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# Disentangling plant- and environment-mediated drivers of active rhizosphere bacterial community dynamics during short-term drought

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1 **Disentangling plant- and environment-mediated drivers of active rhizosphere bacterial community**  
2 **dynamics during short-term drought**

3

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36 **Abstract**

37 Background. Mitigating the effects of climate stress on crops is important for global food security. The  
38 microbiome associated with plant roots, henceforth, the rhizobiome, can harbor beneficial microbes that  
39 alleviate stress impacts. However, the factors influencing the recruitment of the rhizobiome during stress  
40 are unclear. We conducted an experiment to understand bacterial rhizobiome responses to short-term  
41 drought for two crop species: switchgrass and common bean. We used 16S rRNA and 16S rRNA gene  
42 sequencing to investigate the impact of drought severity on the recruitment of active bacterial rhizobiome  
43 members. We included planted and unplanted conditions to distinguish the environment- versus plant-  
44 mediated drivers of the active rhizobiome.

45 Results. Though each crop had a distinct rhizobiome, there were differences in the active microbiome  
46 structure between drought and watered and between planted and unplanted treatments. Despite their  
47 different community structures, the drought rhizobiome dynamics were similar across the two crops.  
48 However, the presence of a plant more strongly explained the rhizobiome variation in bean (17%) than in  
49 switchgrass (3%), with a small effect of plant mediation during drought only observed for the bean  
50 rhizobiome. The switchgrass rhizobiome was stable despite differences in the rhizosphere metabolite  
51 profiles between planted and unplanted treatments. Specifically, steroidal saponins and diterpenoids  
52 were enriched in drought, planted switchgrass soils.

53 Conclusions. We conclude that rhizobiome benefits to resist short-term drought are crop-specific, with the  
54 possibility of decoupling of plant exudation and rhizobiome responses, as we observed in switchgrass.  
55 We propose bacterial taxa uniquely associated with common bean plants during the short-term drought,  
56 which could be further evaluated to determine any plant benefit during drought.

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60 **Keywords**

61 **Rhizosphere microbiome, rhizosphere metabolome, drought stress, indicator species, common**  
62 **bean, switchgrass, 16sRNA:16SrRNA gene ratios**

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## 75 **Background**

76 The idea that beneficial microbes can be managed to improve crop performance is building momentum  
77 with new research directions [1-4]. It is well known that plant-associated microorganisms can promote  
78 plant performance when conditions are unfavorable [5]. For example, microbes can help plants acquire  
79 limiting nutrients such as nitrogen and phosphorus [7]. In turn, plants can shape their microbiome by  
80 secreting metabolites including sugars in their root exudates to support microbiome recruitment and  
81 maintenance [6]. Furthermore, different plant genotypes can have distinct metabolite compositions (e.g.  
82 [8]), with potential consequences for microbiome recruitment (e.g., [9]). In the bioenergy feedstock  
83 switchgrass (*Panicum virgatum*), there were different metabolite profiles for upland and lowland ecotypes  
84 [8], with in-vitro studies showing that differential metabolite accumulation across ecotypes can contribute  
85 to different microbiome compositions [9].

86  
87 The increased frequency and intensity of droughts have become major challenges for crop production  
88 globally [10, 11]. During drought, soil loses its moisture content, which can be exacerbated by erratic  
89 rainfall and temperature fluctuations [12]. There also are changes in other soil and plant properties, such  
90 as root exudation [13], with concurrent changes in the composition and function of the local soil and root-  
91 associated microbiome. Microorganisms can also play direct or indirect roles in plant drought tolerance.  
92 For example, some plant growth-promoting bacteria (PGPB) exude exopolysaccharides which can retain  
93 the soil moisture content at the beginning of drought [14]. They can also produce antioxidant enzymes  
94 during a drought that can support plant response to reactive oxygen species that results from drought -  
95 related stress [15]. However, beneficial and detrimental strains that affect plant growth under drought  
96 conditions have been identified (for example, in sorghum [16]). Drought can increase the relative  
97 abundance of phyla such as Firmicutes, Chloroflexi, and Actinobacteria, while phyla such as  
98 Bacteroidetes and Planctomycetes are relatively depleted [13].

99  
100 Drought responses of the rhizosphere and endosphere microbiomes have been extensively studied in  
101 crops such as rice [17, 18] and sorghum [19]. These studies have shown that drought can cause taxonomic  
102 enrichment of bacteria that may help plants recover during stress while also affecting the temporal  
103 assembly of the root microbiome. A recent study used random forest models to show that different  
104 drought regimes altered the microbiome succession as compared to the watered controls [17]. In this  
105 study, the degree of difference in the microbiome was directly proportional to the duration of drought, and  
106 the delay in microbiome succession persisted even after watering resumed, with enduring changes in the  
107 root microbiome observed as many as 62-84 days after the drought ended [17].

108

109 Because of the dry soil conditions and typically low microbial biomass associated with them, it remains  
110 technically challenging to assess the active microbial members of the rhizosphere during drought, and few  
111 studies have done so [19]. As the dormant bacterial pool in soils and rhizospheres is substantial [20-22],  
112 separating the responses of active from dormant members may be insightful for targeting the most  
113 responsive populations for plant benefit. Additionally, different plants have different inherent tolerances  
114 to dry conditions. Comparing the rhizosphere microbiomes across differently drought-sensitive crops may  
115 also provide insights into the members that especially can benefit sensitive plants. Furthermore, while  
116 changes in plant root exudation during drought can shape the rhizosphere microbiome response [23-25], it  
117 is unclear how much of that response is mediated by the host or attributable to the environmental  
118 conditions of the drought that impact the microbes directly. Drought conditions include low moisture  
119 availability, changes in physical soil structure, and connectivity via soil pores, all of which can directly  
120 impact microbes. Understanding whether responses are host- or environment-mediated could inform the  
121 separate targets for microbiome modification via host and soil management. Plant changes in rhizosphere  
122 exudates during drought stress could serve as signals to reactivate and recruit from the dormant pool,  
123 though this has not been thoroughly considered. Finally, host-mediated microbiome engineering has been  
124 employed in crops, such as wheat, to select beneficial microbial communities that promote plant tolerance  
125 to drought stress [26].

126  
127 In rhizosphere soils or similar environments containing ample amounts of energy-rich substrates, it is  
128 thought that much of the microbial community is active rather than dormant [27, 28]. Evidence from <sup>13</sup>C-  
129 PLFA (phospholipid-derived fatty acids) shows that Gram-positive bacteria assimilated <sup>13</sup>C more actively  
130 in the rhizosphere than in bulk soil [29]. Studies have also shown that Gram-negative bacteria in the  
131 rhizosphere actively assimilated root-derived carbon more successfully than Gram-positive bacteria [30-  
132 33]. For example, activity staining of bacterial cells from rhizosphere soil revealed that as much as 55%  
133 of them were active, with further evidence suggesting that rhizosphere soils have approximately 20%  
134 more active cells than bulk soils [28, 34]. However, the active rhizosphere microbiome pool can change  
135 during stress events, with members shifting to dormancy in response to the changing environment or  
136 climate, for instance, low moisture availability. The contribution of the resuscitated or active community  
137 becomes more prominent during and in the immediate aftermath of stress, when environmental conditions  
138 fluctuate, and prevalent members shift in competitiveness. Thus, it is critical to understand active  
139 community dynamics over short-term stress exposure.

140  
141 Several knowledge gaps exist about microbiome activation and recruitment during drought. First, the  
142 factors affecting the recruitment of the active rhizobiome during drought are unclear; specifically, the role

143 of the environment and plant as drivers in that assembly. Second, there is limited knowledge as to the  
144 effect of the duration of drought on rhizobiome assembly and how that depends on or interacts with  
145 drought conditions. Finally, how these microbiome dynamics differ across different plant families with  
146 different drought tolerances and to what extent the microbiome response is attributable to the host or  
147 environment needs to be understood.

148

149 To address these knowledge gaps, we conducted a greenhouse experiment using the annual legume  
150 common bean (*Phaseolus vulgaris*, var. Red Hawk) and the perennial grass switchgrass (*Panicum*  
151 *virgatum*, var. Cave-in-Rock). These crops substantially differ in physiology and root architecture, as  
152 bean has a taproot system, and switchgrass has a dense rhizome that extends several meters belowground.  
153 Furthermore, switchgrass genotypes are relatively more consistently drought-resistant, whereas bean  
154 genotypes have more variation in their drought tolerance [35-37]. The objectives of this experiment were  
155 to understand the active bacterial rhizobiome immediate assembly and short-term recruitment over a  
156 gradient of drought severity. For both plants, we compared planted to unplanted conditions using  
157 agricultural field soils previously planted with one of the two crops (and thus expected to have a legacy  
158 from their rhizobiomes) and partitioned the influences of plant-mediated versus environment-mediated  
159 rhizobiome responses to drought. We addressed two hypotheses. First, we hypothesized that active  
160 rhizobiomes respond to drought and change progressively with drought severity. Second, we  
161 hypothesized that the host plant mediates the responses of the active rhizobiome to drought via  
162 compositional changes in root metabolites.

163

164

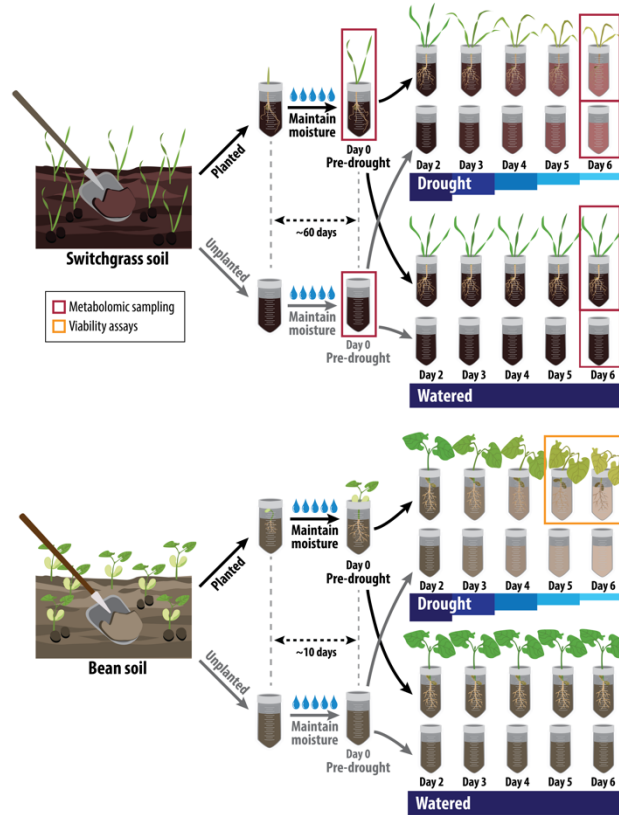
## 165 **Methods**

166

### 167 *Experimental design*

168

169 We performed a greenhouse experiment with common bean (*Phaseolus vulgaris* var. Red Hawk) and  
170 switchgrass (*Panicum virgatum* var. Cave-in-Rock). The experimental design consisted of two crop levels  
171 (bean, switchgrass), two planting levels (planted, unplanted), two moisture levels (drought, watered), and  
172 five destructive plant and rhizosphere sampling points correlating with increased drought severity. The  
173 drought gradient included a pre-drought baseline that was watered, and then destructive sampling over the  
174 next six days, with the day 2 samples experiencing the lowest drought severity and day 6 samples  
175 experiencing the highest. Five replicates were collected at each time point for each combination of the  
176 factors tested (**Figure 1**).



177

178 **Figure 1. The experimental design used in the greenhouse study. Day 2 to day 6 correspond to**  
179 **increased drought severity.**

180

181 *Soil sample collection*

182

183 Bean soil was collected from Montcalm Research Center on September 6, 2018, at Stanton, MI  
184 (43.350885, -85.177044), and was most recently planted with common bean (*Phaseolus vulgaris* var. Red  
185 Hawk) that year. Switchgrass samples were collected from the Great Lakes Bioenergy Research Center  
186 switchgrass plots at Lux Arbor Reserve (42.475224, -85.444979) in Denton, MI, on August 27, 2018.  
187 This site has been under continuous switchgrass (*Panicum virgatum* var. Cave-in-rock) cultivation since  
188 2011. Switchgrass soil was collected and homogenized from six sites randomly situated throughout the  
189 plots. All sampling materials were sterilized with ethanol before use and between samples. A shovel was  
190 used to collect bean and switchgrass soils to a depth of 10 cm. Large rocks and root fragments were  
191 removed from the soil. Within three hours (for bean soils) and five hours (for switchgrass soils), soils  
192 were transported to the lab and stored at 4°C until sieving. Soil nutrient analysis was completed for the  
193 field soils at the Michigan State University Soil and Plant Nutrient Laboratory (**Table S1**).

194

195 Soil samples were sieved (4mm mesh) over three to five days immediately after the sample collection day  
196 and stored at 4°C until needed for the experiment. The drought experiment commenced after eight months  
197 and six months of soil storage at 4°C, for bean and switchgrass, respectively.

198

#### 199 *Seed germination*

200

201 Approximately 250 switchgrass seeds (Sharp Bros. Seed of Mo., Inc) were placed in 50 ml conical tubes.  
202 10 ml of 5% bleach was added to the tubes. The tubes were placed on a shaker at 200 rpm for 20 min. The  
203 bleach/water mix was decanted, and 10 ml of deionized water was added. The tubes were agitated again  
204 at 200 rpm for 5 minutes, then decanted. The deionized water rinse was repeated two more times.

205 Approximately 60 switchgrass seeds were placed onto a wet filter paper (90 mm diameter) per sterile  
206 Petri plate. Seeds were moistened with deionized water, plates were wrapped in parafilm, covered with  
207 foil to block light, and incubated at 32°C incubator for three days. After three days, 1 ml of deionized  
208 water was supplemented to each plate, which was re-wrapped and returned to 32°C for an additional day.

209

210 Bean seeds were obtained from the Dry Bean Breeding and Genetics program at Michigan State  
211 University. Approximately 590 seeds were placed in a 1L Erlenmeyer flask, and 1L of sterilization  
212 solution was added (0.1% Tween-20, 10% bleach, and 90% DI water) to the seeds. The seeds were  
213 soaked for 15 min at room temperature and inverted every few minutes. The seeds were washed five  
214 times with deionized water, and 12 seeds were placed onto Petri dishes lined with moistened filter paper.  
215 Plates were wrapped in parafilm and incubated at room temperature in the dark. Over the next three days,  
216 2 ml of Milli-q water was added to each plate. After four days, approximately 60% of the switchgrass and  
217 bean seeds germinated and had an emerged radicle, and these were advanced for use in the experiment.

218

#### 219 *Growth tube preparation and planting*

220

221 Growth tubes were made by modifying 50 ml conical tubes to better control soil water content during the  
222 experiment. An ethanol-cleaned drill bit was used to drill a hole in the bottom of 50-ml conical tubes. A  
223 hole was also drilled in every cell of the tube racks used to hold the 50 ml tubes for switchgrass and bean  
224 plants. Small squares of Kimwipe® tissue papers (5cm \* 5cm) were cut, each rolled, and one rolled sheet  
225 was used to plug the drain hole in each 50 ml conical tube (**Figure S1**). Autoclaved perlite was combined  
226 with sieved field soil (50:50 v/v) to fill the prepared tubes to the top. Soils were watered with 10 ml of  
227 deionized water, which reduced the total soil volume to approximately 35 ml. Tubes for the planted  
228 treatment received one germinated seed. Unplanted tubes were prepared the same but without a seed.



229 Seeds were planted so that ~1 cm of the seedling was exposed above the soil and at a depth to ensure that  
230 the emerging root would not push the seedling out of the soil. After planting, additional soil: perlite mix  
231 was added to the tube to replace the volume lost after settling and supplemented with 3 ml of deionized  
232 water. Each tube was wrapped in foil to exclude light in the root zone. The greenhouse's daily temperature  
233 range was 21-32°C, with 14 hours of supplemental daytime lighting (400-watt high-pressure sodium  
234 lamp).

235

236 Seedlings were provided sufficient water to achieve healthy growth before initiating the drought.  
237 Switchgrass tubes were watered with 10 ml of deionized water every other day or every two days for two  
238 months until the plants were large enough for the experiment, which was designated as when the roots  
239 had filled the tube. Though this was close to the flowering stage, no flowers were observed. Bean tubes  
240 were watered daily with 10 ml of deionized water for the first five days and then twice daily with 10 ml  
241 for four additional days. After ~9 days of watered conditions, approximately 55 bean plants were sorted  
242 by height as a proxy for total biomass, and similarly sized plants were selected to include in the  
243 experiment. At this vegetative stage, the first trifoliate leaf emerged and started to unfold in all bean  
244 plants, and roots were completely bound within the tube. Before the drought, the planted tubes of both  
245 switchgrass and bean had tube-bound root systems such that all their soil could be directly influenced by  
246 the plant and classified as the rhizosphere. Bean roots were tube-bound within a week, but switchgrass  
247 took about two months. The timing for the start of drought treatment was chosen based on the time  
248 needed for the roots to fill the tube so that the entire soil was plant-influenced rhizosphere. Thus, the  
249 growth stage at the time of drought commencement was different between the two crops.

250

#### 251 *Drought treatment and destructive sampling*

252

253 The same experimental design and sampling strategy were applied to bean and switchgrass (**Figure 1**).  
254 Twenty-five seedlings and 25 unplanted tubes were assigned to either the sufficiently watered (“watered”)  
255 or to the reduced water treatment (“drought”), totaling 100 samples. In addition, five planted and five  
256 unplanted tubes were collected before the drought (“pre-drought,” day 0). These samples were used as a  
257 baseline control to compare to the subsequent drought samples. The drought was initiated on the same  
258 day the pre-drought samples were collected (see **Tables S2** and **S3** for the watering regimes for  
259 switchgrass and bean, respectively). The drought severity was increased over six days by adding  
260 progressively less water. Five replicate soil samples (individual tubes used for destructive sampling) were  
261 collected for each experimental condition from day 2 to day 6 for five post-drought sampling points.

262

263 To ensure that the drought bean plants remained viable (and that our assessment was of the rhizobiome  
264 response to live plants rather than to dead), an additional 36 plants were in parallel subjected to drought to  
265 determine their viability on the final days of the experiment. On day 5, when bean plants were browning  
266 and lacked turgor (day 5), half (18) of the extra plants were provided 15 ml of deionized water in the  
267 morning and the afternoon. All 18 plants were resuscitated, and we observed their leaves returned to full  
268 turgor by the next morning. Thus, the drought plants from day 5 were likely alive when sampled despite  
269 appearing well-desiccated. The same was repeated on day 6 with the remaining drought plants. Thirteen  
270 of the 18 plants resuscitated (leaves back to full turgor) within two days, affirming that most drought  
271 plants on day 6 were viable. As a relatively more drought-tolerant plant, switchgrass did not appear  
272 severely desiccated on days 5 and 6, and the resuscitation check was unnecessary to confirm its viability.

273

#### 274 *Soil, root, and shoot sample collection and gravimetric soil moisture*

275

276 Materials and surfaces were sterilized and/or ethanol-cleaned before use and between samples. All tubes  
277 were destructively sampled. During each sampling, each tube's contents were dumped onto sterilized foil.  
278 The soil was brushed from the root system and homogenized with a spatula. Roots were manually  
279 removed from the soil, and 0.5 g of soil was collected into a 1.5 ml microcentrifuge tube for DNA/RNA  
280 co-extractions. An additional ~6 g soil was retained as a backup. Perlite (>2mm) was avoided. Soils were  
281 flash-frozen in liquid nitrogen and stored at -80°C until nucleic acid extractions. An additional 4 g of soil  
282 was collected to determine gravimetric soil moisture content. After recording the mass of fresh soil  
283 collected, soils were dried in an oven at 50°C for at least three days and then re-weighed. Percent  
284 gravimetric soil moisture was calculated as the percent water mass lost during drying using **Equation 1**  
285 [38].

286

$$287 \text{ Gravimetric moisture content (\%)} = \frac{(\text{Mass of soil}_{\text{wet}} - \text{Mass of soil}_{\text{dry}})}{\text{Mass of soil}_{\text{dry}}} * 100 \quad \text{Equation 1}$$

288

289 After soil collection, the shoot system was cut at the base of the stem and put in pre-labeled envelopes.  
290 The shoots were dried at 50°C for at least five days. Masses taken after drying were used to determine the  
291 biomass of the shoot. Treatment differences in shoot biomass and gravimetric soil moisture were  
292 analyzed with a three-way ANOVA type III test upon satisfaction of normality.

293

294 An additional 30 switchgrass plants (15 watered and 15 drought) were grown for metabolite analysis on  
295 day 6. Soils were homogenized, shoots were sampled as previously described, and root systems were

296 cleaned in deionized water. Then, samples were flash-frozen in liquid nitrogen in 15 ml tubes. Soils of the  
297 unplanted treatments (watered and drought) from day 6, stored at -80°C, were used to compare with the  
298 planted treatments. We also analyzed metabolites from pre-drought soils (four replicates of planted and  
299 five unplanted treatments) stored at -80°C. In addition, ten unplanted soils from day 6 (five drought and  
300 five watered) and nine pre-drought samples (four planted, five unplanted) were analyzed for metabolites.  
301 In summary, 15 switchgrass plants from the day 6 watered and drought treatments each yielded 30  
302 samples for soil, shoot, and root metabolites.

303

#### 304 *Soil DNA/RNA co-extractions*

305

306 DNA/RNA co-extractions were performed using the protocol specified in [39] with minor modifications.  
307 Briefly, 0.5 g of flash-frozen soil was added to 0.7 mm PowerBead® garnet bead tubes (Qiagen,  
308 Germantown, MD, purchased early 2021 before being discontinued). 0.5 ml of CTAB-phosphate buffer  
309 (120 mM, pH 8) and 0.5 ml of phenol:chloroform: isoamyl alcohol solution (25:24:1, nuclease-free,  
310 Invitrogen®) were added to the tube and placed on a bead beater to beat for 30 s. Tubes were then  
311 centrifuged at 12000g for 10 min at 4°C. The top aqueous layer was extracted and placed into a new 1.5  
312 ml microcentrifuge tube. Next, 0.5 ml of chloroform: isoamyl alcohol (24:1, nuclease-free, Sigma®) was  
313 added to the tube and inverted several times to form an emulsion to remove residual phenol. Tubes were  
314 centrifuged at 16000g for 5 min at 4°C, the top aqueous layer extracted and placed into a new 1.5 ml  
315 microcentrifuge tube. Nucleic acids were precipitated by adding two volumes of 30% polyethylene glycol  
316 solution (PEG6000, 1.6M NaCl) and mixing a few times. Tubes were incubated on ice for two hours.  
317 Tubes were then centrifuged at 16000g for 20 min at 4°C. The supernatant was removed, and 1 ml of 70%  
318 ice-cold ethanol was added. Tubes were centrifuged at 16000g for 15 min at 4°C. Ethanol wash was  
319 pipetted out, being careful not to remove the pellet, and placed back in the centrifuge for a final spin for  
320 10 s to collect residual ethanol. The remaining ethanol was pipetted out of tubes, and the pellet was  
321 allowed to air dry. The nucleic acid pellet was resuspended in 30 µl of nuclease-free water. Negative  
322 controls for extractions included tubes to which no soil sample was added, and only included reagents and  
323 the garnet beads to check contamination in the extraction reagents. Two negative controls were processed  
324 alongside the experimental samples for each extraction day. All DNA and RNA samples were quantified  
325 using Qubit® dsDNA BR assay kit and RNA HS assay kit on a qubit 2.0 fluorometer (Invitrogen,  
326 Carlsbad, CA, USA). All nucleic acid raw coextracts (containing DNA and RNA) were visualized using  
327 agarose gel electrophoresis and validated with a band for DNA and RNA [39].

328

#### 329 *DNase treatment and cDNA synthesis*

330

331 The DNA/RNA coextract was used to prepare purified RNA using the Invitrogen TURBO® DNA-free  
332 Kit with minor modifications. 1 µl of 10X TURBO® DNase Buffer and 3 µl of TURBO® DNase enzyme  
333 were added to a 6 µl aliquot of the DNA/RNA coextract. The mixture was then incubated for 30 mins at  
334 37°C, after which 2 µl of a DNase inactivation reagent was added to each tube. The resulting solution  
335 was mixed by flicking the tubes by hand and then incubated at room temperature for 5 mins. The samples  
336 were centrifuged at 2000g for 5 min at room temperature. The purified, DNase-treated RNA samples  
337 were transferred to a clean, sterile tube and immediately processed to make complementary DNA  
338 (cDNA). cDNA was prepared using the Invitrogen® SuperScript III First Strand Synthesis System using  
339 random hexamers per the manufacturer's instructions. cDNA samples were stored at -20°C until further  
340 processing. Negative controls were prepared for the cDNA synthesis step to check for reagent  
341 contamination.

342

#### 343 *PCR and RT-PCR*

344

345 DNA (PCR) and cDNA samples (RT-PCR) were amplified using a standard protocol specified by the  
346 Research Technology Support Facility at Michigan State University. A 15 µl reaction volume was  
347 prepared for each sample to amplify the V4 hypervariable region of the 16S rRNA gene with 2X GoTaq  
348 Green Mastermix, primers 515F (5'- GTGCCAGCMGCCGCGGTAA- 3') and 806R (5'-  
349 GGACTACHVGGGTWTCTAAT-3') [40] and 1 µl template. The final concentration of the GoTaq  
350 Green Mastermix was 1X, and the final concentration of each primer was 0.1 µM. The PCR cycle was run  
351 using the following cycling parameters: initial denaturation at 94°C for 3 min; 30 cycles of denaturation  
352 at 94°C for 45 s, annealing at 50°C for 60 s, and extension at 72°C for 90 s, followed by a final extension  
353 at 72°C for 10 min. Samples were kept at 4°C and immediately visualized on a 1% agarose gel using 100-  
354 bp ladder. All RNA samples purified from DNA/RNA coextracts and used to prepare cDNA were also  
355 used to run PCR and check on 1% agarose gel to ensure there were no contaminating DNA bands in RNA  
356 samples. All PCR reactions included a no-template negative control and an *E. coli* DNA template as a  
357 positive control. All negative controls used for extractions were also included as samples in the PCRs.  
358 Results indicated no contamination from extraction or PCR reagents and consistent amplification  
359 performance with the cycling parameters.

360

#### 361 *Illumina sequencing of the 16S rRNA gene and 16S rRNA*

362

363 Amplicon sequencing was performed at the Genomics Research Technology Support Facility at  
364 Michigan State University. The V4 hypervariable region of the 16S rRNA gene was amplified using dual-  
365 indexed Illumina compatible primers 515F and 806R as described in [41]. PCR products were batch  
366 normalized using an Invitrogen SequelPrep DNA Normalization Plate, and the normalized products  
367 recovered from each of the six plates submitted were pooled. The pools were cleaned up and concentrated  
368 using a QIAquick PCR Purification column followed by AMPureXP magnetic beads; it was quality  
369 controlled and quantified using a combination of Qubit dsDNA HS, Agilent 4200 TapeStation HS  
370 DNA1000, and Invitrogen Colibri Library Quantification qPCR assays.

371

372 Each plate submitted for sequencing had approximately 85 samples (6 plates); each plate was used for one  
373 MiSeq run. The pools from each plate were each loaded onto an Illumina MiSeq v2 standard flow cell,  
374 and sequencing was performed in a 2x250bp paired-end format using a MiSeq v2 500 cycle reagent  
375 cartridge. Custom Sequencing and index primers were added to appropriate wells of the reagent cartridge.  
376 Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54, and the output of RTA was  
377 demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.20.0. In addition to  
378 experimental samples, all negative controls from each extraction day were sequenced, and these samples  
379 were evenly distributed throughout all the MiSeq runs. Two positive controls (mock communities) were  
380 sequenced with each MiSeq run. One positive control was an in-house Mock community prepared in the  
381 Shade Lab [42], and the other positive control was provided by the RTSF at MSU for library preparation.

382

### 383 *Metabolite extraction from plant tissue and soil*

384

385 The switchgrass shoot, root, and rhizosphere soil samples collected from 15 individual plants in the  
386 drought experiment were pooled into seven replicates (with six replicates, each including two samples  
387 and one replicate including three samples (**Figure S2A, B**). The plant tissues were lyophilized and ground  
388 into powders using an automated tissue homogenizer (SPEX SamplePrep, Metuchen, NJ). The plant  
389 metabolites were extracted from the powders using 80% methanol containing 1  $\mu$ M telmisartan internal  
390 standard and normalized by tissue weight to achieve equal concentration as described in [8]. The soil  
391 metabolites were extracted following the same protocol with differences in the soil-to-solvent ratio (1:2,  
392 v/v) and incubation method (mixing on a laboratory tube rocker). Extracts were centrifuged at 4000g for  
393 20 min at room temperature to remove solids. The supernatant was completely dried using a SpeedVac  
394 vacuum concentrator (ThermoFisher, Waltham, MA) and reconstituted using 1/10 the original solvent  
395 volume.

396

397 *Liquid chromatography–mass spectrometry (LC-MS) based untargeted metabolomics*

398 The chromatographic separation and MS analysis for the switchgrass metabolites were performed using a  
399 reversed-phase, UPLC BEH C18, 2.1 mm x 150 mm, 1.7  $\mu\text{m}$  column (Waters, Milford, MA) and an  
400 Electrospray Ionization – Quadrupole Time-of-Flight MS (ESI-QToF-MS, Waters). Mass spectra, under  
401 positive ionization, were acquired in data-independent acquisition (DIA, MS<sup>E</sup>) and data-dependent  
402 acquisition (DDA, MS/MS) modes. The details were described in [8]. The untargeted metabolomics  
403 (DIA) data processing, including retention time (RT) alignment, lock mass correction, peak detection,  
404 adduct grouping and deconvolution, and metabolite annotation, were done using the Progenesis QI  
405 software package (v3.0, Waters) following the protocol in [8]. The identified analytical signals were  
406 defined by the RT and mass-to-charge ratio ( $m/z$ ) information and referred to as the *features*.  
407 Measurement of each feature across the sample panel was filtered by interquartile range, log-transformed,  
408 and scaled for multivariate analyses using R Studio v3.1.1 and R package MetabolAnalyze (scaling  
409 function set to type “pareto”). The log-transformed and pareto scaled (normalized) abundance of features  
410 for soil were used for principal component analysis (PCA). A PLS-DA model was generated using the  
411 MetaboAnalyst 4.0 online tool platform [43] to assess each metabolite feature's variable of importance  
412 (VIP) coefficient. The top 50 features with the highest VIP coefficient were used to visualize in a  
413 heatmap. For this, the features were log-transformed and scaled by row (features) from 0 to 1. This was  
414 completed using R software and then visualized using the heatmap function in the R package  
415 ComplexHeatmap. Distance and clustering methods were set to Euclidean and Ward.D to generate  
416 hierarchical clustering for the heatmap. The DDA (MS/MS) spectra were only used for metabolite mining  
417 using CANOPUS (class assignment and ontology prediction using mass spectrometry) [44] machine  
418 learning function built in the SIRIUS 4 (<https://bio.informatik.uni-jena.de/sirius/>), a computational tool  
419 for systematic compound class annotation. The identification level was denoted for each annotated  
420 metabolite based on the criteria for metabolite identification as per [45].

421

422 *16S rRNA and 16S rRNA gene sequence analysis*

423 Sequence data were analyzed using QIIME2 [46]. All paired-end sequences with quality scores were  
424 compressed and denoised using the DADA2 plugin [47]. The denoising step dereplicated sequences,  
425 filtered chimeras, and merged paired-end reads. The truncation parameters to use with the DADA2 plugin  
426 were determined using FIGARO [48]. FIGARO analyzes error rates in a directory of FASTQ files to  
427 determine the optimal trimming parameters for sequencing pipelines that utilize DADA2. The truncation  
428 length was set to 123 F and 162 R for all the data, with minimum overlap set to 30 base pairs, which  
429 resulted in 93% merging success. All truncation was performed from the 3' end for consistent final read

430 lengths. The DNA and cDNA datasets were separately denoised. The resulting DNA and cDNA count  
431 tables were merged into a single QIIME2 artifact using the feature-table merge command.

432 Similarly, the DNA and cDNA representative sequences were merged into a single QIIME2 artifact using  
433 the feature-table merge-seqs command. The representative sequences from the combined count tables  
434 were clustered at 99% identity de-novo, and the clustered representative sequences were classified using  
435 SILVA v138 [49] to generate the taxonomy file. Ninety-nine percent sequence identity was used to define  
436 OTUs to conservatively account for any potential amplification errors that may have occurred during the  
437 cDNA synthesis from the RNA. The resulting OTU (operational taxonomic unit) table and taxonomy files  
438 were exported to R for ecological analysis.

#### 439 *Designating the active community members*

440

441 All downstream analyses were performed in R version 4.0. The R package decontam [50] was used to  
442 determine the number and identity of contaminants in the dataset (**Figures S3A and S3B**) and remove  
443 them using the prevalence method. Contaminating taxa, mitochondria, and chloroplast sequences were  
444 filtered from the datasets. Based on rarefaction curves, a subsampling depth of 15,000 reads per sample  
445 was selected (**Figure S3C**). After subsampling, 16S rRNA to rRNA gene ratios (hereafter, 16S  
446 rRNA:rRNA gene) were computed from the DNA and cDNA datasets as described in [20]. While we  
447 compared a few methods therein for this dataset (please see Supplemental Information for details), we  
448 ultimately chose the method that applied a 16S rRNA:rRNA gene ratio threshold  $\geq 1$ . The chosen  
449 method was statistically robust in overarching patterns of beta-diversity given the exclusion or inclusion  
450 of phantom taxa (taxa with detected cDNA but not DNA counts). For phantom taxa that were detected in  
451 greater than 5% of samples, the DNA counts = 0 were changed to DNA=1 (as in “method 2” in [20])  
452 (see **Supplemental Materials**). All other phantom taxa were excluded. The DNA OTU table was filtered  
453 to include only sequence counts of active taxa in the samples determined to meet our ratio threshold.  
454 Consequently, while every DNA and cDNA sample of sequence counts was initially rarefied to 15,000  
455 reads, each sample’s active community varied in their total reads (2000-6000). Relativized abundances  
456 were used for ecological statistics.

457

#### 458 *Microbiome data analysis for active community*

459

460 The OTU table of the DNA counts of active taxa, taxonomy table, and metadata files was merged using  
461 the phyloseq package [51] in R. We used Bray-Curtis dissimilarity to determine beta diversity but also  
462 tested weighted UniFrac distance, and results were comparable. Permutational analysis of variance

463 (PERMANOVA) was conducted using the `adonis` function in `vegan` package [52] to assess differences in  
464 community structure by treatment and interactions: drought treatment (watered or drought), drought  
465 severity/sampling (days 2-6), and plant treatment (planted or unplanted). For post hoc tests, pairwise  
466 comparisons between drought levels were computed using the `pairwise.adonis2` function in `vegan`  
467 package. PERMANOVA tests using the `adonis` function in R `vegan` package were also done on metabolite  
468 feature abundances used for multivariate analysis to understand the effects of planting, drought, and  
469 sampling day factors. To understand drought dynamics, we analyzed the Bray Curtis similarity of the  
470 microbiome to the pre-drought samples over the covariate of time for planted and unplanted treatments in  
471 bean and switchgrass. This was visualized using a smoothed conditional means (`geom_smooth` function)  
472 with a linear model. This same approach was also used to assess general, relative fluctuations in  
473 rhizobiome size across samples, as proxied by DNA concentration.

474  
475 Richness was the observed number of OTUs, using the `estimate_richness` function from 100 re-samples of  
476 the community. The normality of alpha diversity metrics was assessed using a Shapiro-Wilk test with a  
477 cut-off of  $W > 0.9$  for normality assumptions. Since data were normally distributed, a parametric three-way  
478 ANOVA test was used to assess the main effects and interaction effects between factors on richness.  
479 Contrasts were set to “sum” before running the ANOVA model to ensure that type III ANOVA tests were  
480 valid. We used non-parametric Kruskal-Wallis tests (`Kruskal.test` in R) to calculate differences in class-  
481 level relative abundances between treatments.

482  
483 We used the `indicspecies` package in R to determine indicator species associated with experimental  
484 conditions. We used the abundance-based counterpart of Pearson’s phi coefficient of association within  
485 the `multipatt` function [53]. To correct the phi coefficient for unequal group sizes the “`func`” parameter  
486 within `multipatt` was set to “`r.g.`” P values were adjusted for false discovery rates. Indicator species were  
487 calculated at the OTU level for each treatment combination and the OTU and family level for the crop-  
488 specific indicators.

489  
490 We created a heatmap visualization of the 50 most abundant and active OTUs in the bean dataset for  
491 indicator species. We used a maximum standardization approach using the `decostand` function in the  
492 `vegan` package in R. We distinguished taxa that changed relative abundance over the drought gradient  
493 from taxa that changed in their detection. For samples without detection of an OTU’s activity or DNA  
494 (e.g., DNA = 0, cDNA = 0), that OTU’s abundance was coded as NA. For samples that had no detection  
495 of an OTU’s activity but had detection of its DNA (e.g., DNA >0, cDNA = 0), that OTU’s abundance was  
496 coded as 0. For samples that detected an OTU’s activity and DNA (e.g., DNA >0, cDNA >0), that OTU’s



497 abundance was the value of its DNA sequence count. We also included a special consideration of  
498 “phantom” OTUs (e.g., DNA =0, cDNA>0), which were assigned a sequence count of 1.

499

500

501

## Results

502

### *Overview*

503

504

505 We conducted a short-term drought experiment over 6 days and assessed the active rhizobiome dynamics  
506 of bean and switchgrass in the greenhouse. Rhizosphere soils were collected from fields recently planted  
507 with each crop, and treatments included planted/unplanted and watered/drought. We collected pre-  
508 drought and post-drought samples over time and with increased drought severity (**Figure 1**).

509

510

### *Efficacy of the short-term drought treatment*

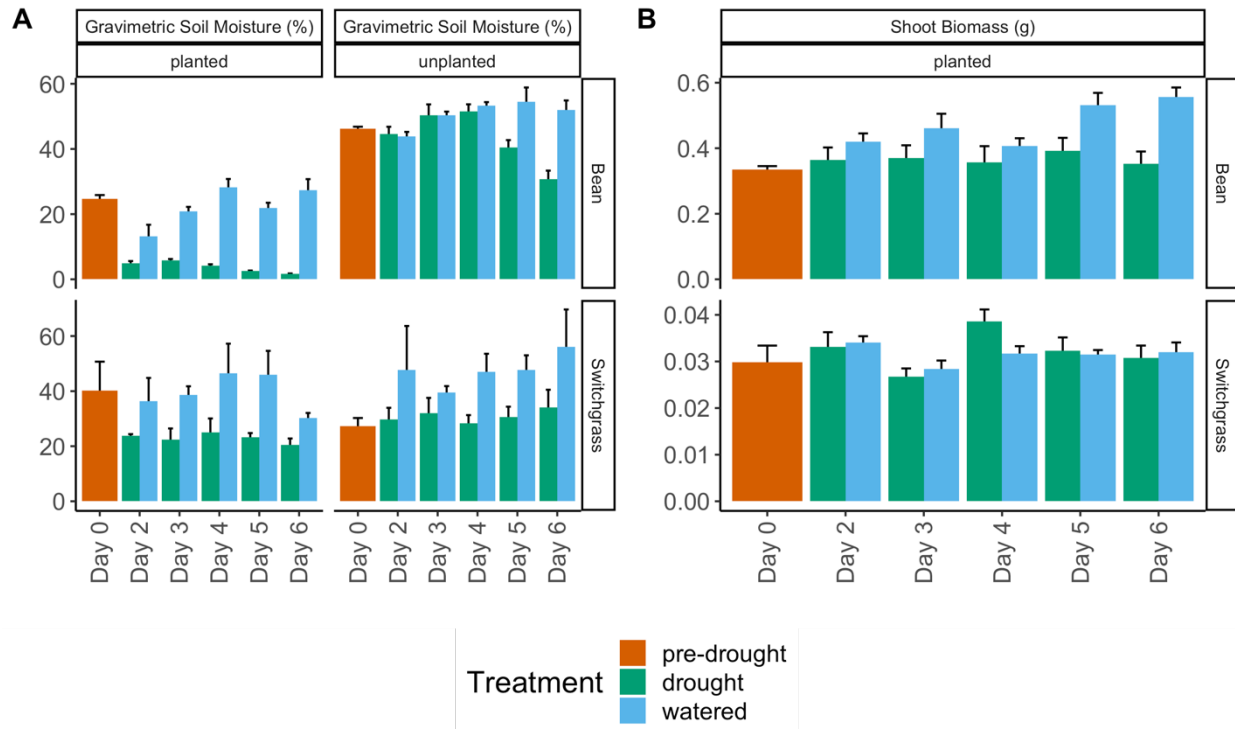
511

512 The watered samples in both bean and switchgrass had relatively high and stable gravimetric soil  
513 moisture content over the different time points for both planted and unplanted conditions (**Figure 2A**). In  
514 bean, the drought samples had a decrease in moisture content over time for both planted and unplanted  
515 soils, as expected. In switchgrass, drought samples had comparable moisture content over time for both  
516 planted and unplanted soils. The final sample (day 6) for switchgrass had the lowest mean soil moisture in  
517 planted soils and the highest mean moisture in unplanted soils. In both bean and switchgrass, the  
518 unplanted drought treatments retained higher soil moisture than the planted ones. There were interaction  
519 effects of drought, sampling day, and plant presence on bean soil moisture (Three-way ANOVA type III  
520 test,  $F=2.84$ ,  $P=0.03$ , **Table S4**), suggesting the effects of each of these factors depended on the levels of  
521 the other factors. In switchgrass, there were main effects of drought on soil moisture (three-way ANOVA  
522 Type III test,  $F=28.43$ ,  $P<0.001$ ) and of plant presence on soil moisture ( $F=6.66$ ,  $P=0.01$ ).

523

524 In bean plants, the shoot biomass was always higher in the watered samples as compared to drought (two-  
525 way ANOVA Type III test,  $F=21.18$ ,  $P<0.001$ ), and the watered plants increased in biomass over time  
526 (**Figure 2B**). There were no differences in switchgrass biomass between watered and drought samples  
527 over time (two-way ANOVA,  $F=0.30$ ,  $P=0.59$ ). Switchgrass shoot biomass fluctuated mildly over time  
528 but had no obvious trend (two-way ANOVA Type III test,  $F= 3.43$ ,  $P=0.02$ , **Table S4**).

529



530

531 **Figure 2: A) Gravimetric soil moisture of bean (sample size (n)=110) and switchgrass (sample size**  
 532 **(n)=110) planted and unplanted soils at different time points for drought and watered samples. B)**  
 533 **Shoot biomass of bean (sample size (n)=55) and switchgrass (sample size (n)=55) at different**  
 534 **sampling days for drought and watered samples.**

535

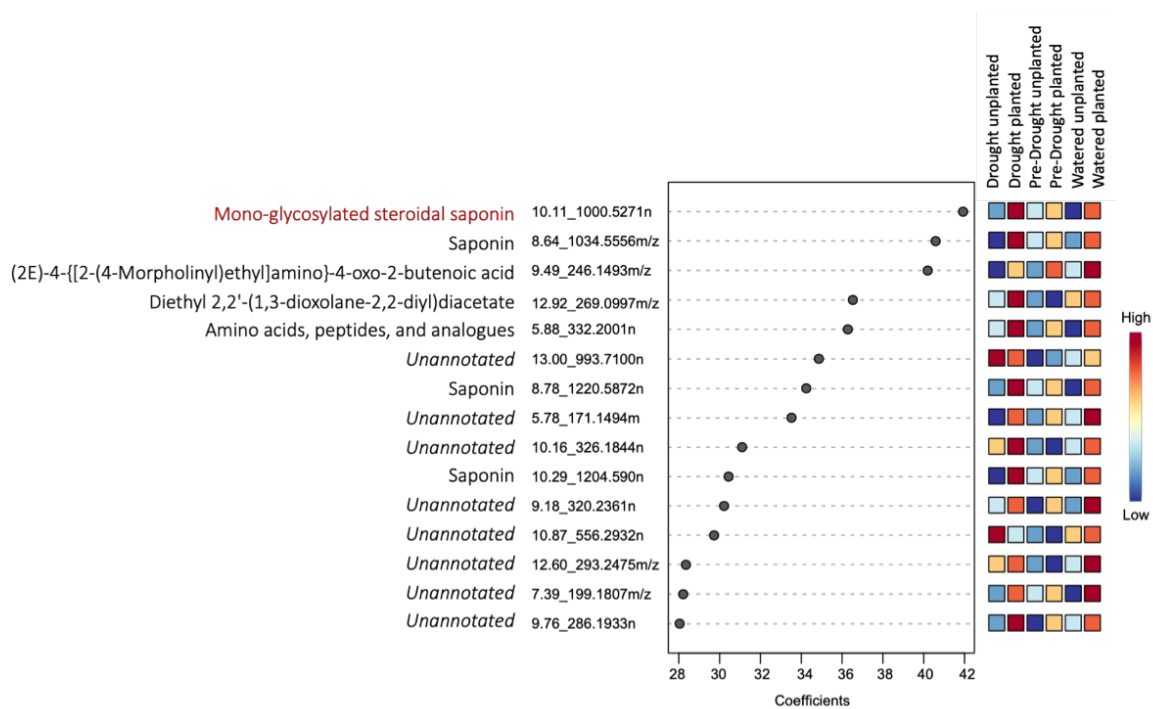
536 *Metabolome responses of drought for switchgrass*

537 Previous work has shown that switchgrass alters its metabolism during drought [54]. Given the absence  
 538 of switchgrass shoot biomass response to the drought, we performed untargeted LC-MS-based  
 539 metabolomics of switchgrass soils to assess any drought-induced changes in plant metabolites that could  
 540 impact its microbiome. In addition, we also profiled the global changes that occurred in the metabolomes  
 541 of switchgrass shoots and roots caused by the drought treatment. The metabolite profiling revealed 3,532  
 542 distinct metabolite features whose maximum abundance among the biological samples was  $\geq 500$  counts  
 543 **(Supplemental Data 1-3).**

544

545 A comparative analysis revealed 1051 root-, 538 shoot- and 231 soil-specific features (**Figure S4A**). The  
 546 root and shoot shared 1551 features, the root and soil shared 883 features, and the leaf and soil shared 750  
 547 features, while 736 features were shared by all three groups (**Figure S4A**). The planted rhizosphere soils  
 548 differed from the unplanted ones and the planted soil collected on day 0 (PERMANOVA, planted versus

549 unplanted,  $F=11.26$ ,  $R^2=0.27$ ,  $P=0.001$ , **Figure S4B**). There also were differences between day 0 and day  
 550 6 soils, indicating an overall effect of time (PERMANOVA  $F=3.55$ ,  $R^2=0.1$ ,  $P=0.02$ ).  
 551 While the scatter plot suggested divergence between the drought and watered metabolomes for planted  
 552 soils (**Figure S4C**), these overarching trends were not statistically supported (PERMANOVA  $F=2.2$ ,  
 553  $R^2=0.15$ ,  $P=0.07$ ). However, several metabolite features strongly differentiated the treatments. Saponins  
 554 most differentiated the switchgrass metabolite soil profiles across the planted and drought samples (the  
 555 metabolite annotation confidence levels can be found in **Supplemental Data 4**). Among the  
 556 discriminating features, *10.11\_1000.5271n* was the most explanatory (highest variable of importance,  
 557 VIP, **Figure 3**). This metabolite feature was previously identified as a mono-glycosylated steroidal  
 558 saponin [8] (**Figure S5**). Some other most important features were also annotated as the specialized  
 559 metabolites, including the root-accumulating diterpenoids with elevated concentrations in drought-treated  
 560 planted soils (**Figure S4D, Supplemental Data 4**). This finding is consistent with the studies  
 561 investigating longer drought exposure [8, 54]. The higher abundances of these specialized metabolites in  
 562 the drought samples from the planted soils suggest that switchgrass could release them into the  
 563 rhizosphere soil when stressed by drought. Additional metabolite results are provided in the  
 564 Supplemental Materials.  
 565



566

567 **Figure 3. VIP coefficient score plot from a PLS-DA model showing the top 15 variables (features)**  
568 **of importance to differentiate the different switchgrass soil types. Feature *10.11\_1000.5271n*, a**  
569 **previously identified switchgrass saponin, had the highest coefficient and is thus the most important**  
570 **detected variable during the drought treatment. At the end of each feature name, ‘m/z’ stands for**  
571 **mass-to-charge ratio and ‘n’ stands for neutral mass.**

572

573 *No detection of relative changes in rhizobiome size over time*

574 To proxy relative changes in microbiome size, we considered the recovered DNA concentration from the  
575 rhizosphere soil before dilution and normalization for sequencing. Linear models suggested that there  
576 were no supported trends in DNA concentrations over time for any of the treatments (**Figure S6**, all  $P >$   
577 0.05)

578

579 *Rhizobiome sequencing summary: total and active bacteria communities*

580 For the total community (DNA) data, total non-chimeric merged reads for both bean and switchgrass were  
581 20,526,147. 19,938,535 reads remained after filtering out mitochondria, chloroplast, and contaminant  
582 OTUs, out of which 10,204,741 and 9,733,794 reads were attributed to switchgrass and bean,  
583 respectively. For the active community (cDNA) data, total non-chimeric merged reads for both bean and  
584 switchgrass were 22,018,663, of which 21,796,243 reads remained after filtering out mitochondria,  
585 chloroplast, and contaminant OTUs, out of which 11,799,699 and 9,996,544 reads were attributed to  
586 switchgrass and bean, respectively. The total number of taxa (DNA) and active taxa (based on DNA/RNA  
587 ratios) were 21,407 and 8,732, for bean samples and 17,331 and 7,539, respectively, for switchgrass  
588 samples.

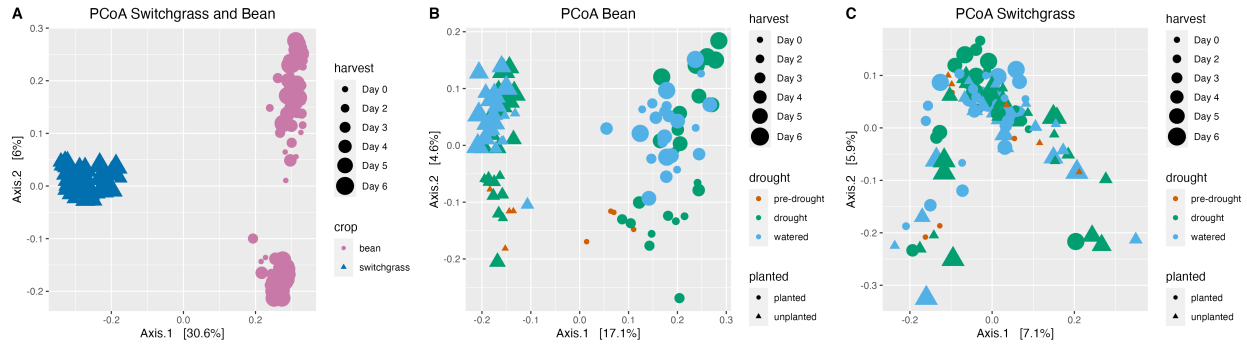
589

590 The microbiome data presented, henceforth, all pertain to the DNA counts of the active community  
591 members (16SrRNA:rRNA gene  $\geq 1$ ). The percent active taxa ranged from 40-60% of the total observed  
592 taxa in a sample. This establishes that an estimated half of the taxa detected in the DNA dataset were not  
593 observed or did not meet our activity criterion in the RNA dataset.

594

595 *Bean and switchgrass had distinct active rhizobiomes*

596 The literature shows that different plants harbor microbiomes [55-57]. Here, we also found different total  
597 (PERMANOVA Pseudo-F=163.02,  $R^2=0.44$ ,  $P=0.001$ ) and active rhizobiome structures for bean and  
598 switchgrass (**Figure S7**, **Figure 4A**, active community PERMANOVA Pseudo-F= 91.21,  $P= 0.001$ ,  $R^2=$   
599 0.30), with 3883 active OTUs shared between the crops. However, the overall beta dispersion was  
600 comparable across bean and switchgrass (PERMDISP  $F=2.95$ ,  $P=0.09$ ).



601

602

603 **Figure 4: A) Principal Coordinate Analysis (PCoA) comparing bean and switchgrass active**  
604 **community composition (sample size (n)=212). PCoA of B) bean (sample size (n)=105) and C)**  
605 **switchgrass (sample size (n)=107) comparing active communities of planted and unplanted soil**  
606 **samples, as well as comparisons across drought treatments: pre-drought, drought, and watered.**  
607 **Samples collected across all sampling days are shown.**

608

609 *Addressing hypothesis :1 Active rhizobiomes respond to drought and change progressively with drought*  
610 *severity.*

611 Effects of drought, sampling day (colinear with drought severity for the drought treatment), and drought:  
612 sampling day interactions were significant and of similar explanatory value for both bean and switchgrass  
613 (**Figure 4B-C**, PERMANOVA all  $P < 0.05$ , see **Table S5**). We further investigated the effect of the  
614 drought treatments (levels: drought, pre-drought, and watered) using post hoc tests. In switchgrass, there  
615 were differences between pre-drought and watered treatments (PERMANOVA Pseudo-F = 1.36,  $R^2 =$   
616  $0.02$ ,  $P=0.03$ ) and between the watered and drought condition (Pseudo-F= 1.55,  $R^2=0.02$ ,  $P=0.004$ ). In  
617 bean, there were differences between the pre-drought and drought condition (Pseudo-F= 1.79,  $R^2=0.03$ ,  
618  $P=0.02$ ), between the pre-drought and watered condition (Pseudo-F= 2.27,  $R^2=0.04$ ,  $P=0.007$ ), and  
619 between the drought and watered condition (Pseudo-F=2.1,  $R^2=0.02$ ,  $P=0.006$ ).

620

621 For the drought treatment in bean samples, planted rhizobiomes had lower richness (number of active  
622 OTUs) than unplanted ones (Three-way ANOVA,  $F=60.12$ ,  $P<0.001$ , **Table S6**). For switchgrass, there  
623 were no richness differences between the levels of different factors tested in the experiment, except for a  
624 weak interaction observed between planted and drought treatments (Three-way ANOVA  $F=4.30$ ,  $P<0.05$ ,  
625 **Table S6**).

626

627 We hypothesized that rhizobiomes in the drought samples would change faster and to a greater extent  
628 than the watered ones. We also hypothesized that the presence of the plant might suppress or stabilize

629 these dynamics due to host-microbiome feedback, resulting in reduced and slower change in planted than  
630 unplanted soils. To test this, we assessed linear models of the active rhizobiome structure (beta diversity)  
631 over time and as compared to the pre-drought condition (**Figure S8**). In these models, the slopes  
632 represent the rates of change, and the intercepts represent their magnitudes. However, the linear models  
633 generally were not supported for changes in beta diversity, suggesting only incremental beta diversity  
634 changes given the short-term drought. The exception was detecting significant linear regression for  
635 changes in beta diversity in the switchgrass watered and planted rhizobiome ( $R^2 = 0.23$ ,  $P = 0.02$ ).

636  
637 While there were no detected differences in beta-dispersion (community variance) among switchgrass  
638 experimental factors, for bean rhizobiomes, there were differences detected among the drought  
639 treatments, with post-hoc tests revealing differences in dispersion between the watered and drought  
640 rhizobiomes (mean dispersion(watered) = 0.41, mean dispersion (drought) =0.44, PERMDISP Pseudo-  
641  $F = 13.56$ , d.f.= 1,  $P = 0.001$ ) and between the pre-drought and drought rhizobiomes (mean dispersion (pre-  
642 drought) = 0.40, mean dispersion (drought) = 0.44 , PERMDISP Psuedo- $F = 8,15$ , d.f.= 1,  $P = 0.01$ ), but  
643 not between the pre-drought and watered (PERMDISP  $P = 0.47$ ).

644  
645 There were statistically supported differences in community structure with drought and time/drought  
646 severity for both plants. Still, it is notable that there were no apparent differences in the temporal  
647 dynamics (rate and magnitude of change) across these treatments. In addition, average distance to the  
648 median in community structure (dispersion) was modestly higher in droughted bean rhizobiomes than in  
649 pre-drought and watered ones. Thus, hypothesis 1 was supported by the data.

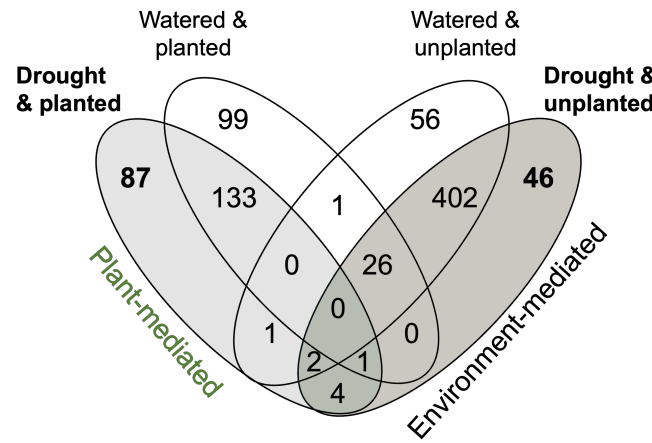
650  
651 *Addressing hypothesis 2: The plant mediates the responses of the active rhizobiome members to drought.*  
652 The most striking difference between the rhizobiomes of the two crops was whether a plant was present.  
653 Though the presence of a plant was a significant explanatory factor for both crops' rhizobiomes, it had the  
654 highest explanatory value of all the factors tested for bean (17% explanatory value, **Figure 4B**) and  
655 relatively low explanatory for switchgrass (3% explanatory value, **Figure 4C, Table S5**). Furthermore,  
656 the interaction between plant presence and drought was only significant in bean rhizobiomes  
657 (PERMANOVA Pseudo- $F = 1.84$ ,  $P = 0.03$ ) and not in switchgrass (PERMANOVA Pseudo- $F = 1.13$ ,  $P =$   
658  $0.18$ , **Table S5**). We conclude that whether the plant mediates rhizobiome responses to short-term  
659 drought depends on the plant. Thus, hypothesis 2 was partially supported and conditional on the plant  
660 species investigated. There were plant-mediated responses to drought for bean rhizobiomes but not for  
661 switchgrass.

662

663 Motivated by the plant-mediated effects of drought for bean, we next investigated the activated taxa that  
664 were uniquely associated with the planted drought condition in bean rhizobiomes. We identified 87 such  
665 taxa using indicator analysis (**Figure 5A**). We reasoned that the responses of these activated taxa are  
666 likely to be mediated, directly or indirectly, by the bean plant during drought.  
667

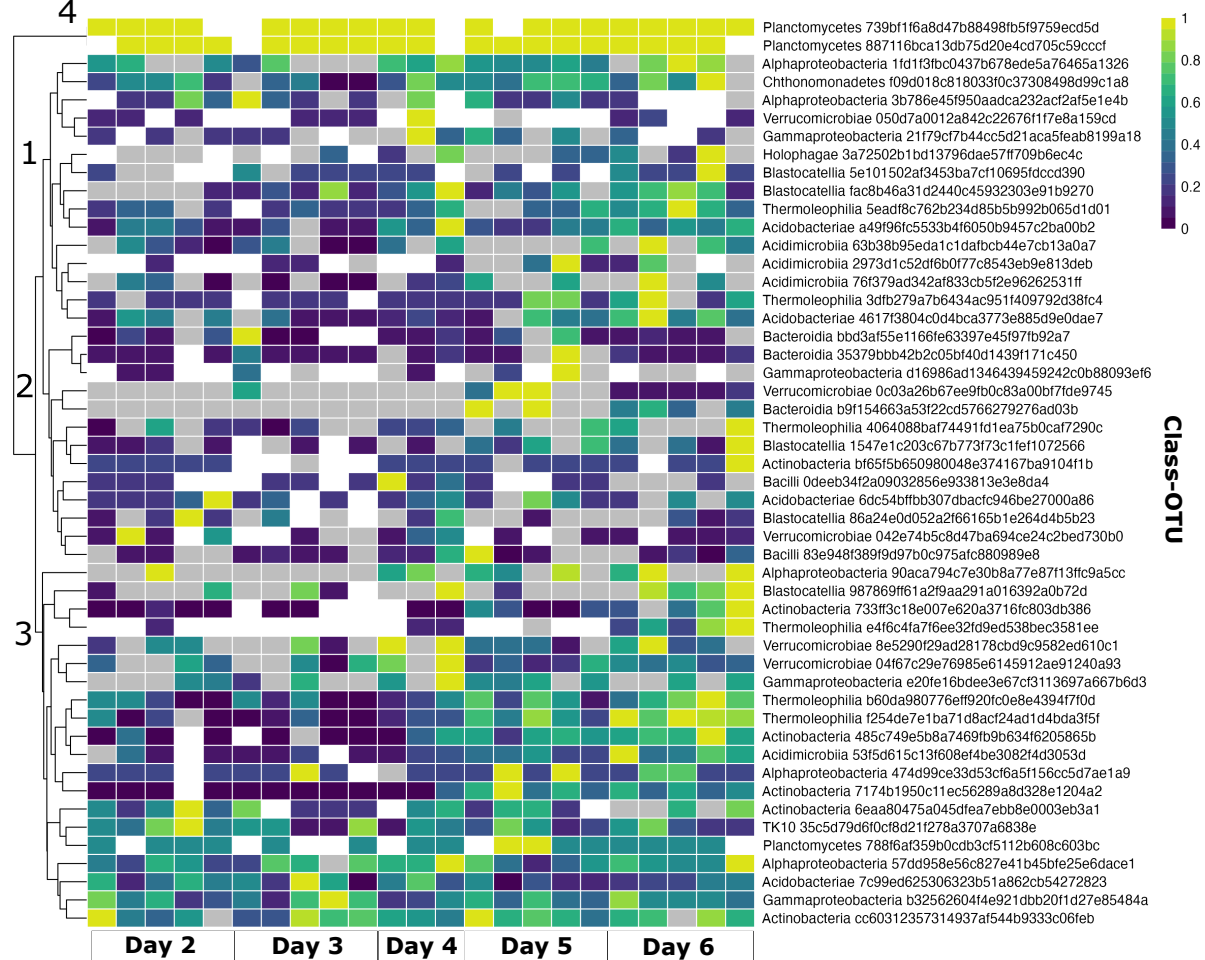
A

Activated Taxa in Bean



Activity dynamics of indicator taxa in bean drought planted condition  
(50 most abundant out of 87 indicators)

B



668

669



670 **Figure 5: A. Venn diagram showing the indicator species associated with each drought/planted**  
671 **condition in common bean (sample size (n)=203). B. heatmap showing the relative abundance**  
672 **dynamics of the top 50 most abundant and active OTUs from the 87 indicators associated with the**  
673 **bean planted drought condition. Inactive taxa are coded as “0” (zero), corresponding to the light**  
674 **gray cells. More abundant OTUs are more yellow, and less abundant are bluer. Taxa not detected**  
675 **in either DNA or RNA dataset is denoted as NA and colored as white in the heatmap. We**  
676 **standardized counts within an OTU relative to the maximum observed abundance value detected**  
677 **for that OTU across samples. Phantom taxa detected in >5% of samples were included. The legends**  
678 **on the right indicate classification at the Class level and the OTUID (sample size (n)=23).**

679

680 Focusing on the most abundant 50 indicators of the planted drought condition, we examined their activity  
681 dynamics over drought severity (**Figure 5B**). Several OTUs increased over time and with drought  
682 severity (enriched in Days 5-6, within clades 1 and 3), including members belonging to the classes  
683 Actinobacteria, Verrucomicrobiae, Acidobacteriae, Acidimicrobiia, Thermoleophilia, and  
684 Alphaproteobacteria. Several OTUs were detected consistently over time despite increasing drought  
685 severity (clade 4), including some belonging to Alphaproteobacteria, Planctomycetes,  
686 Gammaproteobacteria, and Actinobacteria (**Figure 5B**). These planted drought indicator taxa that were  
687 either enriched over time or stable are targets for follow-up research to understand their exact fitness  
688 advantages and potential benefits to the host in the drought environment.

689

690 Notably, while the indicator species analysis revealed several OTUs strongly associated with each  
691 drought and planting level for bean rhizobiomes, no indicators were discovered for switchgrass, further  
692 supporting the previous finding of no interaction of planting and drought for switchgrass.

693

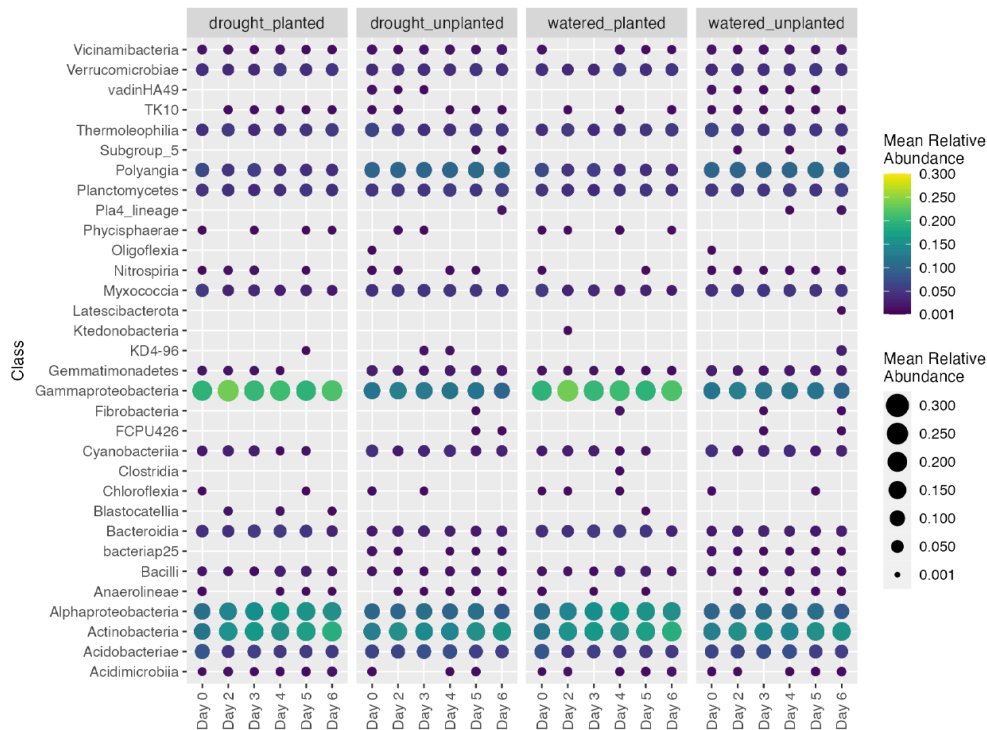
694 In the drought samples planted with bean, we noted that many stable or enriched OTUs belonged within  
695 few classes. Thus, we next compared the overall differences and changes in the relative abundances of  
696 these classes between the treatments for bean rhizobiomes (**Figure 6**). One motivation for this analysis  
697 was to understand what classes the plant retains during drought by comparing the planted and unplanted  
698 drought conditions. Only taxa associated with Blastocatellia were absent in the unplanted drought versus  
699 the planted drought rhizobiomes. However, Gammaproteobacteria (Kruskal-Wallis chi-squared = 23.96,  
700  $df = 1, P < 0.001$ ), Actinobacteria (Kruskal-Wallis chi-squared = 5.89,  $df = 1, P = 0.02$ ) and  
701 Alphaproteobacteria (Kruskal-Wallis chi-squared = 26.75,  $df = 1, P < 0.001$ ) were significantly higher in  
702 relative abundance in the planted compared to the unplanted treatments in bean under drought condition.  
703 Among these, Gammaproteobacteria (Kruskal-Wallis chi-squared = 31.59,  $df = 1, P < 0.001$ ) and

704 Alphaproteobacteria (Kruskal-Wallis chi-squared = 16.65, df = 1,  $P < 0.001$ ) were significantly increased  
 705 in planted compared to unplanted treatments in watered bean treatments samples. At the same time,  
 706 Actinobacteria was not statistically different between planted and unplanted treatments.

707

708 Another motivation was to identify potentially “lost” taxa during a drought that are normally associated  
 709 with the bean. We explored drought-sensitive taxa by comparing the planted drought to the planted  
 710 watered samples, reasoning that if any of these taxa are beneficial for the plant, they could be targets to  
 711 prioritize for recovery after a drought. However, there were no notable absences or decreases of the major  
 712 active classes in the planted soils across watered and drought conditions. Rather, the most notable  
 713 comparative difference among treatment groups was between the planted and unplanted conditions, as  
 714 several classes were only associated with the unplanted soils, including vadinHa49, Subgroup 5,  
 715 Pla4\_lineage, Oligoflexia, FCPU426, and bacteriap25. These classes are potentially selected against or  
 716 non-competitive in the presence of the plant.

717



718

719

720 **Figure 6. Temporal changes in relative abundances of active taxa that were the most abundant**  
 721 **bacterial classes in bean (n=56) pre-drought and drought conditions.**

722

723

## 724 **Discussion**

725

726 Soil microbes can be important in plant drought tolerance [58]. Improving our understanding of root-  
727 associated soil microbial responses during drought stress can help improve a plant’s resilience through  
728 targeted microbiome manipulation. This study used microbiome and metabolomics data to understand  
729 microbial community shifts during short-term drought in two plant species – common bean and  
730 switchgrass. We focused on the subset of the bacterial community that was likely to be active (RNA-  
731 based assessment) during drought by comparing unplanted and planted rhizosphere soils to watered soils.  
732 These comparisons were critical to tease apart plant-mediated responses to drought, which otherwise can  
733 be challenging to explore as the stress of drought independently will alter soil microbial communities and  
734 direct many taxa to dormancy.

735

736 We deliberately included rhizosphere soils from fields with a recent legacy of growing each crop and thus  
737 had an imprint of their typically-associated rhizosphere microbiota. We predicted that in doing so, the  
738 microbiome assembly during short-term drought would be biologically relevant to the field and crop. The  
739 major differences in the active community structures between bean and switchgrass soils were maintained  
740 in drought and watered conditions and over time, with no indication of convergence across the two  
741 communities given the drought. We detected no specific taxa that were generally selected in drought  
742 across the different crops, neither at the OTU or family taxonomic level. While other studies have  
743 suggested that there could be similarities in the drought response of the microbiome across  
744 phylogenetically distant and related host species [59], our study suggests that developing universal,  
745 “drought-supportive” targets for microbiome manipulation could instead require solutions tailored to  
746 different plant families. Despite differences in rhizobiome compositions and different host sensitivities to  
747 drought, we observed that both bean and switchgrass rhizobiomes changed comparably given water  
748 reduction and over time with increasing drought severity. The major difference between the crops was  
749 that the presence of a plant was more important for the bean rhizobiome response to drought. At the same  
750 time, the plant presence was largely inconsequential for the switchgrass rhizobiome. Generally,  
751 switchgrass rhizobiome differences between the planted and unplanted treatments were weak relative to  
752 the bean.

753

754 For the bean rhizobiomes, several OTUs within classes Actinobacteria and Alphaproteobacteria were  
755 enriched in the planted samples compared to unplanted samples during drought. Actinobacteria have  
756 generally been seen to be enriched during drought in plant roots [60, 61] and are well-known for their  
757 versatile capabilities ranging from stress tolerance to bioremediation. We also found that

758 Alphaproteobacteria and Actinobacteria increased relative abundance over drought severity for bean-  
759 planted treatments during drought.

760

761 Physiological and morphological differences between bean and switchgrass could explain the differences  
762 observed in their assembly of the rhizobiome during drought stress. Switchgrass is known to be generally  
763 drought-tolerant [35]. Its extensive and deep root architecture could indicate better resource and water  
764 allocation that benefits switchgrass during drought, making it less reliant on beneficial support from the  
765 plant's microbiome. On the other hand, bean plants could benefit from directed manipulation of the  
766 microbiome during short-term drought, given its wide range of drought susceptibility across genotypes  
767 [37]. A more expansive analysis of the microbiomes of perennial versus annual life histories and among  
768 plants with different physiologies could provide insights into expectations of different microbiome  
769 drought responses. It could be that the rhizosphere microbiomes of perennial plants are more generally  
770 more resistant to stress. Notably, in our study, the metabolome results show that the switchgrass plants  
771 were responsive to the drought and had clear shifts in some expected features of their metabolite profile  
772 as compared to the watered and unplanted conditions. Thus, the metabolite and microbiome changes were  
773 not necessarily coupled at the temporal scale of the experiment (days), nor were they collectively  
774 indicative of the plant-mediated shift in metabolism that subsequently drove a shift in its rhizosphere  
775 microbiome. The observation that the switchgrass microbiome was relatively stable drought could be due  
776 in part to specialized metabolites that switchgrass produces to combat stress.

777

778 Saponins are a group of specialized plant metabolites that can exhibit biological activities such as  
779 antibacterial, antifungal, and cytotoxic properties [9, 62]. Saponins are secreted from plant roots into the  
780 rhizosphere and can alter soil bacterial communities [63]. Saponins also have a role in detoxifying free  
781 radicals [64] thus helping combat the oxidative stress imposed by drought. In this study, saponins were  
782 increased in concentration in the planted switchgrass rhizospheres under drought. In other studies,  
783 saponin concentrations also increased in leaves of different plant species under water-deficit conditions  
784 [65, 66] [2], suggesting similar impacts of drought in other plant. Actinobacteria are known to degrade  
785 saponins in the rhizosphere. They are also known as assimilators of saponins, which can explain the co-  
786 occurrence of saponins with Actinobacterial OTUs in our study [63]. In addition, the increased  
787 accumulations of diterpenoids were also observed in the planted rhizosphere soils stressed by drought.  
788 This is consistent with a recent metabolomics study that showed that diterpenoids are also enriched in  
789 switchgrass roots after a longer period of drought stress (five weeks) [54].

790

791 We identified several bean OTUs that selectively increased only with increasing drought severity. This  
792 included OTUs belonging to classes Alphaproteobacteria, Blastocatellia, Actinobacteria,  
793 Thermoleophilia, Gammaproteobacteria, Verrucomicrobiae, Acidobacteriae, and Acidimicrobiia. These  
794 short-term stress responders are critical for further exploration as they may be potential bioinoculant  
795 targets.

796

797 Our study suggests that considering the active members of the rhizobiome provides complementary  
798 insights into the ecology of drought and can suggest different responses of taxa. This study is different in  
799 its focus on detecting active taxa by rRNA:rRNA gene ratios. Other studies typically consider in  
800 aggregate the total rhizobiome community using DNA-based sequencing, including active, inactive, and  
801 deceased members. From these DNA-based studies, the literature reports that soils with limited moisture  
802 and under drought conditions have reduced the richness of Proteobacteria and Verrucomicrobia [67].  
803 However, in our study, members of these groups were active and abundant in the rhizobiome of drought,  
804 unplanted bean rhizosphere soil. Out of the 46 bean taxa uniquely detected in the unplanted drought,  
805 several OTUs belonging to these phyla were detected as active, persistent, and in some cases, relatively  
806 high abundance. This included, for example, two Verrucomicrobiae OTUs  
807 (OTU#543e7a460e393e133062fbfae2f5675e and OTU#37184df09489f56490af1670195a8be5) as well as  
808 a Gammaproteobacteria OTU (OTU#da195c41827a582bf1b4bd7cb9007452) and Alphaproteobacteria  
809 OTU (OTU#fa5570e95914a27603f466f8d4789a26).

810

811 Furthermore, though Actinobacteria are widely detected in drought soil [68-70], out of the 46 unique taxa  
812 for the unplanted drought samples, we only found one OTU belonging to Actinobacteria (OTU#  
813 78b14ac22ebd6a508a7f122a42beb87a). Interestingly, we did not find this Actinobacteria OTU in the  
814 planted drought samples in bean rhizobiomes, though several other OTUs were detected from  
815 Actinobacteria. It could be that Actinobacteria are persistent but perhaps dormant or not highly active  
816 under some drought conditions and that specific OTUs have distinct responses during drought treatments  
817 that could change under the influence of a plant. Thus, broad generalizations at class or family levels  
818 remain challenging and specific cases must be evaluated at deeper taxonomic resolution.

819

820 The RNA: DNA ratio method also has limitations that must be carefully considered in interpreting these  
821 results. For example, we set a ratio threshold of 1 for the detection of active taxa, which removed several  
822 taxa from consideration and, as such, retained only those that met our criteria. Thus, we could be missing  
823 the detection of additional active OTUs, especially among rare taxa. Other studies have applied RNA:  
824 DNA gene ratios ranging from 0.5-2 [28], with five considered overly conservative [20]. However, after

825 the included taxa were designated, it was important to use the DNA sequence counts to designate taxon  
826 relative abundances (and not the RNA counts) to reduce bias due to the expected variation in transcription  
827 from taxon to taxon [71].

828

829 Another consideration of this study is the intense and short duration of the drought exposure,  
830 accumulating over six days. Our goal was to understand immediate drought response and selection of  
831 members in the rhizosphere, so we deliberately chose a shorter timeframe and pulse event to avoid the  
832 inclusion of longer-term assembly dynamics. As the rhizobiome assembly continues with drought and re-  
833 wetting, its activity dynamics are expected to change. Thus, this work presents the immediate  
834 consequences of short-term rhizobiome drought, and additional work is needed to understand longer-term  
835 consequences.

836

### 837 **Conclusions**

838 This study assessed the active rhizobiome responses to short-term drought for two different crops:  
839 switchgrass and bean. Both plants' active rhizobiomes changed given the drought and over time with  
840 drought intensity, but the response was more muted for switchgrass than for bean. Using unplanted  
841 controls, we determined that there were plant-mediated effects of drought on the active rhizobiome only  
842 for bean plants and not for switchgrass, suggesting that different plants have different reliance on or  
843 engagements with or their rhizobiomes in drought. There was unique recruitment of taxa to drought bean  
844 plants, and we could distinguish among the plant- and environment-responsive taxa. This work identifies  
845 rhizobiome taxa that may be recruited by or lost by bean plants during drought, which can be targeted to  
846 improve understanding and tested for plant benefits.

847

### 848 **Declarations**

#### 849 **Ethics approval and consent to participate**

850 Not applicable

851

#### 852 **Consent for publication**

853 Not applicable

854

#### 855 **Availability of data and materials**

856 Sequence data for this study has been deposited to NCBI Sequence Read Archive under project number  
857 PRJNA862978. Scripts for analyzing the microbial community for this study can be found on GitHub at  
858 [https://github.com/ShadeLab/PAPER\\_DroughtRhizobiome\\_Bandopadhyay\\_2023](https://github.com/ShadeLab/PAPER_DroughtRhizobiome_Bandopadhyay_2023).

859

860 **Competing interests**

861 The authors declare that they have no competing interests.

862

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870

871 **Authors contributions**

872 AS and AWB designed the experiment. SB, XL, AWB conducted experiments. SB, XL, and AS  
873 generated figures. SB, XL, RLL and AS analyzed the data. SB prepared the first draft. All authors read  
874 and approved the final manuscript.

875

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1070 Figure Legends

1071

1072 **Figure 1.** The experimental design used in the greenhouse study. Day 2 to day 6 correspond to increased  
1073 drought severity for the 6-day drought treatment.

1074

1075 **Figure 2.** A) Gravimetric soil moisture of bean (sample size (n)=110) and switchgrass (sample size  
1076 (n)=110) planted and unplanted soils at different sampling days for drought and watered conditions. B)  
1077 Shoot biomass of bean (sample size (n)=55) and switchgrass (sample size (n)=55) at different sampling  
1078 days for drought and watered conditions.

1079

1080 **Figure 3.** VIP coefficient score plot from a PLS-DA model showing the top 15 variables (features) of  
1081 importance to differentiate the different switchgrass soil types. Feature *10.11\_1000.5271n*, a previously  
1082 identified switchgrass saponin, had the highest coefficient and is thus the most important detected  
1083 variable during the drought treatment. At the end of each feature name, ‘m/z’ stands for mass-to-charge  
1084 ratio and ‘n’ stands for neutral mass.

1085

1086 **Figure 4.** A) Principal Coordinate Analysis (PCoA) comparing bean and switchgrass active community  
1087 composition (sample size (n)=212). PCoA of B) bean (sample size (n)=105) and C) switchgrass (sample  
1088 size (n)=107) comparing active communities of planted and unplanted soil samples, as well as  
1089 comparisons across drought treatments: pre-drought, drought, and watered.

1090

1091 **Figure 5.** A) Venn diagram showing the indicator species associated with each drought/planted condition  
1092 in common bean (sample size (n)=203). B) Heatmap showing the relative abundance dynamics of the top  
1093 50 most abundant and active OTUs from the 87 indicators associated with the bean planted drought  
1094 condition. Inactive taxa are coded as “0” (zero), corresponding to the light gray cells. More abundant  
1095 OTUs are more yellow, and less abundant are bluer. Taxa not detected in either DNA or RNA dataset is  
1096 denoted as NA and colored as white in the heatmap. We standardized counts within an OTU relative to  
1097 the maximum observed abundance value detected for that OTU across samples. Phantom taxa detected in  
1098 >5% of samples were included. The legends on the right indicate classification at the Class level and the  
1099 OTUID (n=23).

1100

1101 **Figure 6.** Dot plots demonstrate the temporal changes in relative abundances of active taxa that were the  
1102 most abundant bacterial classes in bean (n=56) pre-drought and drought conditions.

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1106 Supplemental Tables

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1108 **Table S1.** Chemical and physical analysis of field-collected soils for bean and switchgrass.

1109

1110 **Table S2.** Water addition schedule by treatment for switchgrass.

1111

1112 **Table S3.** Water addition schedule by treatment for bean plants.

1113

1114 **Table S4.** Differences in gravimetric soil moisture and shoot biomass by experimental factors as assessed  
1115 by a three-way ANOVA with interactions (Type III). The F-statistics are reported with significance  
1116 indicated at  $p < 0.05^*$ ,  $p < 0.01^{**}$ , and  $p < 0.001^{***}$ .

1117

1118 **Table S5.** Permuted analysis of variance analysis to determine differences in microbiome structure given  
1119 experimental treatments (a.k.a. factors). Sums of squares, degrees of freedom, R-squared values, and  
1120 Pseudo F values are reported. The significance of each test is indicated at  $p < 0.05^*$ ,  $p < 0.01^{**}$ , and  
1121  $p < 0.001^{***}$ .

1122

1123 **Table S6.** Differences in the number of observed active OTUs (richness) across experimental factors, as  
1124 assessed using a three-way ANOVA with interactions (Type III tests). F values are reported, and  
1125 significance is indicated at  $p < 0.05^*$ ,  $p < 0.01^{**}$ , and  $p < 0.001^{***}$ .

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1127

1128 Supplemental Figures

1129

1130 **Figure S1.** Photo of the experimental setup in the greenhouse for A) switchgrass (inset- tube showing  
1131 switchgrass roots grown to fill to the inside edge of the tube) and B) bean.

1132

1133 **Figure S2.** Sample collection for the switchgrass untargeted metabolomics. **A.** Sample panel with the  
1134 numbers of replicates indicated. **B.** The drought and watered switchgrass tissue and planted soil replicates  
1135 were combined from the samples collected from individual plants.

1136

1137 **Figure S3.** Sequencing quality control checks. (A) Library sizes of true samples (blue) and negative  
1138 control (red) samples for both DNA and cDNA-sequenced samples. (B) OTUs were designated as either  
1139 true or false contaminants based on their prevalence in true samples and negative controls using package  
1140 decontam in R. (C) Rarefaction curves for all DNA and cDNA samples (pre-rarefaction). Curves start to  
1141 plateau out at 13-15k reads (n=438).

1142

1143 **Figure S4.** The untargeted metabolomics revealed metabolite differences between the soil types. A. Venn  
1144 diagram documenting comparative metabolite profiles and the number of specific and shared features  
1145 identified in switchgrass root, shoot, and soil. B. PCA scores plot of the metabolomes of different soil  
1146 types showing the principal components PC1 and PC3. C. 3-D scatter plot of PCA scores plot of the  
1147 metabolomes of different soil types showing PC1, 2, and 3. E. Heatmap showing relative abundances of  
1148 the top 50 PLS-DA most important features (vertical axis) across the biological samples (horizontal axis).  
1149 For the hierarchical clustering of features, Euclidean and Ward.D were used as the distance measure and  
1150 clustering method, respectively (sample size root (n)=30, shoot (n)=30, soil(n)=49).

1151

1152 **Figure S5.** MS/MS spectra for the feature *10.11\_1000.5271n*, obtained by the positive LC-MS analysis,  
1153 DDA mode. Top trace: survey scan; bottom trace: MS/MS. The molecular ions and sapogenin core  
1154 fragment ions were annotated. M, molecular ion.

1155

1156 **Figure S6.** Recovered DNA concentrations of samples by crop, treatment, and time point. DNA was  
1157 measured using Qubit 2.0 with the dsDNA BR assay kit.

1158

1159 **Figure S7.** Stacked bar plots depicting the Class-level, active bacterial community composition by  
1160 treatment for bean and switchgrass. All samples collected are shown (n=212).

1161

1162 **Figure S8.** For each experimental treatment, changes in Bray Curtis similarity to the pre-drought  
1163 community over time and/or with increasing severity. Sampling day is indicated by the symbol size and  
1164 treatment by colors (blue is planted, green is unplanted) and line type (solid is drought, and dashed is  
1165 watered). The linear regressions were insignificant for any treatment except for the watered, planted  
1166 switchgrass (blue, dashed line on the right panel).

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1168

#### 1169 **Supplemental Data**

1170 **Data 1.** The metabolite features identified from Cave-in-Rock shoot tissues (with abundance  $\geq 500$ ) by  
1171 the positive mode LC-MS analysis.

1172

1173 **Data 2.** The metabolite features identified from Cave-in-Rock root tissues (with abundance  $\geq 500$ ) by the  
1174 positive mode LC-MS analysis.

1175

1176 **Data 3.** The metabolite features identified from all soil samples (with abundance  $\geq 500$ ) by the positive  
1177 mode LC-MS analysis.

1178

1179 **Data 4.** The top 50 PLS-DA important metabolite features (obtained by positive mode LC-MS analysis).

1180 Annotations were performed by searching the online mass spec databases through the Progenesis QI or

1181 using the CANOPUS machine function built in the SIRIUS 4 (<https://bio.informatik.uni-jena.de/sirius/>)

1182 (Materials and Methods). The ones annotated by CANOPUS are in boldface. <sup>a</sup> Compounds were

1183 previously reported in [54]; <sup>b</sup> Compounds were previously reported in [8]. <sup>c</sup> Identification level (A; B; C)-

1184 (A) standard or NMR; (B(i)) confident match based on MS/MS and (B(ii)) confident match using in-silico

1185 MS/MS approaches and (B(iii)) partial match based on MS/MS and (C(i)) confident match based on MSn

1186 and (C(ii)) confident match using in-silico MSn approaches and (C(iii)) partial match based on MSn; (D)

1187 MS only. These are based on the criteria for metabolite identification in [45].