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1 **The plant circadian clock influences rhizosphere community structure and function**

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20 **Abstract**

21 Plants alter chemical and physical properties of soil, and thereby influence rhizosphere microbial
22 community structure. The structure of microbial communities may in turn affect plant
23 performance. Yet, outside of simple systems with pairwise interacting partners, the plant genetic
24 pathways that influence microbial community structure remain largely unknown, as are the
25 performance feedbacks of microbial communities selected by the host plant genotype. We
26 investigated the role of the plant circadian clock in shaping rhizosphere community structure and
27 function. We performed 16S rRNA gene sequencing to characterize rhizosphere bacterial
28 communities of *Arabidopsis thaliana* between day and night time points, and tested for
29 differences in community structure between wild-type (Ws) *vs*. clock mutant (*toc1-21*, *ztl-30*)
30 genotypes. We then characterized microbial community function, by growing wild-type plants in
31 soils with an overstory history of Ws, *toc1-21* or *ztl-30* and measuring plant performance. We
32 observed that rhizosphere community structure varied between day and night time points, and
33 clock misfunction significantly altered rhizosphere communities. Finally, wild-type plants
34 germinated earlier and were larger when inoculated with soils having an overstory history of
35 wild-type in comparison to clock mutant genotypes. Our findings suggest the circadian clock of
36 the plant host influences rhizosphere community structure and function.

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39 **Introduction**

40 In comparison to unvegetated soils, the presence of plants dramatically affects the
41 structure of soil microbial communities. Plant roots affect the physical as well as chemical
42 environment through the exudation of carbon into the rhizosphere zone, which immediately
43 surrounds the roots (Bais *et al.*, 2006; Jones *et al.*, 2009; Dennis *et al.*, 2010). Rhizosphere
44 microbial community structure is dynamic and changes over the course of plant development
45 (Lundberg *et al.*, 2012), in part due to changes in exudation (Chaparro *et al.*, 2014). Although
46 much is known about rhizosphere assembly dynamics on longer time scales, there is currently
47 little information regarding assembly dynamics on shorter, diurnal time scales. Further, while
48 plant exudation may "feed-down" and affect microbial community structure, rhizosphere
49 communities can "feed-up" and affect plant performance, by increasing plant access to nutrients
50 (Çakmakçı *et al.*, 2001; Chen *et al.*, 2002; Richardson *et al.*, 2009; Richardson and Simpson,
51 2011), relieving abiotic stress (Zolla *et al.*, 2013), suppressing pathogens (Mendes *et al.*, 2011,
52 2013), altering phenology (Wagner *et al.*, 2014; Panke-Buisse *et al.*, 2014), and promoting plant
53 growth (Bashan, 1998; Lugtenberg and Kamilova, 2009; Henning *et al.*, 2016). Some plant
54 species, such as many legumes, have developmental genetic mechanisms that attract explicitly
55 beneficial nitrogen-fixing rhizobia taxa (Bravo *et al.*, 2016). The extent to which plants may
56 attract complex beneficial communities remains largely unclear.

57 The use of experimental genetic lines available in plant model species may reveal
58 specific genetic paths that affect microbial community structure. Comparing mutant *vs*. wild-type
59 plants of *Arabidopsis thaliana*, Lebeis *et al.* (2015) observed that salicylic acid, an immune
60 signaling molecule, altered rhizosphere bacterial community structure. This finding suggests that
61 genes regulating physiological traits, such as immune response, may play a role in shaping

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62 rhizosphere communities. Genes regulating additional physiological traits such as gas-exchange
63 and specifically carbon assimilation may also be worth examining, due to their effects on the
64 photoassimilate pool available for allocation to root growth and for carbon exudation into the
65 rhizosphere. More generally, the comparison of phenotypes between single-locus mutant
66 genotypes with those expressed by wild-type genotypes removes potentially confounding effects
67 of variation segregating elsewhere in the genome, and enables isolation of pathway-specific
68 effects. Naturally occurring large-effect alleles at causal loci could in some cases play a role in
69 shaping microbial community structure in natural plant populations or could be manipulated in
70 crop species to improve plant growth.

71 Changes in the presence-absence (or abundance) of just a few microbial taxa can affect
72 plant performance, due to the vast number of root-associated microbial cells and functions
73 (Henning *et al.*, 2016). Across diverse ecosystems, community structure and function are related
74 (Tilman *et al.*, 1997; Talbot *et al.*, 2014), including in plant-rhizosphere associations and in cases
75 where microbial community membership changes by one to few taxa. For instance, Henning et
76 al. (2016) observed that the addition of single bacterial taxa to the rhizosphere of *Populus*
77 *trichocarpa* led to drastic changes in plant growth traits. Similarly, Zolla et al. (2013) observed
78 differences in drought response by *Arabidopsis thaliana* plants grown in soil that differed in
79 community structure as a consequence of overstory history. Thus, differences in rhizosphere
80 community structure can lead to differences in rhizosphere community function as estimated
81 from plant performance.

82 In the current study, we tested the role of the plant circadian clock in determining
83 rhizosphere community structure and function, where function was measured as plant
84 performance. The circadian clock regulates up to 30% of the transcriptome, and affects diverse

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85 processes including patterned fluxes of carbon into (stomatal conductance, carbon assimilation)
86 and out of (exudation) the plant on a diurnal scale (Watt and Evans, 1999; Harmer *et al.*, 2000;
87 McClung, 2006; Covington *et al.*, 2008; Badri and Vivanco, 2009; Harmer, 2009; Greenham and
88 McClung, 2015). We hypothesized that the circadian clock could shape rhizosphere community
89 structure on a diurnal scale, if community structure responds to diurnally patterned fluxes of
90 carbon into the rhizosphere, that is, we anticipated that microbial community structure might
91 vary over the course of 24 hrs. We further hypothesized that rhizosphere communities of plant
92 genotypes harboring clock mutations could differ from wild-type plants, due to differences in
93 physiological phenotypes. Specifically, mutations in the clock genes *TIMING OF CAB*
94 *EXPRESSION 1* and *ZEITLUPE* lead plants to express altered clock period, or the duration of
95 one circadian cycle (Millar *et al.*, 1995; Kim *et al.*, 2005). As a consequence of altered clock
96 function, clock mutants express distinct physiological phenotypes, including reduced carbon
97 assimilation, chlorophyll content, and stomatal conductance (and thus root water uptake) relative
98 to wild-type plants under 24 hr environmental cycles (Dodd *et al.*, 2004, 2005). Clock
99 misfunction may influence rhizosphere communities, if for instance the reduced flux of carbon
100 into plants influences the flux of carbon exudation (Thornton *et al.*, 2004) or if shifts in plant
101 water use alter soil water potential and nutrient availability and hence the rhizosphere
102 environment (Matimati *et al.*, 2014). Finally, if rhizosphere community structure is altered by
103 mutations in clock genes, then we hypothesize there may be differences in community function
104 in the form of plant performance, in which microbial communities shaped by wild-type
105 genotypes may lead to improved plant performance in comparison to microbial communities
106 found in association with clock mutant genotypes.
107

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108 **Materials and Methods**

109

110 Plant Material and Growth Conditions

111 To investigate the role of the circadian clock in shaping rhizosphere community structure and
112 function, we used the *Arabidopsis thaliana* accession, Wassilewskija (Ws, CS2360), and two
113 circadian clock period mutants in the Ws background, *TIMING OF CAB EXPRESSION* 1 (*toc1-*
114 *21*) and *ZEITLUPE* (*ztl-30*). *toc1-21* is a short period mutant (~ 20 hours), while *ztl-30* is a long
115 period mutant (~28 hours) in free-running conditions (Kevei *et al.*, 2006; Fujiwara *et al.*, 2008).
116 Many prior studies have shown that the resonance between endogenous and environmental
117 cycles affects plant phenotypes and performance (Dodd *et al.*, 2005; Yerushalmi and Green,
118 2009; de Montaigu *et al.*, 2015; Salmela *et al.*, 2016); the current experiments extend prior
119 research to test effects of the plant host clock on the rhizosphere microbiome.

120 For each experiment, seeds were surface sterilized using 15% bleach, 0.1% *Tween*, and
121 84.9% RO H_2O solution, cold stratified in the dark in 1ml of RO H_2O for five days at 4^0C, and
122 placed in RO H_2O to germinate in a Percival PGC-9/2 growth chamber (Percival Scientific,
123 Perry, IN, USA) to ensure synchronous germination. Throughout this study, the growth chamber
124 environment was set to 12/12 light-dark cycle (lights came on at 7 A.M. and turned off at 7
125 P.M.), 22^0C/18^0C day-night temperature cycles, 40% relative humidity, and photosynthetic
126 photon flux density = 350 μmol photons m^{-2} s^{-1}. Upon the observation of root radicles, seedlings
127 were aseptically transferred to 2 inch in diameter pots filled with a mixture of sterilized potting
128 media (N ~ 400 ppm, P ~ 90 ppm) and microbial inoculate. To generate our sterilized media,
129 Redi-Earth Potting Mix (Sungro Horticulture, Agawam, MA, USA) was autoclaved twice for 60
130 minutes. Next, 2ml of microbial inoculate was added to each pot. The microbial inoculate was

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131 created by mixing 360ml of RO H_2O with 40g of soil from the Catsburg region in Durham,
132 North Carolina, USA (36.062294^{0}N, -78.849644^{0}W) and filtered through 1 000µm, 212µm,
133 45µm sieves, to remove soil nematodes that might negatively impact plant performance (van de
134 Voorde *et al.*, 2012). The Catsburg region has a well-documented history of *A. thaliana*
135 occurrence, which has been naturalized in this region (Mauricio, 1998). Soil from the Catsburg
136 region and our sterilized potting mix was characterized at the Colorado State Soil-Water-Plant
137 Testing Lab (Fort Collins, CO, USA); of greatest relevance to microbial growth, the Catsburg
138 and potting soils had similar pH values (5.4 *vs.* 5.3, respectively). Following germination,
139 seedlings were thinned to one plant per pot, and pots were watered at 7 A.M. daily.
140
141 <u>Experimental Design</u>
142 *Experiment 1: Temporal Changes in Rhizosphere Community Structure*. To determine if
143 rhizosphere bacterial communities are diurnally dynamic, replicates of wild-type Ws plants were
144 grown for four weeks as described above. Starting at 6 A.M. on July 21 and ending at 6 A.M. on
145 July 22, 10 replicates were selected at random and harvested every 6 hrs for rhizosphere soil by
146 separating the roots from the rosette (N = 50), removing closely adhering soil particles from the
147 roots as described in Bulgarelli et al. (2012), and storing the samples at -80°C.
148
149 *Experiment 2: Candidate Drivers of Rhizosphere Community Structure*. To characterize the
150 effects of circadian period misfunction on rhizosphere bacterial community structure, 10
151 replicates of Ws, *toc1-21*, and *ztl-30* genotypes were planted in a fully randomized design and
152 grown for four weeks. Rhizosphere samples were collected as described above at 6 P.M. on July
153 21 and stored at -80°C. All samples were collected prior to visible signs of bolting, or the

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154 transition from a vegetative to a reproductive state, to avoid confounding effects of plant
155 developmental stage (Lundberg *et al.*, 2012; Chaparro *et al.*, 2014). At the end of this
156 experiment, we collected additional rhizosphere soil from 4 replicates of each of the three
157 genotypes to generate the inoculum for *Experiment 3*.
158
159 *Experiment 3: Rhizosphere Community Feedbacks on Plant Performance*. To test if rhizosphere
160 microbiomes assembled by the three plant genotypes had differential effects on plant
161 performance, we synchronously germinated seeds of the Ws genotype and planted these seeds in
162 sterilized soil media inoculated with soil slurry generated by the Ws, *toc1-21*, or *ztl-30* genotypes
163 and collected at the end of *Experiment 2* (N = 60; 20 replicates × 3 inoculates). To determine the
164 effects of the rhizosphere microbiome treatment on plant performance, rosette diameter was
165 measured weekly for three weeks. In a second experiment, we allowed seeds to germinate
166 naturally in sterilized soil media inoculated with the same soil slurries ($N = 60$; 20 replicates x 3
167 inoculates). For this experiment, seeds were checked daily for germination as estimated from the
168 first observation of cotyledons.
169
170 <u>DNA Extraction and Amplicon Sequencing</u>
171 To extract microbial DNA, rhizosphere samples were placed into 15ml Nunc Conical Centrifuge
172 Tubes (Thermo Scientific, Waltham, MA, USA) containing 3ml of phosphate-buffered saline
173 (PBS), and then agitated for 15 minutes to separate soil particles from plant roots as described in
174 Bulgarelli *et al.* 2012. Plant roots were then removed with sterilized forceps and the samples
175 were centrifuged for 15 minutes at 3000 rcf. The supernatant was discarded, and 0.25g of the
176 pellet was put into bead tubes from the Mobio Power Soil DNA Isolation Kit (Mobio

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177 Laboratories, Carlsbad, CA, USA) using sterilized disposable spatulas. DNA was extracted from
178 each sample following the manufacturer's instructions. With each round of extractions, a soilless
179 blank was included as a negative control. At the end of each round, PCR was performed to
180 ensure sufficient DNA yields and reagent sterility.

181 DNA extracts were sent to the Marine Biological Laboratories (Woods Hole, MA, USA)
182 for amplicon library preparation of the V4V5 region of the 16S rRNA gene using the 518F and
183 926R primers (Huse *et al.*, 2014). Sequencing was performed on the Illumina MiSeq platform
184 (Illumina, San Diego, CA, USA) as described in Nelson et al. (2014). Sequence reads were
185 demultiplexed and quality filtered (Phred score ≥ 20, chimera removal by ChimeraSlayer) using
186 QIIME 1.9.1, uclust was used to perform open reference OTU picking at 97% sequence
187 similarity using the Greengenes database (ver. 13.8), and all singletons were removed to avoid
188 the possibility that a sequencing error was called as an OTU (Caporaso *et al.*, 2010; Bokulich *et*
189 *al.*, 2012; McDonald *et al.*, 2012; Edgar, 2010; Haas *et al.*, 2011). We rarefied to 100 000 reads
190 per sample to ensure common sampling effort. All sequences have been deposited into the Short
191 Read Archive (SRA) under accession number SRA579608.

192

193 Sequencing data analyses

194 To describe rhizosphere community structure, we generated Jaccard (presence-absence analysis)
195 and Bray-Curtis (abundance analysis) dissimilarity matrices, and Shannon diversity estimates in
196 QIIME (Caporaso *et al.*, 2010). For *Experiment 1,* we used adonis to determine if rhizosphere
197 community structure differed between day (6 P.M.) and night (6 A.M.) time points. To test if
198 shifts in community structure were consistent between day and night time points, we used
199 Pearson's correlation coefficients to compare the percent change in OTU abundance between 6

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200 A.M. and 6 P.M. on July 21 and the percent change in OTU abundance between 6 P.M. on July
201 21 and 6 A.M. on July 22, for OTUs with more than 100 reads per sample to avoid potentially
202 confounding effects of low-abundance taxa. For *Experiments 2 & 3*, we used one-way ANOVAs
203 and Tukey's HSD *post hoc* comparison tests using the *car* and *agricolae R* packages to
204 characterize differences in Principal Coordinates between genotype along axis 1 and to
205 determine differences in plant performance between soil treatments (Fox *et al.*; de Mendiburu,
206 2016). Moreover, OTUs were split into common (> 500 reads) or rare (< 500 reads) categories,
207 and presence-absence analyses and abundance analyses were performed again on the split
208 datasets to determine if effects of clock genotype were detected using common or rare microbial
209 taxa alone. Further, sequence data was reanalyzed without rarefaction using the R package
210 *Phyloseq*, to determine if results were consistent in the absence of rarefaction (McMurdie *et al.*,
211 2014). Results were similar regardless of rarefaction, that is, the effect of host plant genotype
212 was significant for both binary Jaccard (p = 0.001) and Bray-Curtis dissimilarity (p = 0.001)
213 analyses with and without rarefaction; here, we present the results of analyses based on
214 rarefaction. All plots were generated using the R package *ggplot2* (Wickham, 2009).

215 To identify OTUs that explain observed differences in plant performance due to soil
216 overstory history in *Experiment 3*, we used the *indicspecies* package for indicator value analysis
217 in R 3.0.3 and *LefSe* on the galaxy web platform (Cáceres and Legendre, 2009; Dufre□ne and
218 Legendre, 1997; Segata *et al.*, 2011; R Core Team, 2013). Indicator value analysis (IVA) has
219 been used commonly in ecological studies to ascertain species that underlie treatment or site
220 differences (Dufréne and Legendre 1997), and is used here to test which OTU(s) is(are) specific
221 to a given level of a factor (e.g., present / abundant in the rhizospheres of Ws replicates and
222 absent from *toc1-21* and *ztl-30* rhizospheres). Notably, the calculation of IVA weights presence-

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223 absence and abundance, and as such may be sensitive to rare taxa. *LefSe* performs linear
224 discriminant analysis on sequence data to identify marker taxa that underlie treatment
225 differences, and is weighted preferentially by abundance differences of more common taxa.
226 Because rare OTUs contributed to microbiome differences between host plant genotypes, we
227 used both IVA and *Lefse*. Finally, we coarsely estimated microbial community size by dividing
228 the quantity of extracted DNA using a Qubit (ThermoFisher Scientific Waltam, MA, USA) by
229 the mass of soil used for each extraction to determine if microbial community size influenced
230 plant performance in *experiment 3*.
231
232 **Results**
233
234 <u>Sequencing Results</u>
235 For *Experiment 1*, after quality filtering, chimera removal, OTU picking, outlier sample filtering
236 and rarefaction to 100 000 reads per sample (**Supplemental Figure 1a**), there was a total of 3
237 700 000 high quality reads out of 10 250 881 raw reads. For *Experiment 2*, after similar
238 processing, but rarefaction to 116 000 reads per sample (**Supplemental Figure 1b**) there was a
239 total 2 668 000 high quality reads out of 6 487 790 raw reads. The number of reads after each
240 processing step can be found in **Supplemental Tables 1** and **2.**
241
242 <u>Experiment 1: Temporal Changes in Rhizosphere Community Structure.</u>
243 We found significant differences in rhizosphere community structure between the communities
244 collected at 6 P.M. (day) and both of the two 6 A.M. (night) collections (P = 0.001 contrasting 6
245 P.M. with the first collection at 6 A.M. on July 21 or P = 0.008 contrasting 6 P.M. with the

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246 collection at 6 A.M. on July 22; **Figure 1a**). The percent change in OTU abundance between 6
247 A.M. on July 21 and 6 P.M. was positively correlated with the percent change in OTU
248 abundance between the 6 A.M. on July 22 and 6 P.M. time points ($r = 0.423$, $P < 0.001$). This
249 relationship suggests that the abundance of many common OTUs shift in a similar manner
250 between day and night time points (**Figure 1b**).
251
252 Experiment 2: Candidate Drivers of Rhizosphere Community Structure
253 Rhizosphere community composition (presence *vs*. absence of taxa), abundance, and diversity
254 differed among genotypes (**Figure 2, Supplemental Figure 2**). From Jaccard presence-absence
255 analysis, Ws, *toc1-21*, and *ztl-30* rhizosphere communities were significantly different from one
256 another ($P < 0.001$; **Figure 2a**). PCo1 describes the effect of progressive clock changes between
257 short (lowest PCo values) *vs*. wild-type (intermediate values) *vs*. long (highest values)
258 endogenous period lengths of the plants on rhizosphere community composition. Bray-Curtis
259 relative abundance analysis revealed differences between the rhizosphere communities of the
260 three clock genotypes, where *toc1-21* communities were different from both Ws ($P = 0.02$) and
261 *ztl-30* ($P = 0.04$) communities, while Ws and *ztl-30* communities were not significantly different
262 from one another ($P = 0.39$; **Figure 2b**). This result suggests that a period length shorter than 24
263 hrs specifically alters abundances of OTUs within the rhizosphere community. Likewise, the
264 *toc1-21* rhizosphere communities showed significantly reduced richness and evenness based on
265 Shannon's diversity index in comparison to Ws ($P = 0.03$; **Figure 2c**).
266 To clarify the contributions of rare *vs*. common OTUs to host plant genotype differences,
267 we analyzed the data when culled to different minimum read numbers. When culling to a
268 minimum read number of 1 000 for an OTU (or approximately 1% of the community), Jaccard

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269 and Bray Curtis dissimilarities were significant, indicating that common taxa contribute at least
270 partially to observed differences among the three host plant genotypes in the presence-absence of
271 taxa (**Figure 3a**) and to differences in OTU abundance between *toc1-21* and both Ws and *ztl-30*
272 (**Figure 3b**). Communities culled to OTUs with between 1-500 reads showed significant
273 differences in both composition and abundance, indicating that rare microbial taxa respond to
274 plant genotype (**Figure 3cd**). In particular, when data for rare OTUs are analyzed, the distinction
275 between Ws *vs*. *ztl-30* becomes significant (p = 0.001) (cf **Figure 2b** *vs*. **Figure 3d**).
276 Combined, the IVA and *Lefse* analyses identified a total of 13 indicator OTUs associated
277 with the Ws rhizosphere (**Figure 4**), 12 indicator OTUs associated with the *toc1-21* rhizosphere
278 (**Supplemental Tables 7, 9**), and 12 indicator OTUs associated with the *ztl-30* rhizosphere
279 (**Supplemental Table 8, 9**). Because IVA is more sensitive to rare taxa, the two methods select
280 somewhat different OTUs as biomarkers of host plant genotype. Notably however, there is
281 significant taxonomic overlap between the OTUs identified by IVA and *Lefse*. That is, taxa
282 identified by IVA are phylogenetically related to those identified by *Lefse*, or vice versa. For
283 instance, of the 13 indicator OTUs associated with the Ws rhizosphere, six taxa were members of
284 the phylum Acidobacteria (IVA: DS-100;o_;f_;g_, llb;f_;g_, PAUC26;f_;g_; *Lefse*:
285 Acidobacteria, Solibacterales;f_;g_, iii1_15;f_;g_) and two taxa were members of the
286 Chloroflexi (Indicator species analysis: Anaerolineae;o_;f_;g_, *Lefse*: Chloroflexi). From
287 previous studies, both phyla and two taxa, *Agromyces* and *Cellulomonas*, have been previously
288 described as growth promoting (Egamberdiyeva and Höflich, 2002; Kuffner *et al.*, 2008; Chen *et*
289 *al.*, 2014; Kielak *et al.*, 2016). Finally, community size as estimated from DNA per unit soil
290 mass did not significantly differ across clock genotypes (P = 0.11).
291

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292 Experiment 3: Rhizosphere Community Feedbacks on Plant Performance.

293 Soil overstory history had a significant influence on early plant performance (**Figure 5**). Wild-
294 type plants grown in a soil with a history of Ws plants had significantly larger rosette diameters
295 than plants grown in soils with a history of *toc1-21* and *ztl-30* after 1 week (19.4% and 14.4%,
296 respectively; P = 0.002) and 2 weeks of growth (10.8% and 8.3%, respectively; P = 0.04).
297 However, at the end of three weeks of growth, Ws plants grown in soils conditioned by each of
298 the clock genotypes were only marginally different in size (P = 0.11). In a germination
299 experiment of similar design (in which seedlings were not transplanted but instead germinated
300 directly on soil), Ws seeds in pots with Ws inoculum germinated an average of 5.2 days earlier
301 than seeds planted into pots with *toc1-21*(P = 0.002) inoculum and 5.7 days earlier than those
302 planted into *ztl-30* (P = 0.024) inoculated pots (P < 0.001; **Figure 5b**).

303

304 **Discussion**

305

306 The rhizosphere microbiome has been referred to as the "second genome" of plants or the
307 extended phenome (Berendsen *et al.*, 2012). In part, these names reflect the role of the
308 rhizosphere microbiome in determining plant performance. Empirical studies suggest complex
309 feedbacks between plants and microbes, under which plant species may modulate rhizosphere
310 community structure via carbon exudation and under which microbes may alter plant phenotypes
311 directly or via ecosystem services such as nutrient accessibility (Bulgarelli *et al.*, 2013). The
312 mechanisms by which different plant genotypes may influence rhizosphere community structure
313 remain largely unclear, as are the effects on plant performance of rhizosphere microbiomes
314 selected by the plant host genotype (Heath and Tiffin, 2007; Panke-Buisse *et al.*, 2014; Lebeis *et*

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315 *al.*, 2015). Understanding plant-rhizosphere microbiome interactions is agroecologically relevant
316 because rhizosphere communities can strongly influence plant fitness and biomass, which can in
317 turn inform evolutionary studies of adaptation, conservation, and agronomic practices (Pérez-
318 Jaramillo *et al.*, 2016). In this study, we tested the role of the plant circadian clock as a mediator
319 of plant-rhizosphere microbiome interactions. We hypothesized that 1) rhizosphere community
320 structure may be temporally dynamic, if rhizosphere taxa may respond to diurnally patterned
321 fluxes of carbon, water, or nutrient availability into the rhizosphere (or other diurnally patterned
322 plant phenotypes). 2) We further hypothesized that clock misfunction would play a role in
323 shaping community structure, because differences in plant physiology attributable to genotype
324 would lead to differences in rhizosphere community structure. 3) Finally, we hypothesized that
325 differences in rhizosphere community structure attributable to plant genotype could lead to
326 differences in community function with regards to plant performance.

327 The composition of plant-associated microbiomes is known to shift on long time scales,
328 such as across seasons or across developmental stages of the plant host (Lundberg *et al.*, 2012;
329 Chaparro *et al.*, 2014; Wagner *et al.*, 2016). The short duration of many microbial life cycles
330 means that microbial community composition may also respond to more rapid changes in the
331 environment. Yet, it remains unclear if the community composition of microbes found in
332 association with plants changes on short timeframes, such as across day-night transitions. We
333 observed diurnally patterned shifts in rhizosphere community structure. For instance, we
334 observed shifts in community structure between day (6 P.M.) and night (6 A.M.) time points, and
335 we observed that these shifts in community structure were consistent between the day and both
336 night time points (**Figure 1**). Difference in community structure observed between the two time
337 points may reflect the effects of day *vs.* night conditions and carbon, water, or nutrient

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338 availability in the rhizosphere. Several prior studies have shown that the concentration of certain
339 exudates varies over the course of day (Badri and Vivanco, 2009; Watt and Evans, 1999). For
340 instance, Iijima *et al.* (2003) observed higher rates of mucilage exudation at night, while other
341 studies have observed higher prevalence of flavonoids and catechin during day conditions
342 (Hughes *et al.*, 1999; Iijima *et al.*, 2003; Tharayil and Triebwasser, 2010). Further, rhizosphere
343 water is depleted diurnally, depending on root and soil hydraulics (Sperry *et al.*, 1998), and the
344 transpiration stream increases nutrient flow (Matimati *et al.*, 2014), potentially depleting soil
345 nutrients in the rhizosphere zone. Therefore, rhizosphere taxa and populations may vary in
346 abundance depending on soil resource availability, leading to our observed differences in
347 community structure between day and night time points. Future experiments should be designed
348 to tease apart the relative influence of root exudates, water dynamics, and nutrient uptake within
349 the rhizosphere on microbes.

350 Specific plant genes play important roles in shaping rhizosphere community structure
351 (Bravo *et al.*, 2016), and here we observed that circadian clock genes significantly influence
352 rhizosphere community structure. In the current study, plant genotype explained 19.1% of the
353 variation in community composition (presence *vs*. absence of taxa), 21.7% of the variation in
354 community relative abundances, and brought about differences in community diversity between
355 short (*toc1-21*) *vs*. longer (Ws, *ztl-30*) period genotypes (**Figure 2**). These differences in
356 community structure explained by clock genotype surpass variation explained by genotype in
357 previous studies of the influence of plant genotype on rhizosphere community structure
358 (Lundberg *et al.*, 2012; Bulgarelli *et al.*, 2012; Peiffer *et al.*, 2013; Lebeis *et al.*, 2015). Our
359 results thus suggest that circadian clock misfunction has a strong influence on rhizosphere
360 community structure. The large percent variance explained may arise from the pervasive

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361 transcriptomic and phenotypic effects of clock misfunction on the plant host, or potentially the
362 microbial inoculant used here is one that amplifies the effect of host genotype, as demonstrated
363 in other studies (Weinert *et al.*, 2011; Peiffer *et al.*, 2013).

364 *TIMING OF CAB EXPRESSION 1* had a particularly pronounced impact on rhizosphere
365 community structure, because strong mutant alleles in this gene led to changes in community
366 composition, abundance, and diversity (**Figure 2**). On the other hand, disruption of *ZEITLUPE*
367 had less of an influence on shaping rhizosphere community structure, with its effect limited to
368 differences in OTU presence-absence relative to Ws (**Figure 2**). One possible explanation for the
369 asymmetric effects of clock misfunction is that long period lengths theoretically enable better
370 phase adjustment to dawn, such that period lengths shorter than 24 hrs may have more
371 detrimental fitness consequences in nature (or in this case lead to greater deviations in
372 rhizosphere microbial community structure) in comparison to period lengths greater than 24 hrs
373 (Johnson and Kondo, 1992; McClung, 2006; Kevei *et al.*, 2006; Hotta *et al.*, 2007). Regardless
374 of the exact mechanisms, clock misfunction and the mismatch between endogenous plant cycles
375 and exogenous cycles affected aspects of microbial community structure.

376 As in any ecosystem, there is a link between community structure and function (Tilman
377 *et al.*, 1997). Several studies have illustrated this relationship in plant-rhizosphere microbiome
378 interactions, where differences in plant performance can be attributed to differences in
379 rhizosphere community structure (Mendes *et al.*, 2011; Zolla *et al.*, 2013; Mendes *et al.*, 2013;
380 Wagner *et al.*, 2014). Here, differences in community structure brought about by mutations in
381 circadian clock genes led to differences in plant performance among wild-type Ws plants grown
382 in soils with differing plant genotype overstory histories (**Figure 5**). Ws plants performed best
383 when exposed to an inoculum from soils in which wild-type rather than clock mutant genotypes

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384 had been grown. We observed differences in the timing of germination, where Ws seeds sown in
385 soils with a history of Ws occurrence germinated earlier. In comparison to untreated soil,
386 autoclaved soil, such as that used here, differs in both chemical and physical properties and
387 reflects a novel and possibly more stressful environment for plants (Trevors, 1996; Brulé *et al.*,
388 2001; Lau and Lennon, 2011); differences in germination observed here may therefore reflect
389 that the microbes from Ws-conditioned soil enable normal germination under the novel
390 autoclaved soil conditions (rather than an acceleration of germination timing under natural
391 conditions *per se*) (Lau and Lennon, 2011; Mahmood *et al.*, 2014). Beyond germination timing,
392 we observed that wild-type plants were also larger when grown in soils with a history of Ws
393 rather than mutant genotype growth. These findings from two experiments in which soils were
394 independently conditioned by Ws *vs*. mutant genotypes suggest first that plants can select
395 explicitly beneficial soil communities that improve initial offspring performance, and second that
396 disruption of these communities by mutations in clock genes adversely affects initial offspring
397 phenology and growth.

398 While additional research is required to ascertain causality, community composition
399 patterns and indicator analyses provide hypotheses as to which OTUs may lead to these
400 differences in performance (Cáceres and Legendre, 2009; DeAngelis *et al.*, 2015). Here, we
401 identified 13 indicator OTUs associated with Ws rhizosphere. Parallel to the differences in
402 microbial community structure among host plant genotypes (**Figures 2 and 3**), one possibility is
403 that rare OTUs underlie differences in plant performance observed between the Ws and *ztl-30*
404 rhizosphere microbiomes, while rare and common OTUs could contribute to plant performance
405 differences observed between the Ws and *toc1-21* microbiomes. Rare OTUs could affect plant
406 performance via so-called indirect effects, such as facilitation of or competition with explicitly

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407 plant growth-promoting microbes, while common microbes could promote plant growth through
408 direct interactions (Saleem *et al.*, 2016). Specifically in regard to the indicator species analyses
409 (IVA and *Lefse*), the presence and abundance of Acidobacteria (Kielak *et al.*, 2016), Chloroflexi
410 (Chen *et al.*, 2014), *Cellulomonas* (Egamberdiyeva and Höflich, 2002), and *Agromyces* (Kuffner
411 *et al.*, 2008) in the Ws rhizosphere may explain the differences in plant size between rhizosphere
412 treatments, as these OTUs have been previously associated with plant growth promotion.
413 In sum, we have shown that the plant circadian clock shapes rhizosphere community
414 structure, particularly the presence of rare taxa. Further, this plant genetic driver of community
415 assembly also influences community function, as estimated from plant performance. Because
416 community structure may shift in response to day and night conditions, future characterizations
417 of the rhizosphere should account for differences in community structure due to the timing of
418 rhizosphere collection. Moreover, more work needs to be done investigating the role of other
419 pertinent clock genes and loci that regulate plant physiology, as these may also shape
420 rhizosphere community structure and function.
421
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427
428 **Conflict of Interest**
429 The authors declare no conflicts of interest.

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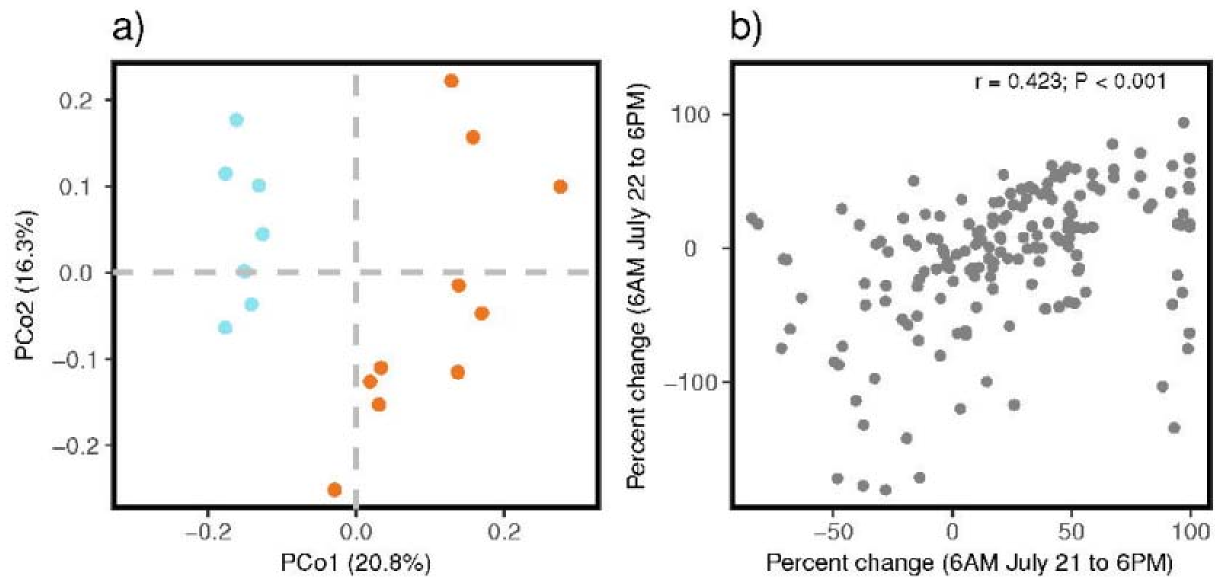
637 **Figure Legends**



638

639 **Figure 1:** Day *vs.* night influences rhizosphere community structure. **a)** Principal Coordinate
640 Analysis of Bray Curtis dissimilarities ($n = 17$). Differences between the 6 P.M. (day, orange)
641 and 6 A.M. (night, blue) time points were significant at P = 0.001. **b)** Bivariate relationship
642 between percent change in OTU abundance from 6 A.M. to 6 P.M. July22 and percent change in
643 OTU abundance from 6 A.M. on July 21 to 6 P.M. ($r = 0.423$; $P < 0.001$).

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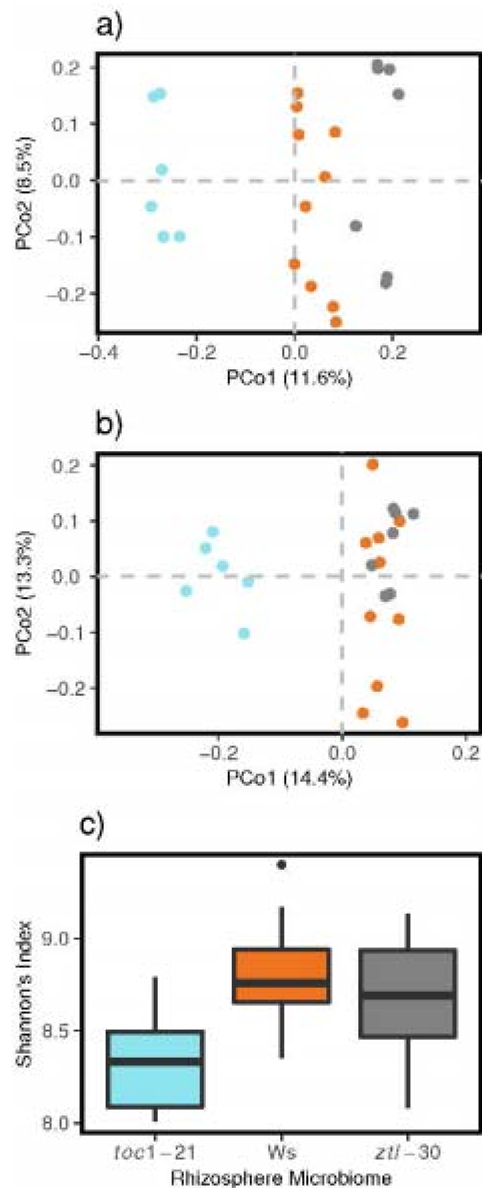

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645 **Figure 2:** Clock function in *A. thaliana* alters rhizosphere community composition, abundances,
646 and diversity. (**a**) Principal coordinate analysis of Jaccard dissimilarities, where rhizosphere
647 communities of Ws are represented by orange circles, *toc1-21:* blue circles, and *ztl-30*: gray
648 circles (n = 23). Rhizosphere community composition differs significantly between clock
649 genotypes (P = 0.001). (**b**) Principal coordinate analysis of Bray-Curtis dissimilarities (n = 23).
650 OTU abundances differ significantly between *toc1-21* and both the Ws and *ztl-30* genotypes (P =
651 0.001). (**c**) Mean Shannon diversity index. The top and bottom of boxes represent the 75th and

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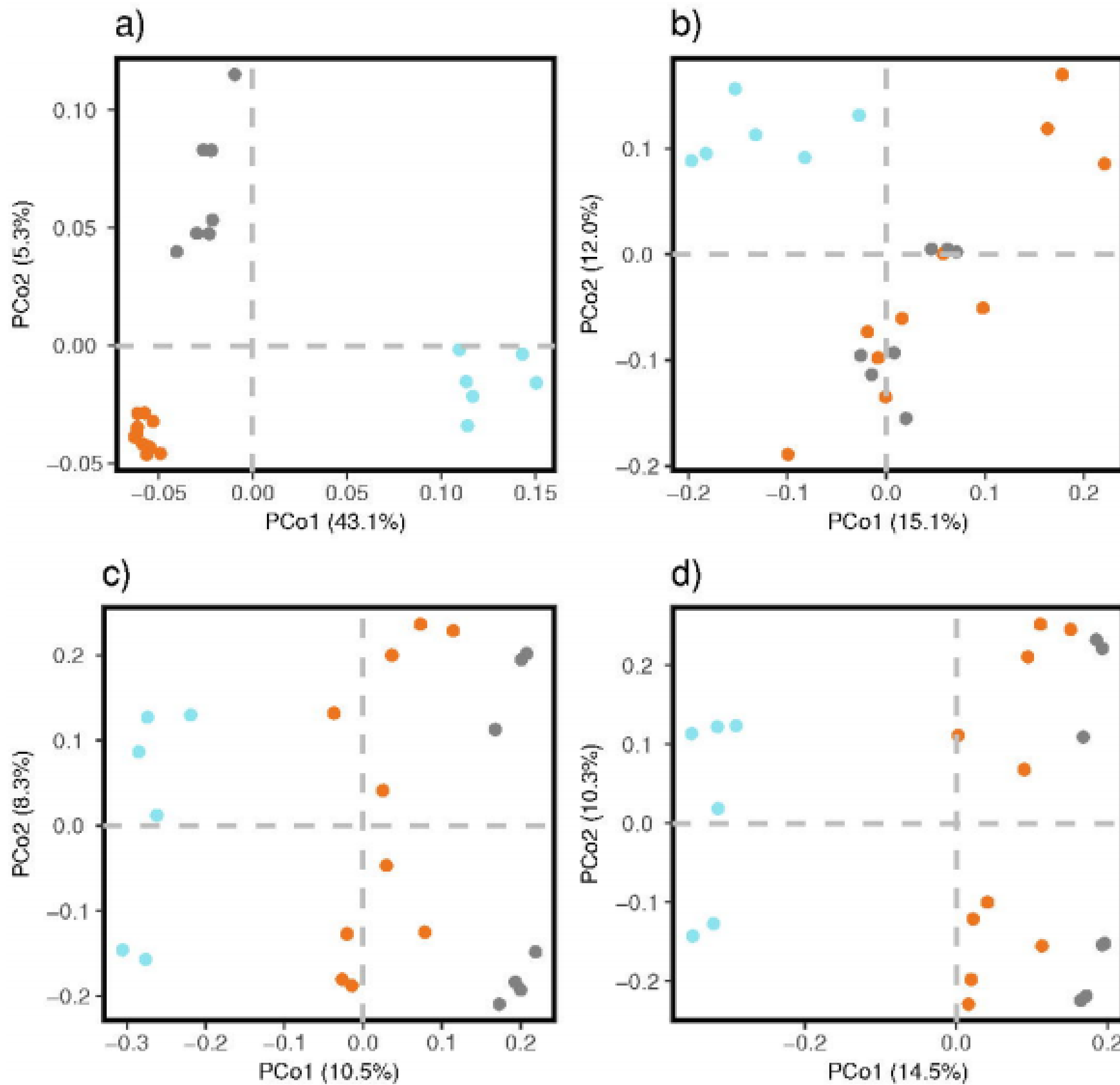
652 25th percentiles, respectively. Whiskers represent 1.5 times the interquartile range. One-way
653 ANOVA and Tukey's *post hoc* comparisons indicate significant differences between *toc1-21* and
654 both the Ws and *ztl-30* genotypes (P = 0.03).



655

656 **Figure 3:** Plant genotype influences both common (> 500 reads) and rare (1-500 reads)
657 rhizosphere taxa, but differences between the Ws and *ztl-30* rhizosphere communities are
658 attributable to rare taxa. (**a**) Principal coordinate analysis of Jaccard dissimilarities of common
659 taxa, where rhizosphere communities of Ws are represented by orange circles, *toc1-21:* blue

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660 circles, and *ztl-30*: gray circles (n = 23). Rhizosphere community composition differs
661 significantly between clock genotypes (P = 0.001). (**b**) Principal coordinate analysis of Bray-
662 Curtis dissimilarities of common taxa (n = 23). OTU abundances differ significantly between
663 *toc1-21* and both the Ws and *ztl-30* genotypes (P = 0.001). (**c**) Principal coordinate analysis of
664 Jaccard dissimilarities of rare taxa (n = 23). Rhizosphere community composition differs
665 significantly between clock genotypes (P = 0.001). (**d**) Principal coordinate analysis of Bray-
666 Curtis dissimilarities of rare taxa (n = 23). OTU abundances differ significantly between Ws and
667 both *toc1-21* and *ztl-30* genotypes (P = 0.001).
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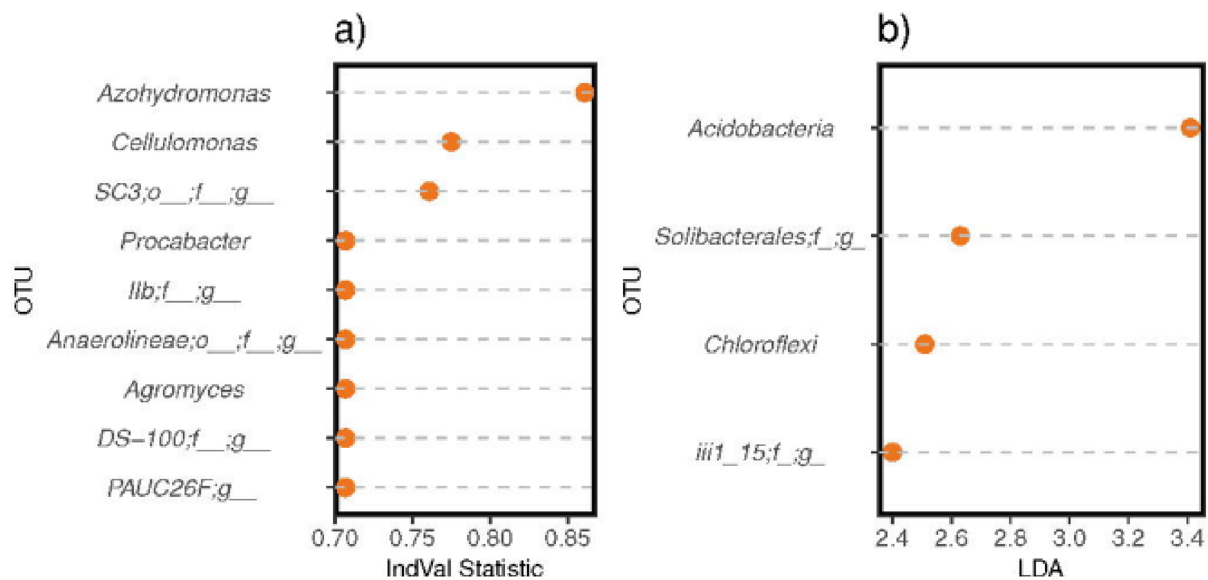

669

670 **Figure 4**: Indicator taxa based on (**a**) Indicator value analysis and **(b)** *Lefse*.

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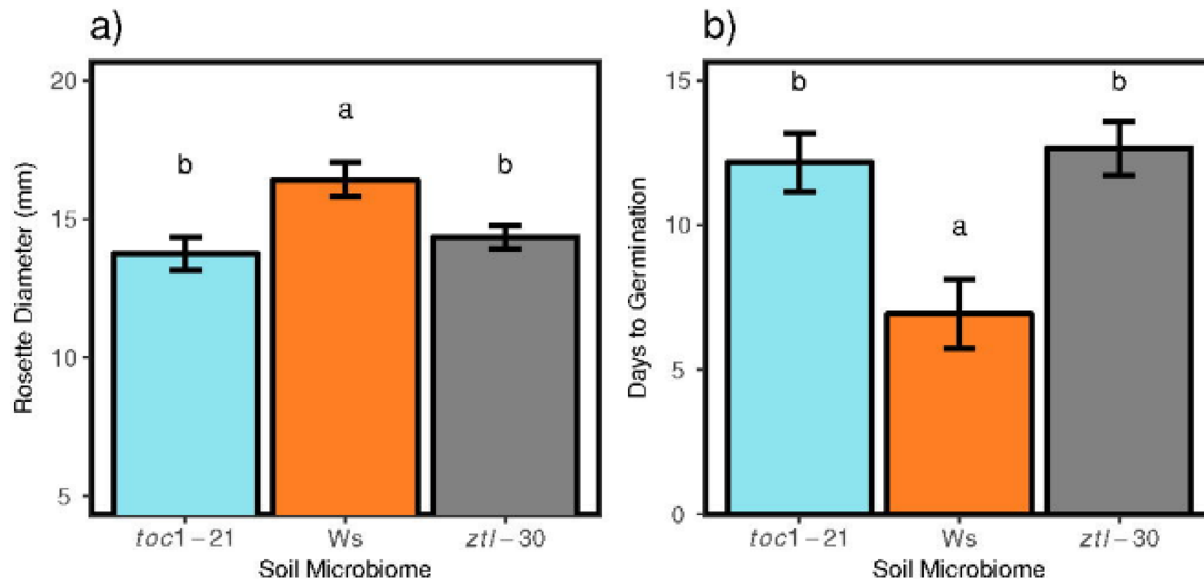

671

672 **Figure 5:** Effects of soil overstory history on plant growth. Letters denote significant differences
673 between soil treatments based on Tukey's Honest Significant Differences *post hoc* comparisons.
674 (**a**) At Week 1 (n = 60), plants grown in a soil with a history of Ws had significantly larger
675 rosette diameters than plants grown in soils with a history of *toc1-21* or *ztl-30* (P = 0.002). (**b**) In
676 a separate experiment where seeds were not germinated synchronously (n = 35), seeds sowed
677 onto a soil with a history of Ws occurrence germinated significantly earlier than seeds sown into
678 soils with a history of *toc1-21* or *ztl-30* genotypes (P < 0.001).