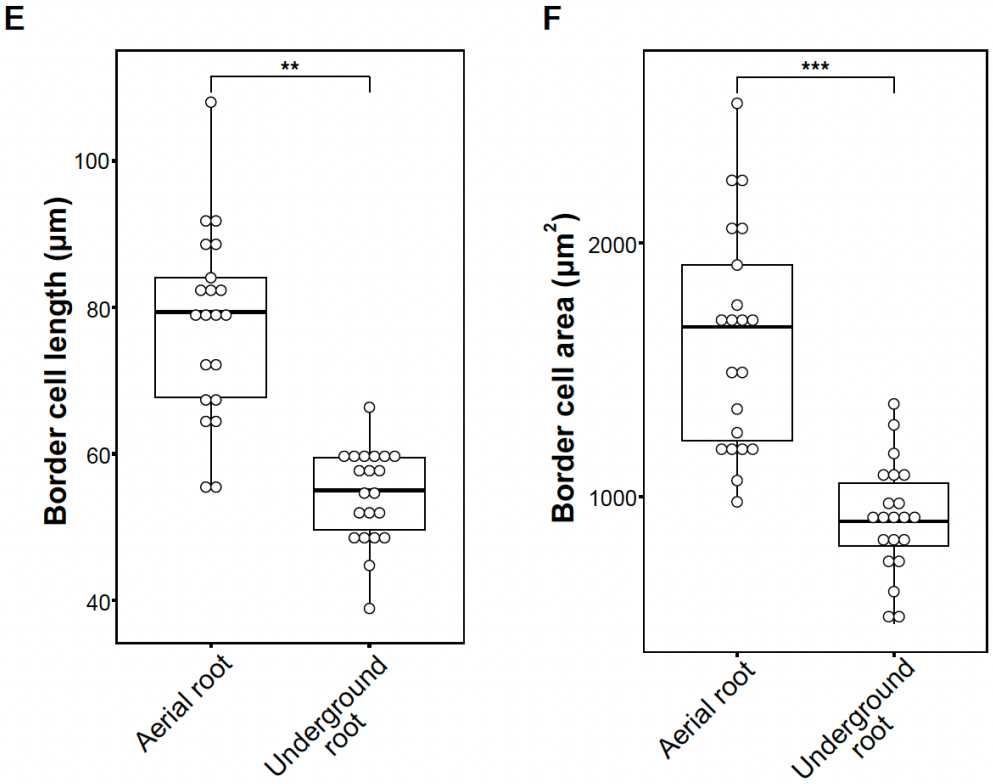


Suppl. Fig 2. Sorghum screening of selected genotypes in two locations.

A) Number of nodes in different sorghum accessions grown at the University of Florida during 2021 (n = 5). Generally, two groups can be observed, one with nodes with aerial roots and another without. B) Number of nodes in different sorghum accessions grown at the University of Wisconsin during 2022 (n = 3). Only specific accessions developed fewer aerial roots compared

with the conditions in Florida. C) Diameter (mm) of aerial roots of different sorghum accessions grown at the University of Florida (n = 5). The accessions with aerial roots have thick diameters. D) Diameter (mm) of aerial roots in different sorghum accessions grown at the University of Wisconsin during 2022 (n = 3). Thickness was still observed in the accession with aerial roots. E) Number of aerial roots at the top node in different sorghum accessions grown at the University of Florida (n = 5). F) Number of aerial roots at the top node in different sorghum accessions grown at the University of Wisconsin during 2022 (n = 3). * Data from these accessions were obtained under a low nitrogen regimen. A subset of data for the Florida plots were obtained from the Wolf *et al.* study [28].


Underground root border cells - S.bicolor
Aerial root border cells - S.bicolor
Bright field
SYTO 13 combined with propidium iodine
A
B
C
D

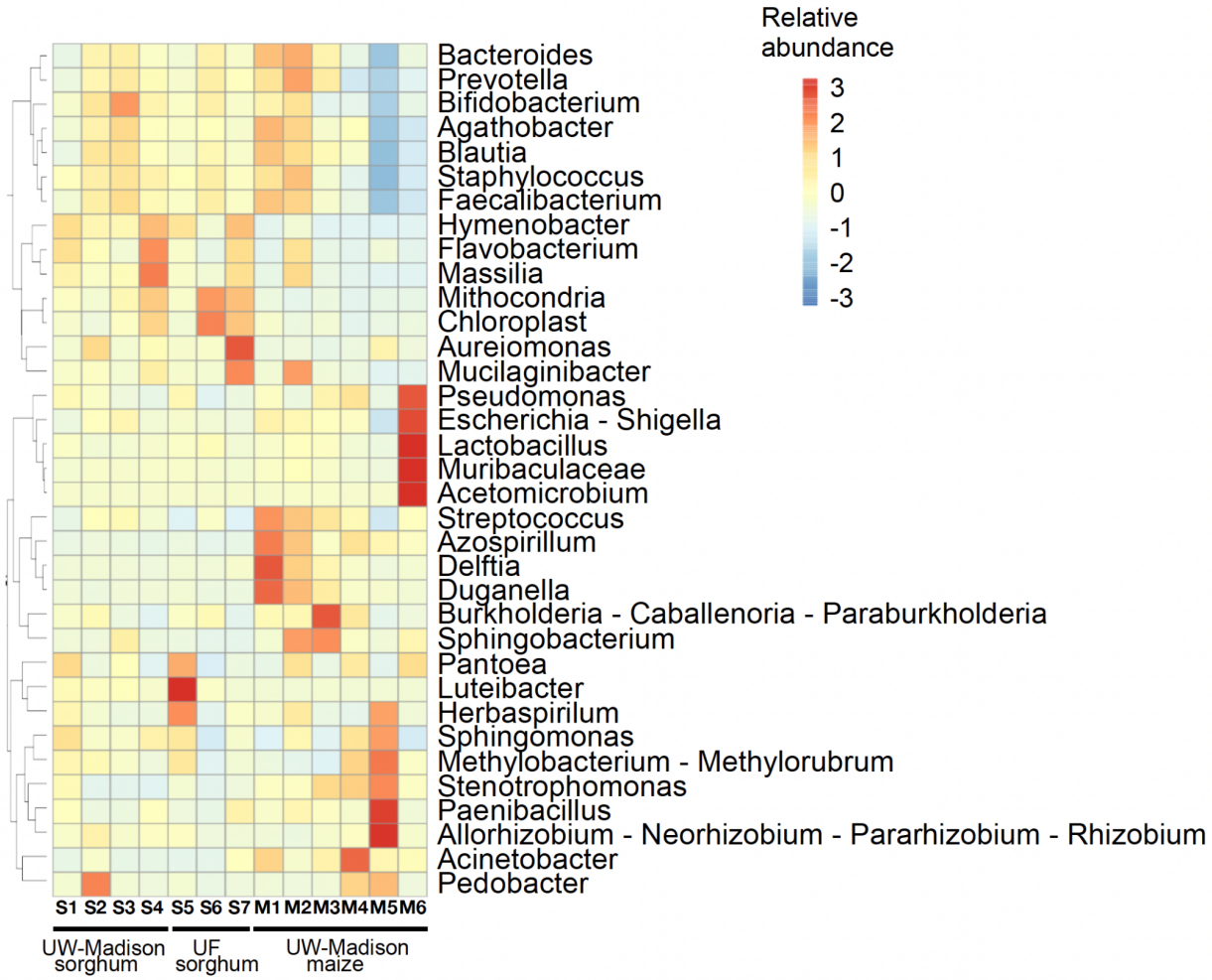

E
F
Border cell length (µm)
Border cell area (µm²)
**
***
100
80
60
40
2000
1000
Aerial root
Underground root

916

917 **Suppl. Fig 3. Border cells in sorghum mucilage.**

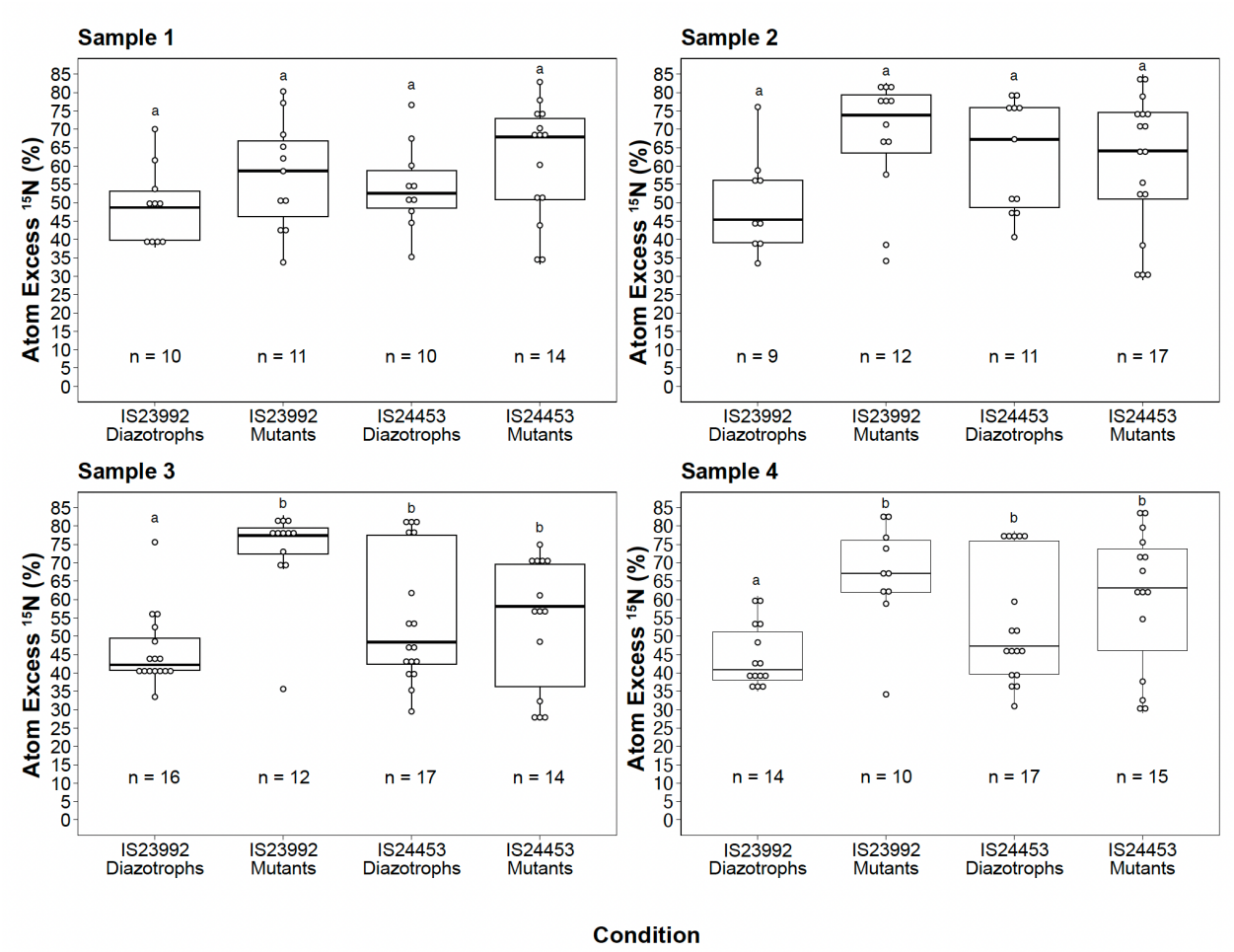
918 A) Border cells from sorghum underground roots (S=scale bar 100 μm). Border cells have a bean
919 shape. B) Border cells from sorghum aerial roots (scale bar 100 μm). Irregular cells are observed
920 in a more elongated form. C) Live-dead viability staining in border cells from sorghum
921 underground roots (scale bar 100 μm). The presence of live cells was recorded using the green
922 fluorescence of their nuclei. D) Live-dead viability staining in border cells from sorghum aerial
923 roots (scale bar 100 μ m). Live cells were identified using the green fluorescence of their nuclei.
924 E) Length comparison between border cells in μm. Border cells from mucilage were more
925 elongated than border cells from underground roots. F) Area comparison between border cells in
926 μm^2. ** and *** denote $p < 0.01$ and < 0.001, respectively, based on a Wilcoxon test ($n = 20$).

927



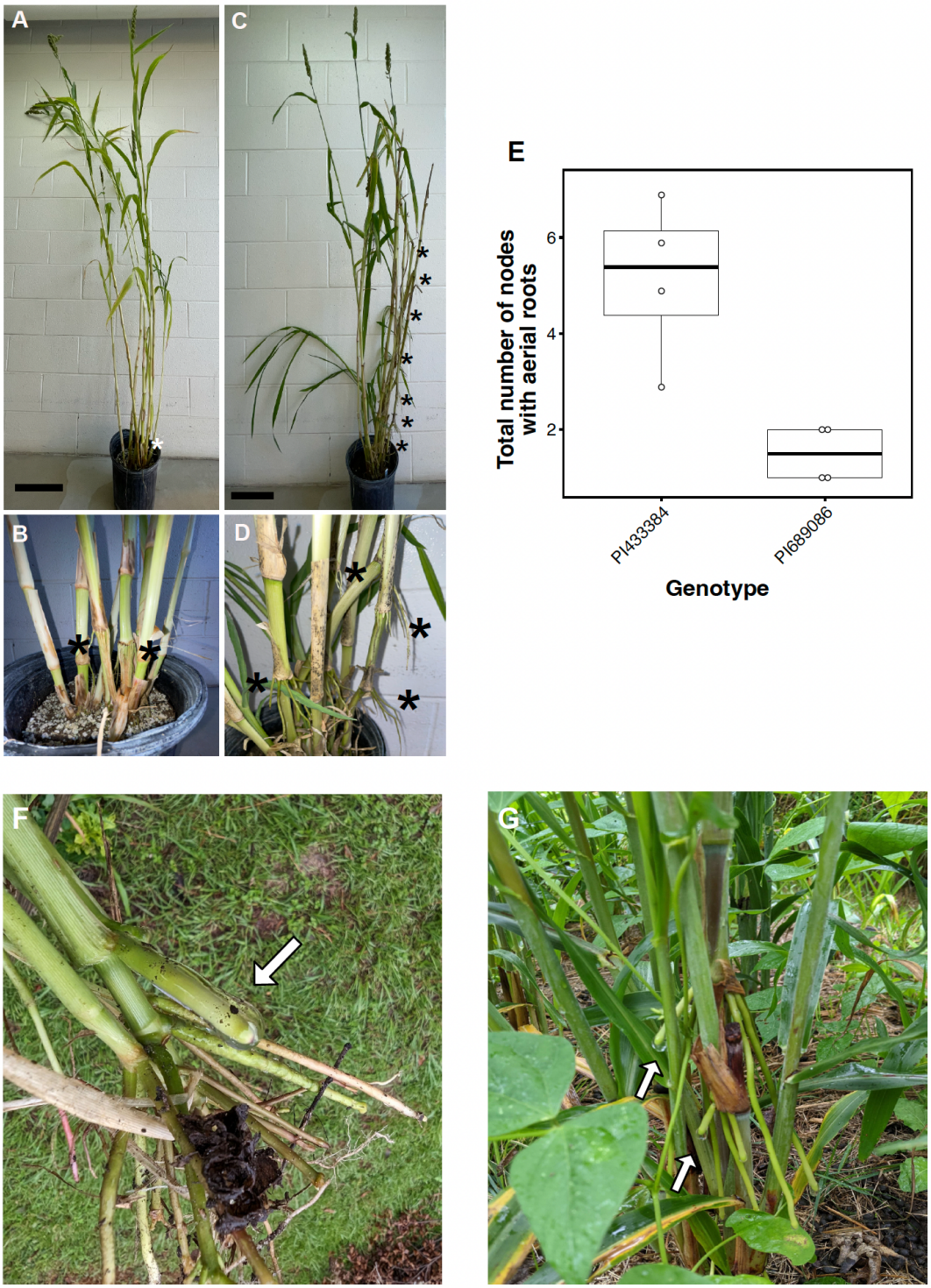
Suppl. Fig 4: Bacterial genera from different sorghum and maize mucilage.

The hierarchical clustering heatmap represents the microbiome community composition at the genus level for 13 samples obtained from the mucilage of sorghum (S) and maize (M). The samples have been grouped through clustering based on their community composition. The microbiome of each sample was obtained from different genotypes and locations, thus showing unique profiles.



Suppl. Fig 5. ^{15}N isotope dilution experiment on aerial roots of sorghum accessions from leaf samples.

Accessions IS23992 and IS24453 were inoculated with the nine strains from the ARA experiment (diazotrophs), and three mutant strains *Azospirillum brasilense* FP10, *Azotobacter vinelandii* DJ100 Δ*nifH*, *Azorhizobium caulinodans* ORS571 Δ*nifA*. The third topmost leave was analyzed. The samples were collected before inoculation (sample one - stage three) and every two weeks (sample 3 to 4 - stage 4 to 6). An ANOVA was conducted using the package multcompViewto determine the factors contributing to differences in means.



Suppl. Fig 6. Aerial roots in other grass species

A preliminary screening in *Setaria* (*Setaria italica* and *Setaria magna*) and other species of the Sorghum clade revealed the presence of aerial roots. A) *Setaria magna* accession PI689086

displayed few aerial roots. Scale bar: 20 cm. B) Close-up of an aerial root at the base of *Setaria italica* accession PI689086. C) The *Setaria magna* accession PI433384 exhibited more aerial roots. Scale bar: 20 cm. D) Close-up of an aerial root at the base of *Setaria magna* accession PI433384. E) Quantification of aerial roots in Setaria accessions PI433384 and PI689086. F) Johnsongrass (*Sorghum halapense*) grown in Tallahassee, Florida, displayed aerial roots with mucilage. G) Sudangrass (*Sorghum bicolor* × *Sorghum arundinaceum*) grown in western North Carolina displayed aerial roots with mucilage. Asterisks mark the nodes with aerial roots; white arrows indicate aerial roots with mucilage.

958 **Supplementary Table 1.** Sorghum accessions to evaluate aerial root formation in greenhouse and

959 field experiments

960

Accession	Collection	Origin
IS10757	Mini core	Chad
IS11026	Mini core	Ethiopia
IS15170	Mini core	Cameron
IS15466	Mini core	Cameron
IS15478	Mini core	Cameron
IS17348	ICRISAT	Ethiopia
IS23992	Mini core	Yemen
IS24453	Mini core	South Africa
IS25089	Mini core	Ghana
IS28023	ICRISAT	Yemen
IS28025	ICRISAT	Yemen
IS28033	ICRISAT	Yemen
IS28262	ICRISAT	Yemen
IS29078	ICRISAT	Yemen

961

962

963 **Supplementary Table 2.** Mucilage sugar composition of accession IS11026 in two independent

964 experiments. Monosaccharide composition was determined by GC-MS. * data obtained from 1.64

965 mg sample; ** data obtained from a 2.16 mg sample; nd = not detected.

	Replicate 1*		**Replicate 2****	
Monosaccharide	**Amount (μg)**	**% by mole**	**Amount (μg)**	**% by mole**
Arabinose	163.9	19.77	254.4	20.66
Rhamnose	nd	-	nd	-
Ribose	nd	-	nd	-
Fucose	124.9	13.77	224.6	16.68
Xylose	21.1	2.54	34.1	2.77
Glucuronic Acid	75.9	7.08	121.8	7.65
Galacturonic acid	nd	-	nd	-
Mannose	67.8	6.81	87.1	5.89
Galactose	484.9	48.73	674.0	45.59
Glucose	12.9	1.29	11.4	0.77
N-Acetyl Mannosamine	nd	-	nd	-
N-Acetyl Galactosamine	nd	-	nd	-
N-Acetyl Glucosamine	nd	-	nd	-
Total	951.4	100	1,407.4	100

966

967

968 **Supplementary Table 3.** Mucilage samples from maize and sorghum for primary metabolism

969 analysis

Genotype	Plant	Volume (mg)
IS 29091 biological replicate 1	Sorghum	1500
IS 29091 biological replicate 2	Sorghum	200
IS 29091 biological replicate 3	Sorghum	1000
IS 2245 biological replicate 1	Sorghum	150
IS 2245 biological replicate 2	Sorghum	75
IS 2245 biological replicate 3	Sorghum	250
CIMMYT-BANK-017456 biological replicate 1	Maize	750
CIMMYT-BANK-017456 biological replicate 2	Maize	750
CIMMYT BANK 017456 biological replicate 3	Maize	500
CIMMYT BANK 017456 biological replicate 4	Maize	500
GRIN AMES 19897 biological replicate 1	Maize	400
GRIN AMES 19897 biological replicate 2	Maize	500
GRIN AMES 19897 biological replicate 3	Maize	250
GRIN AMES 19897 biological replicate 4	Maize	100

970

971

972 **Supplementary Table 4.** Top metabolites in aerial root mucilage. Peak (mz/rt) is the mass by
973 charge (mz) divided by the retention time (rt). d.value denotes the comparison between sorghum
974 and maize, where a positive value signifies that the metabolite is more abundant in sorghum than
975 maize, and negative values indicate higher abundance in maize. Other acronyms include standard
976 deviation (stdev), raw p-value (rawp), and q-value (q.value) for statistical analysis.

	Peak (mz/rt)	d.value	stdev	rawp	q.value
1	4850	8.6391	22.282	0	0
2	montanic acid	5.9485	1276.4	0.00011905	0.0032409
3	1064	5.825	2328.1	0.00011905	0.0032409
4	4901	-5.7521	469.11	0.00015873	0.0032409
5	168	5.3462	3634.3	0.00015873	0.0032409
6	Pinitol	-5.1539	375.29	0.0002381	0.0040512
7	Octadecanol	4.7778	248.11	0.00043651	0.0049514
8	N-acetylaspartate dyethilester	4.7568	18986	0.00043651	0.0049514
9	84161	4.7356	631.58	0.00043651	0.0049514
10	505155	4.5206	19490	0.00059524	0.0057391
11	328824	-4.4684	166.07	0.00063492	0.0057391
12	505146	4.4321	19227	0.0006746	0.0057391
13	100956	-4.2263	150.2	0.00087302	0.0068558
14	levoglucosan	4.0558	195.21	0.0011111	0.0081023
15	dodecanol	3.7188	293.75	0.001746	0.011883
16	1725	3.676	14665	0.0019841	0.01263
17	14724	3.6622	143.57	0.0021032	0.01263
18	azelaic acid	3.5732	108.45	0.0025397	0.014404
19	103182	3.5634	85.184	0.0026984	0.014499
20	21885	3.4903	1123.9	0.0032937	0.015836
21	thymine	-3.4522	216.9	0.0034127	0.015836
22	lignoceric acid	3.4511	465.46	0.0034127	0.015836
23	127661	3.4037	393.86	0.0037698	0.016733
24	2551	3.3414	130.98	0.0043254	0.018399
25	putrescine	-3.2171	1115.5	0.0057937	0.023659
26	threonine	-3.1399	2661.8	0.0071429	0.028046
27	169611	-3.1161	750.57	0.0074603	0.028208
28	42357	3.0631	321.56	0.0080159	0.028218
29	87877	3.0619	710.68	0.0080159	0.028218
30	4-hydroxymandelic acid	3.0201	290.67	0.0084921	0.028898
31	1-octacosanol	2.8541	963.42	0.012143	0.035571
32	isopalmitic acid	2.8421	218.03	0.01246	0.035571
33	4-hydroxybenzoic acid	2.8215	5841.1	0.012937	0.035571
34	adipic acid	2.8159	1130.8	0.013175	0.035571
35	47197	2.7985	335	0.013611	0.035571
36	131202	2.7821	258.41	0.014048	0.035571

981 **Supplementary Table 5.** Maize and sorghum accession for microbiome study. Number of

982 replicates is indicated in the sample ID.

Sample ID	Genotype	Plant	Location
S1 (4 samples)	IS 2245	sorghum	West Madison
S2 (3 samples)	IS 2245*	sorghum	West Madison
S3 (4 samples)	IS 23992	sorghum	West Madison
S4 (4 samples)	IS 29092	sorghum	West Madison
S5 (4 samples)	IS 2902	sorghum	UF
S6 (4 samples)	IS 2902*	sorghum	UF
S7 (4 samples)	IS 2245	sorghum	UF
M1 (4 samples)	CIMMYT-BANK-017456	maize	West Madison
M2 (4 samples)	CIMMYT-BANK-017456	maize	West Madison
M3 (4 samples)	CIMMYTA-BANK-014019	maize	West Madison
M4 (4 samples)	GRIN AMES 19897	maize	West Madison
M5 (3 samples)	GRIN AMES 19897	maize	West Madison
M6 (4 samples)	GRIN AMES 19897	maize	West Madison

983 *Samples were obtained from a low nitrogen pilot study

984

985 **Supplementary Table 6.** AMOVA for each sample comparison. S = sorghum
986 sample, M = maize sample, and df = degrees of freedom. Statistically
987 significant differences are indicated with asterisks: * p ≤ 0.05; ** p ≤
988 0.01, and *** p ≤ 0.001
989

Group	Sum of Square	df	Mean Square	Fixation Index	p-value
S1-M3	0.144504	1	0.144504	5.1731	0.005**
M4-M5	0.0855831	1	0.0855831	2.22751	0.095
S2-M4	0.0864243	1	0.0864243	3.0695	0.038*
M2-M5	0.115087	1	0.115087	3.71158	0.029*
S6-S7	0.0614475	1	0.0614475	0.486006	0.61
S3-M5	0.180132	1	0.180132	5.09055	0.026*
S1-S6	0.184116	1	0.184116	1.95325	0.263
S5-M6	0.450519	1	0.450519	5.24901	0.01**
S3-M1	0.054311	1	0.054311	1.92444	0.114
S2-M3	0.186426	1	0.186426	7.09958	0.03*
S2-S7	0.266473	1	0.266473	4.39073	0.068
S4-S6	0.22921	1	0.22921	2.53538	0.198
M4-M6	0.138883	1	0.138883	3.15016	0.05*
S2-S6	0.137483	1	0.137483	1.29883	0.413
S5-M2	0.223533	1	0.223533	3.187	0.107
M2-M4	0.0199211	1	0.0199211	0.70151	0.589

M3-M5	0.0460803	1	0.0460803	1.26165	0.341
M1-M5	0.1314	1	0.1314	3.8839	0.013*
S7-M6	0.740479	1	0.740479	10.4001	0.02*
S6-M2	0.225729	1	0.225729	2.42344	0.184
S4-M6	0.183315	1	0.183315	5.21219	0.011*
S4-M1	0.0626517	1	0.0626517	2.86947	0.023*
S3-S4	0.0656918	1	0.0656918	2.8403	0.072
S2-S4	0.0574829	1	0.0574829	3.29319	0.066
S4-S7	0.436925	1	0.436925	8.27984	0.029
S5-M3	0.407831	1	0.407831	5.45696	0.017
S5-M1	0.270468	1	0.270468	3.73097	0.03*
M1-M2	0.0309953	1	0.0309953	1.26133	0.189
S4-S5	0.231663	1	0.231663	3.43716	0.019**
S3-M3	0.16078	1	0.16078	5.27755	0.005**
S5-S7	0.0721351	1	0.0721351	0.697439	0.438
S4-M2	0.0571673	1	0.0571673	2.93463	0.028*
M1-M3	0.115701	1	0.115701	3.96639	0.003**
S1-S5	0.163211	1	0.163211	2.29049	0.184
S4-M5	0.0948554	1	0.0948554	3.42189	0.034*
S7-M5	0.549234	1	0.549234	7.74059	0.04*
S2-S5	0.111242	1	0.111242	1.4217	0.269
S7-M4	0.436884	1	0.436884	7.08226	0.001***
S1-M4	0.0736667	1	0.0736667	2.49594	0.089

S7-M3	0.655788	1	0.655788	10.9105	0.007**
S3-M2	0.0690644	1	0.0690644	2.66988	0.034*
M3-M6	0.0679816	1	0.0679816	1.59931	0.201
S3-S7	0.418749	1	0.418749	7.07855	0.011*
M5-M6	0.10333	1	0.10333	2.07341	0.044*
S2-M1	0.0962802	1	0.0962802	4.08539	0.032*
M2-M6	0.201935	1	0.201935	5.3267	0.014*
S5-M4	0.245334	1	0.245334	3.21468	0.023*
S5-M5	0.348851	1	0.348851	3.94134	0.027*
S4-M4	0.0506137	1	0.0506137	1.97263	0.121
S6-M6	0.437969	1	0.437969	4.02419	<0.001*
S4-M3	0.108391	1	0.108391	4.50182	0.012*
S2-M2	0.0652358	1	0.0652358	3.14503	0.025*
S6-M1	0.272552	1	0.272552	2.85402	0.048*
S1-S3	0.0566845	1	0.0566845	2.10059	0.052*
M1-M6	0.221378	1	0.221378	5.49822	0.015*
S1-S4	0.0218245	1	0.0218245	1.05958	0.349
M2-M3	0.0991976	1	0.0991976	3.69911	0.033*
S2-M5	0.16442	1	0.16442	5.24333	0.105
S1-M5	0.123894	1	0.123894	3.83004	0.016*
S6-M3	0.381372	1	0.381372	3.90187	0.007**
S1-M6	0.217747	1	0.217747	5.57941	0.009**
S2-M6	0.264406	1	0.264406	6.68187	0.015*

S3-S6	0.219448	1	0.219448	2.26721	0.309
S1-M2	0.0740045	1	0.0740045	3.17115	0.014*
S6-M4	0.23653	1	0.23653	2.38145	0.211
S7-M1	0.485294	1	0.485294	8.38696	0.007**
S1-S2	0.0290423	1	0.0290423	1.31515	0.245
M1-M4	0.0441517	1	0.0441517	1.43577	0.138
M3-M4	0.0719655	1	0.0719655	2.18114	0.125
S1-S7	0.390742	1	0.390742	6.90035	0.006**
S5-S6	0.0159133	1	0.0159133	0.11281	0.878
S6-M5	0.325414	1	0.325414	2.80247	0.157
S3-S5	0.194684	1	0.194684	2.63844	0.07
S2-S3	0.0518463	1	0.0518463	2.06391	0.13
S3-M6	0.241142	1	0.241142	5.80253	0.023*
S1-M1	0.0850429	1	0.0850429	3.31029	0.016*
S7-M2	0.406276	1	0.406276	7.31907	0.032*
S3-M4	0.0837043	1	0.0837043	2.61203	0.001***
All samples S1-S2-S3-S4-S5-S6-S7-M1-M2-M3-M4-M5-M6	2.31435	12	0.192863	3.6472	<0.001***

991 **Supplementary Table 7.** Diazotrophs isolated from the sorghum mucilage.

992

Strain	Sorghum Genotype	Phylum/Class	Closest type strain	Length (bp)	Similarity (%)	Accession Number
1.1	IS_29091	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas lactitubi* M15^T	1495	99.78	NR_179509.1
1.2	IS_29091	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas lactitubi* M15^T	1320	100	NR_179509.1
1.3	IS_29091	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas lactitubi* M15^T	1333	100	NR_179509.1
1.6	IS_29091	*Pseudomonadota/Gammaproteobacteria*	*Klebisiella michiganensis* W14^T	1319	99.7	MT572941.1
1.7	IS_29091	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas lactitubi* M15^T	1377	99.49	NR_179509.1
1.9	IS_29091	*Pseudomonadota/Gammaproteobacteria*	*Klebisiella oxitoca* JCM 1665^T	1286	99.61	LC133345.1
2.1	IS_31706	*Pseudomonadota/Betaproteobacteria*	*Pseudacidovorax intermedius* CC-21	1298	100	NR_044241.1
2.2	IS_31706	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^T	1310	100	NR_026163.1
2.5	IS_31706	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^T	1355	99.78	NR_026163.1
2.6	IS_31706	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas rhizophila* e-p10^T	1356	99.93	NR_028930.1
3.1	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Novosphingobium resinovorum* NCIMB 8767^T	1236	100	NR_044045.1
3.2	IS_15744	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas lactitubi* M15^T	1353	99.78	NR_179509.1

3.3	IS_15744	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas lactitubi* M15^{T}	1250	100	NR_179509.1
3.5	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Novosphingobium resinovorum* NCIMB 8767^{T}	1220	100	NR_044045.1
3.6	IS_15744	*Actinomycetota/Actinomycetes*	*Microbacterium neimengense* 7087^{T}	1330	99.55	NR_118272.1
4.2	IS_31706	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^{T}	1388	99.77	NR_026163.1
4.4	IS_31706	*Pseudomonadota/Gammaproteobacteria*	*Phytobacter diazotrophicus* DSM 17806^{T}	1441	99.74	KY288669.1
4.6	IS_31706	*Pseudomonadota/Alphaproteobacteria*	*Sphingobium limneticum* 301^{T}	1369	99.7	NR_109484.1
5.1	IS_31706	*Pseudomonadota/Alphaproteobacteria*	*Agrobacterium fabacearum* CNPSo 675^{T}	1295	100	MN741112.1
5.2	IS_31706	*Pseudomonadota/Gammaproteobacteria*	*Pseudoxanthomonas winnipegensis* NML 130738^{T}	1352	99.93	MH795530.1
5.3	IS_31706	*Bacteroidota/Flavobacteriia*	*Epilithonimonas hungarica* CHB-20p^{T}	1265	99.84	NR_044354.1
5.4	IS_31706	*Pseudomonadota/Alphaproteobacteria*	*Sphingobium yanoikuyae* HAMBI 1842^{T}	1317	96.82	LT899948.1
6.1	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Agrobacterium fabacearum* CNPSo 675^{T}	1290	100	MN741112.1
6.11	IS_15744	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas nematodicola* W5^{T}	1365	99.93	NR_181111.1

6.2	IS_15744	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas cyclobalanopsidis* TPQG1-4^T	1356	99.85	MN036524.2
6.3	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Novosphingobium kaempferiae* Sx8-5^T	1298	99.54	CP089301.1
6.4	IS_15744	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas cyclobalanopsidis* TPQG1-4^T	1356	99.7	MN036524.2
6.7	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Agrobacterium divergens* LMG 31531^T	1350	100	AM403584.1
6.8	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Agrobacterium fabacearum* CNPSo 675^T	1292	100	MN741112.1
6.9	IS_15744	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas nematodicola* W5^T	1377	99.85	NR_181111.1
7.2	IS_31706	*Actinomycetota/Actinomycetes*	*Microbacterium oleivorans* BAS69^T	1379	98.7	NR_042262.1
7.3	IS_31706	*Pseudomonadota/Gammaproteobacteria*	*Pseudomonas campi* S1-A32-2	1284	98.68	MT415401.1
7.6	IS_31706	*Bacillota/Bacilli*	*Bacillus amyloliquefaciens* DSM 7^T	1389	100	FN597644.1
7.8	IS_31706	*Actinomycetota/Actinomycetes*	*Microbacterium oleivorans* BAS69^T	1329	98.87	NR_042262.1
8.1	IS_29314	*Pseudomonadota/Alphaproteobacteria*	*Azospirillum humicireducens* SgZ-5^T	1181	96.21	CP015285.1
8.4	IS_29314	*Pseudomonadota/Gammaproteobacteria*	*Pseudomonas bharatica* CSV86^T	1331	99.77	MN866057.2
10.2	IS_29091	*Actinomycetota/Actinomycetes*	*Microbacterium neimengense* 7087^T	1291	99.84	NR_118272.1
10.3	IS_29091	*Bacteroidota/Chitinophagia*	*Filimonas endophytica* SR 2-06^T	1292	98.14	NR_145918.1
10.4	IS_29091	*Bacteroidota/Chitinophagia*	*Filimonas zeae* 772^T	1329	97.9	NR_149799.1
10.5	IS_29091	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^T	1349	100	NR_026163.1

10.6	IS_29091	*Pseudomonadota/Alphaproteobacteria*	*Agrobacterium divergens* LMG 31531^{T}	1365	100	AM403584.1
10.7	IS_29091	*Pseudomonadota/Alphaproteobacteria*	*Agrobacterium fabacearum* CNPSo 675^{T}	1231	100	MN741112.1
11.1	IS_15744	*Actinomycetota/Actinomycetes*	*Microbacterium binotii* CIP 101303^{T}	1330	100	NR_044290.1
11.1	IS_15744	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^{T}	1338	99.18	NR_026163.1
11.2	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Sphingobium yanoikuyae* HAMBI 1842^{T}	1297	99.92	LT899948.1
11.4	IS_15744	*Pseudomonadota/Gammaproteobacteria*	*Pseudomonas sediminis* PI11^{T}	1145	94.53	KP319033.1
11.7	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Azospirillum palustre* B2^{T}	1232	99.35	NR_178321.1
11.8	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Sphingobium yanoikuyae* HAMBI 1842^{T}	1311	100	LT899948.1
12.2	PI_534063	*Pseudomonadota/Alphaproteobacteria*	*Rhizobium cellulosilyticum* ALA10B2	1183	99.32	NR_043985.1
12.4	PI_534063	*Pseudomonadota/Betaproteobacteria*	*Herbaspirillum seropedicae* Z67^{T}	1388	99.71	CP011930.1
12.6	PI_534063	*Actinomycetota/Actinomycetes*	*Microbacterium neimengense* 7087^{T}	1378	99.78	NR_118272.1
12.7	PI_534063	*Pseudomonadota/Betaproteobacteria*	*Herbaspirillum seropedicae* Z67^{T}	1341	99.85	CP011930.1
12.9	PI_534063	*Pseudomonadota/Betaproteobacteria*	*Herbaspirillum seropedicae* Z67^{T}	1281	99.84	CP011930.1
13.1	IS_9745	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^{T}	1379	99.78	NR_026163.1
13.3	IS_9745	*Actinomycetota/Actinomycetes*	*Microbacterium hominis* DSM 12509^{T}	1325	98.19	NR_042480.1

13.4	IS_9745	*Actinomycetota/Actinomycetes*	*Microbacterium hominis* DSM 12509^{T}	1360	99.76	NR_042480.1
13.5	IS_9745	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas terrae* R-32768	1361	99.34	NR_042569.1
13.7	IS_9745	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^{T}	1305	99.16	NR_026163.1
13.8	IS_9745	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^{T}	1322	99.17	NR_026163.1
13.9	IS_9745	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas lactitubi* M15^{T}	1339	99.78	NR_179509.1
14.1	IS_29091	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^{T}	1270	98.98	NR_026163.1
14.2	IS_29091	*Pseudomonadota/Gammaproteobacteria*	*Pseudomonas turukhanskensis* IB1.1^{T}	1331	98.5	NR_152710.1
14.3	IS_29091	*Pseudomonadota/Gammaproteobacteria*	*Enterobacter cancerogenus* FDAARGOS 1428^{T}	1287	99.92	CP077290.1
14.4	IS_29091	*Actinomycetota/Actinomycetes*	*Microbacterium aerolatum* CCM 4955^{T}	1289	99.07	MT760116.1
14.5	IS_29091	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^{T}	1373	99.05	NR_026163.1
14.6	IS_29091	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^{T}	1355	99.12	NR_026163.1
14.7	IS_29091	*Pseudomonadota/Gammaproteobacteria*	*Pseudomonas turukhanskensis* IB1.1^{T}	1299	100	NR_152710.1

14.9	IS_29091	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^{T}	1315	100	NR_026163.1
15.1	IS_13971	*Actinomycetota/Actinomycetes*	*Microbacterium laevaniformans* DSM 20140^{T}	1270	99.21	MN543870.1
15.2	IS_13971	*Pseudomonadota/Alphaproteobacteria*	*Novosphingobium resinovorum* NCIMB 8767^{T}	1257	100	EF029110.2
15.5	IS_13971	*Pseudomonadota/Alphaproteobacteria*	*Devosia riboflavina* NBRC 13584^{T}	1191	100	NR_113618.1
15.7	IS_13971	*Pseudomonadota/Alphaproteobacteria*	*Ketogulonicigenium vulgare* DSM 4025T	1259	100	DQ915606.1
15.8	IS_13971	*Pseudomonadota/Alphaproteobacteria*	*Devosia riboflavina* NBRC 13584^{T}	1347	99.85	NR_113618.1
15.9	IS_13971	*Pseudomonadota/Betaproteobacteria*	*Pseudacidovorax intermedius* CC-21	1213	96.83	NR_044241.1
16.1	IS_15744	*Actinomycetota/Actinomycetes*	*Microbacterium oleivorans* BAS69^{T}	1269	99.13	NR_042262.1
16.2	IS_15744	*Actinomycetota/Actinomycetes*	*Microbacterium oleivorans* BAS69^{T}	1276	98.82	NR_042262.1
16.3	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Sphingobium yanoikuyae* HAMBI 1842^{T}	1355	99.92	LT899948.1
16.4	IS_15744	*Actinomycetota/Actinomycetes*	*Microbacterium binotii* CIP 101303^{T}	1319	100	NR_044290.1
16.5	IS_15744	*Actinomycetota/Actinomycetes*	*Microbacterium oleivorans* BAS69^{T}	1377	98.77	NR_042262.1
16.7	IS_15744	*Actinomycetota/Actinomycetes*	*Microbacterium neimengense* 7087^{T}	1370	99.89	NR_118272.1
17.2	IS_29091	*Pseudomonadota/Alphaproteobacteria*	*Agrobacterium divergens* LMG 31531^{T}	1231	100	AM403584.1
17.3	IS_29091	*Pseudomonadota/Alphaproteobacteria*	*Novosphingobium kaempferiae* Sx8-5^{T}	1193	99.92	CP089301.1

17.4	IS_29091	*Pseudomonadota/Gammaproteobacteria*	*Phytobacter diazotrophicus* DSM 17806^{T}	1288	99.74	KY288669.1
17.5	IS_29091	*Pseudomonadota/Alphaproteobacteria*	*Agrobacterium divergens* LMG 31531^{T}	1291	98.99	AM403584.1
17.6	IS_29091	*Pseudomonadota/Gammaproteobacteria*	*Pseudomonas turukhanskensis* IB1.1^{T}	1331	98.81	NR_152710.1
18.1	IS_31706	*Pseudomonadota/Gammaproteobacteria*	*Enterobacter cancerogenus* FDAARGOS 1428^{T}	1247	99.68	CP077290.1
18.2	IS_31706	*Pseudomonadota/Alphaproteobacteria*	*Agrobacterium larrymoorei* ATCC 51759^{T}	1230	100	MT780298.1
18.3	IS_31706	*Pseudomonadota/Alphaproteobacteria*	*Sphingobium yanoikuyae* HAMBI 1842^{T}	1320	100	LT899948.1
18.4	IS_31706	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas maltophilia* IAM 12423	1295	99.85	MN240936.1
18.6	IS_31706	*Pseudomonadota/Alphaproteobacteria*	*Novosphingobium kaempferiae* Sx8-5^{T}	1311	99.39	CP089301.1
18.7	IS_31706	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas lactitubi* M15^{T}	1404	99.52	NR_179509.1
18.8	IS_31706	*Pseudomonadota/Alphaproteobacteria*	*Azospirillum palustre* B2^{T}	1267	98.97	NR_178321.1
18.9	IS_31706	*Pseudomonadota/Alphaproteobacteria*	*Azospirillum humicireducens* SgZ-5^{T}	1298	99.03	CP015285.1
19.2	IS_31706	*Bacteroidota/Flavobacteriia*	*Epilithonimonas hungarica* CHB-20p^{T}	1339	99.71	NR_044354.1
19.4	IS_31706	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas cyclobalanopsidis* TPQG1-4^{T}	1403	99.86	MN036524.2
19.6	IS_31706	*Bacteroidota/Chitinophagia*	*Siphonobacter intestinalis* 63MJ-2	1335	99.1	NR_165683.1

19.7	IS_31706	*Pseudomonadota/Gammaproteobacteria*	*Klebsiella variicola* DSM 15968	1410	99.65	CP010523.2
20.1	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Sphingobium yanoikuyae* HAMBI 1842^{T}	1311	100	LT899948.1
20.2	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Sphingobium yanoikuyae* HAMBI 1842^{T}	1299	100	LT899948.1
20.3	IS_15744	*Actinomycetota/Actinomycetes*	*Microbacterium testaceum* DSM 20166^{T}	1373	99.16	NR_026163.1
20.4	IS_15744	*Pseudomonadota/Alphaproteobacteria*	*Agrobacterium pusense* NRCPB10^{T}	1291	99.92	NR_116874.1
21.1	IS_29091	*Pseudomonadota/Alphaproteobacteria*	*Shinella oryzae* Z-25^{T}	1341	98.44	NR_029103.1
21.3	IS_29091	*Pseudomonadota/Gammaproteobacteria*	*Stenotrophomonas lactitubi* M15^{T}	1293	99.61	NR_179509.1

993

994 **Supplementary Table 8.** Diazotrophs that have been used for biological nitrogen fixation

995 experiments. References to the origin of these strains and the source of isolation are provided.

Diazotroph	Phenotype	Reference	Source*
Azospirillum brasilense FP2 [#]	Fixer strain	(Pedrosa and Yates 1984)	Wheat, maize and forage grass (*Poa pratense*)
Azospirillum baldaniorum Sp 245[T#]	Fixer strain	(Baldani et al. 1983)	Wheat
Klebisiela variicola A3[#]	Fixer strain	This study	Sorghum mucilage
Klebisiela michiganensis A2 [#]	Fixer strain	This study	Maize mucilage
Azorhizobium caulinodans ORS571[T#]	Fixer strain	(Dreyfus and Dommergues 1981)	Nodules in *Sesbania rostrata*
Stutzerimonas stutzeri A1501 [#]	Fixer strain	(Qiu et al. 1981)	Rice
Paraburkholderia silvatlantica SRMrh-20[T#]	Fixer strain	(Perin et al. 2006)	Maize
Azotobacter vinelandii DJ[#]	Fixer strain	(Setubal et al. 2009)	Ubiquitous
Herbaspirillum seropedicae SmR1[#]	Fixer strain	(Pedrosa et al. 2011)	Maize, sorghum, rice, wheat and sugarcane
Azospirillum brasilense FP10	Non-fixer strain	(Pedrosa and Yates 1984)	Wheat, maize and forage grass (*Poa pratense*)
Azotobacter vinelandii DJ100 Δ*nifD*	Non-fixer strain	(Robinson et al. 1986)	Ubiquitous
Azorhizobium caulinodans ORS571 Δ*nifA*	Non-fixer strain	(Ryu et al. 2020)	Nodules in *Sesbania rostrata*

* Plant(s) where it has been isolated.

[#] For ARA experiment

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