Rhizosphere-colonizing bacteria persist in the protist microbiome

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Running title: Rhizosphere colonization by protist-associated bacteria

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Abstract

Soils contain diverse predatory protists that affect the abundance and behavior of rhizosphere bacteria, including bacteria that may benefit plant health. Protists also harbor their own bacterial microbiomes, including transient and extracellular associates, but it is not known whether the protist microbiome affects the plant rhizosphere. To address this question, we profiled the bacterial microbiomes of eight evolutionarily diverse rhizosphere protist isolates after two years of continuous laboratory culture. We then compared the protist culture microbiomes to maize rhizosphere communities six weeks after protist inoculation. Introduction of protists enriched 13 protist-associated bacterial amplicon sequence variants (ASVs) in the rhizosphere, which comprised ~10% of the rhizosphere bacterial community. Additional bacterial ASVs ranked highly in abundance in both rhizosphere and protist microbiomes; together, a median 47% of the protist microbiome was enriched or abundant in the rhizosphere. Inoculation with some of the protist cultures positively affected root biomass traits, but a protist mixture had no effect, indicating that the impact of protist holobionts on plant growth is context-dependent. Isolates of protist-associated bacteria had both positive and negative effects on protist growth in culture, suggesting that the bacteria use multiple strategies to survive in proximity to predators. This study demonstrates that even after prolonged laboratory culture, evolutionarily diverse rhizosphere protists host bacterial microbiomes dominated by plant-colonizing bacteria that impact the rhizosphere microbiome after inoculation. The findings suggest that protists may contribute to the rhizosphere as part of the soil microbial seedbank, and identify bacterial groups that may be important to the plant-protist interaction.

Importance

Understanding the impact of predatory protists on the plant microbiome will be essential to deploy protists in sustainable agriculture. This study shows that eight rhizosphere protist isolates hosted diverse and distinct bacterial communities, and that a large proportion of these bacteria could be found colonizing the maize root environment long after protists were introduced. This study demonstrates that maize rhizosphere bacteria can persist for years in the protist microbiome, indicating that extremely diverse eukaryotes could help select and maintain rhizosphere bacteria in the absence of the plant.

44 **Introduction**

45 Microbial eukaryotes including fungi, nematodes, and protists are integral to the plant microbiome.
46 Single-celled protists- eukaryotes that cannot be classified as plant, animal, or fungus- are ubiquitous in
47 soil, and are often predators of bacteria and fungi (1). Protists may stimulate plant growth, and this was
48 historically often attributed to the stimulation of nutrient cycling (2). Consensus has grown that
49 predators also benefit plant growth and disease resilience through selective predation. Culling prey
50 bacteria can increase the competitive fitness of predation-resistant bacteria, including plant growth
51 promoting strains of *Bacillus*, *Sphingomonas, Pseudomonas*, and *Azospirillum* (3). Although few species
52 of rhizosphere bacteria are known to benefit from protists, network association studies have identified
53 hundreds of positive correlations among protist and bacterial groups in the rhizosphere (4–6). This
54 suggests that a much wider diversity of rhizosphere bacteria may have positive ecological associations
55 with predators than is currently established. Given that correlations could arise from niche constraints,
56 environmental factors, or random chance (7), systematic studies are needed to understand which
57 rhizosphere-colonizing bacteria are positively selected by protists.

58 Rhizosphere eukaryotes interact with bacteria, but they also host their own bacterial microbiomes
59 that shape their interaction with plants. For example, nematode endosymbionts detoxify plant
60 secondary metabolites, endofungal *Rhizobium* and *Burkholderia* spp. promote plant beneficial and
61 pathogenic fungi, and *Fusarium*-colonizing *Pantoea* inhibit virulence by producing antifungal compounds
62 (8–11). Highly cosmopolitan multi-kingdom colonists, including some strains of *Bacillus* and
63 *Pseudomonas* bacteria, may even be transferred from fungi or nematodes to plants (12, 13). Protists
64 host intracellular and extracellular bacterial communities through a wide range of mutualistic and
65 antagonistic mechanisms (14). The model amoebae *Dictyostelium discoideum* hosts both prey and
66 predation-resistant bacteria, which may be obligate or transient residents (15). Green algae
67 microbiomes parallel those of plant roots, and include many bacteria that can colonize and benefit both
68 organisms (16). No studies to our knowledge have characterized bacteria associated with other groups
69 of rhizosphere protists, but an understanding of such communities could point to synergisms that
70 benefit the plant. For example, nitrogen-fixing *Sinorhizobium meliloti* is a transient resident of ciliate
71 cells, and association with protists increases the bacteria's distribution and nodulation capacity (17, 18).

72 We previously observed that inoculating plants with a defined protist consortium resulted in a
73 rhizosphere bacterial community composed of bacteria similar to those in the protist inoculum (19).
74 Protist inoculation also alleviated a negative growth effect of field soil bacteria, and this impact was
75 dependent on bacteria in the protist culture. These findings led us to hypothesize that the protist

76 microbiome could help establish a beneficial rhizosphere microbiome. In this study, we identify bacterial
77 taxa found in long-term culture with rhizosphere protist isolates. (Although the nature of their
78 interaction with protists is not known, we term these "protist-associated bacteria" for brevity). Second,
79 we tracked abundance of those bacterial taxa after protist inoculation onto plants. We asked, 1) Are
80 protist-associated bacteria enriched in the rhizosphere after protist inoculation? 2) Do the microbiome
81 effects of protist inoculation vary among different protists? 3) Is bacterial establishment in the
82 rhizosphere affected by protist inoculum diversity? Finally, 4) how do protist-associated bacteria affect
83 protist growth? We find that bacterial taxa associated with evolutionarily diverse protists can colonize
84 the rhizosphere, suggesting that protists could affect rhizosphere health through shared microbiota.
85

86 **Results**

87 *Characterization of bacteria associated with rhizosphere protist isolates*

88 Protists were previously isolated from independent maize root samples at two sites (20), and
89 maintained in laboratory culture for over two years without addition of live bacteria. Eight protist
90 isolates were selected for microbiome characterization, of which five are known to colonize the maize
91 rhizosphere after inoculation to roots (*Colpoda*, *Cercomonas, Ochromonas*, *Thaumatomonas*, and
92 Chrysophyceae sp., Table S1) (19). Three isolates were selected to represent the groups Amoebozoa and
93 Rhizaria (*Flamella* and *Nuclearia* sp.), and *Allapsa*, a genus with significance to plant health (5). Sequence
94 profiling of 16S rRNA genes in twice-washed protist cultures identified 30 to 75 bacterial genera in each
95 protist microbiome (Table S1). Protist culture microbiomes had lower Shannon diversity indices than
96 bacteria cultured from bulk and rhizosphere soils collected at the original protist isolation sites, which
97 we sequenced concurrently and used as bacterial inoculum for plants (Table S1).

98 We identified the 30 most abundant bacterial genera, belonging to 15 orders, across inoculum
99 samples (henceforth referred to as "dominant genera", Fig. 1). While each microbiome was distinct,
100 there were five core dominant genera detected in all protist microbiomes (*Pseudomonas*, *Variovorax*,
101 *Sphingomonas*, *Reyranella*, and *Bradyrhizobium*), and eight additional genera represented in six of eight
102 communities. However, there were no core ASVs of *Sphingomonas* or *Reyranella*, and two core ASVs of
103 *Variovorax* (Table S2), showing that the core genera include bacterial strain diversity. Members of
104 Burkholderiales and Hyphomicrobiales were ubiquitously abundant in the protist microbiomes; nine of
105 the dominant bacterial genera represented these two orders. The results indicate that while each protist

106 culture enriched a distinct subset of bacteria after isolation from rhizosphere soil, certain bacterial taxa
107 broadly persisted in and dominated microbiomes of diverse rhizosphere protists.
108
109 *Establishment of protists and protist-associated bacteria in the rhizosphere*
110 To study the fate of protist-associated bacteria in the maize rhizosphere, we inoculated maize
111 with the eight protist cultures singly and in mixture, and co-inoculated these with a bulk soil bacterial
112 community cultured from our Lockwood Farm field site (BSC1, Fig. 2). To determine the effects of the
113 bacterial community, additional plants were inoculated with bulk soil communities from Griswold (BSC2)
114 or a rhizosphere soil community from the Lockwood site (RSC, Fig. 2), and co-inoculated with the eight--
115 protist mixture. Rhizosphere microbial communities were profiled by amplicon sequencing of the 16S
116 and 18S rRNA genes after six weeks.
117
118 <u>*Colpoda* sp., but not other inoculated protists, were consistently abundant after six weeks.</u>
119 ASVs corresponding to four of the eight inoculated protists were detected in the rhizosphere
120 samples after six weeks (Table S3); all were found to establish in the maize rhizosphere three weeks after
121 inoculation in our previous growth chamber study: *Cercomonas* sp. (UC19), *Ochromonas* sp. (UC48),
122 *Thaumatomonas* sp. (UC70), and *Colpoda* sp. (UC22) (19). In this study, the former three protists were
123 detected inconsistently at six weeks, and at low read frequency (<0.01% of eukaryotic reads). In contrast,
124 the *Colpoda* isolate ASVs consistently dominated protist sequence in inoculated pots, comprising an
125 average of 20% of the eukaryotic reads (Table S3). Maize protist communities are highly temporally
126 dynamic (21), this result suggests that *Colpoda sp*. is uniquely persistent as a plant inoculant.
127 Bacterial communities were highly variable within each bacterial treatment, and bacterial
128 inoculation did not significantly affect rhizosphere diversity or plant biomass (Fig. S1). The lack of effect
129 of bacterial inoculation suggested that bacteria from non-inoculum sources, such as the open
130 greenhouse environment or maize seed, primarily determined the rhizosphere bacterial community
131 composition.
132
133 <u>The maize rhizosphere consistently recruited specific protist-associated bacteria.</u>
134 Thirteen bacterial ASVs were significantly enriched in the rhizosphere after inoculation with
135 individual protist cultures, even when the corresponding protists were not detected (Fig. 3A, Fig. S2).
136 Bacterial taxa enriched in the rhizosphere typically comprised greater than 0.5% of the bacterial
137 community in the corresponding protist cultures (Fig. 3A, Table S4), and nine of the enriched ASVs

138 represented dominant culture genera identified in Fig. 1. A median 8.4% of the reads in each protist
139 microbiome corresponded to ASVs enriched in the rhizosphere (0.8%-44%); over 17% of the microbiome
140 associated with *Thaumatomonas*, *Flamella,* and *Allapsa* were rhizosphere-enriched. Some ASVs that
141 were relatively rare in the protist microbiomes were also enriched, including *Brevundimonas* and an
142 unclassified Burkholderiaceae. Many highly abundant protist-associated microbiome members, such as
143 *Variovorax* (Fig. 1), were not enriched in the rhizosphere, indicating that high abundance in association
144 with the protist was not predictive of rhizosphere enrichment.
145 Eight (61%) of the enriched bacterial ASVs were enriched by only one protist treatment. In
146 contrast, a *Sphingomonas* ASV (*Sphingomonas*-α) was broadly enriched by four of the eight protist
147 cultures and comprised up to 14.3% of the rhizosphere microbiome after inoculation. *Sphingomonas*-α
148 was detected in all other protist microbiomes except that of *Nuclearia*. Collectively, these results
149 demonstrate that a substantial proportion of the rhizosphere protist microbiome can colonize the
150 rhizosphere, even after prolonged laboratory cultivation of protist isolates.
151
152 <u>Inoculating with multiple protists increased the abundance of protist-associated bacteria in the</u>
153 <u>rhizosphere.</u>
154 Nine of the thirteen protist-enriched bacterial ASVs were also enriched in the rhizosphere after
155 inoculation with an eight-protist mixture (Fig. 3A), indicating that inoculating diverse protist cultures has
156 an additive effect in enriching protist-associated bacteria. The abundance of eight protist-associated
157 bacterial taxa did not significantly differ whether single or mixed protists were inoculated (Fig. S2), even
158 though the mixture contained a one-eighth dilution of each culture. However, the more diverse
159 inoculum had a negative effect on the relative abundances of *Sphingomonas*-α, *Mucilaginibacter*,
160 unclassified Burkholderiaceae sp., and two uncultured Planctomycetales sp., which were significantly
161 lower in the mixed than single treatments (Fig. S2). Mixed protist inoculation resulted in an increased
162 overall abundance of protist-associated bacteria in the rhizosphere (Fig. 3B). After inoculation with
163 mixed protists, the thirteen protist-enriched bacterial ASVs comprised a median 9.9% of the bacterial
164 reads in the rhizosphere, compared with 3.5% of reads after inoculation with single protists.
165
166 <u>Treatment with some protist cultures benefited root growth when inoculated individually, but</u>
167 <u>not in mixture.</u>
168 We previously observed that plants inoculated with an antibiotic-treated protist culture had
169 reduced root biomass compared with plants treated with a microbiome-replete protist (19), indicating

170 that protist-associated bacteria were required to increase root growth. Therefore, we hypothesized that
171 inoculation with mixed protist cultures would have a greater biomass effect than individual cultures due
172 to the greater number and diversity of protist-associated bacteria enriched on the root. Among all data
173 combined, plants inoculated with single protists had greater root:shoot biomass ratio than uninoculated
174 plants, and showed a nonsignificant trend toward greater root biomass, but plants inoculated with the
175 protist mixture did not (Fig. 4A-B). Among single protist treatments, *Thaumatomonas* culture-treated
176 plants had increased root:shoot biomass ratios compared to the negative control, and *Flamella* and
177 *Allapsa* treatments had increased root biomass (Fig. 4C-D). *Flamella* and *Allapsa* had the greatest
178 bacterial richness among the protist cultures cultures (Table S1) and with *Thaumatomonas* had the
179 greatest abundance of rhizosphere-enriched bacteria (Table S4), suggesting that bacterial richness or
180 dominance of rhizosphere bacteria in the protist microbiome could be associated with greater biomass
181 impact. However, no significant biomass phenotype was observed in plants treated with the protist
182 mixture (Fig. 4C-D). Hence, the increased microbiome effect of the protist mixture did not translate to an
183 increased plant biomass effect, and instead may have precluded the growth effects associated with
184 *Thaumatomonas*, *Flamella*, and *Allapsa* treatments. Given the complex interactions among protists and
185 bacteria, the taxonomic composition of enriched bacteria, rather than the overall richness or proportion
186 of rhizosphere colonizers, may be more important for plant growth.
187
188 <u>Protist inoculation was less impactful in the presence of a rhizosphere bacterial community</u>
189 To determine whether the microbiome effect of protists was influenced by the surrounding
190 bacterial community, we co-inoculated protist mixtures with bacterial communities cultured from three
191 soil sources. Bacterial enrichment patterns following protist inoculation were similar among plants
192 treated with bulk soil communities or no bacterial treatments; seven ASVs were enriched in multiple
193 treatments (Fig. 5A). However, when protists were co-inoculated with rhizosphere bacterial community
194 obtained from mature maize plants (RSC), only one ASV was enriched. We hypothesized that the
195 rhizosphere soil community was a source of some protist-associated bacteria, and therefore they were
196 not enriched by the protist inoculations. By comparing RSC-inoculated plants with controls, we
197 confirmed that inoculation with RSC was sufficient to increase the rhizosphere abundance of four
198 protist-associated ASVs in the rhizosphere (Fig. S3A). RSC-treated plants also had a higher combined
199 abundance of protist-enriched taxa than bulk soil communities, and protist inoculation of RSC-treated
200 plants did not increase the total abundance of protist-associated bacteria their abundance as observed

201 in other treatments (Fig. S3B). This supports our previous finding that protist inoculation may substitute
202 for rhizosphere soil in enriching certain bacterial taxa in the environment (19).
203 We asked whether the rhizosphere environment was required for enrichment of protist-
204 associated bacteria, or if protists alone were sufficient to enrich their abundance in soil. In unplanted
205 pots, only *Brevundimonas* and *Pedobacter* ASVs showed increased abundance after protist inoculation,
206 while others were only enriched in the presence of the plant (Fig. 5B). This result indicates that the
207 protist-associated bacteria are specifically enriched by the rhizosphere. Unclassified Burkholderiaceae α
208 was not consistently detected in unplanted pots (Fig. 5B, Fig. S3C), indicating that this taxon may
209 obligately require either plant or protist hosts for environmental persistence.
210
211 *Seventeen additional bacterial ASVs were abundant in both rhizosphere soil and protist cultures*
212 We detected diverse rhizosphere bacterial communities on both inoculated and untreated plants
213 (Fig S1, Table S5), which could mask potential enrichment of some bacteria by protist inoculation. To
214 determine whether other bacterial taxa colonized both rhizosphere and protist microbiomes, we
215 identified ASVs in high rank abundance in both protist culture (top 20) and rhizosphere samples of the
216 plants inoculated with the corresponding single protist (top 100). Thirty ASVs met these criteria, of which
217 17 had not been enriched by protist inoculation (Table 1, Table S6). Shared abundant ASVs included
218 representatives of dominant genera that were abundant in protist cultures, including *Azospirillum*,
219 *Pseudomonas*, and *Bradyrhizobium*. Notably, a *Variovorax* sp. ASV that was highly abundant in most
220 protist microbiomes was also usually among the top 20 most abundant taxa on maize (Table S6).
221 Together, ASVs that were enriched or abundant on plants comprised a median 46.8% of the reads in the
222 protist culture microbiomes (Table S4, range 6.6%-67.8%). This shows that several rhizosphere protist
223 cultures were dominated by candidate rhizosphere-colonizing bacteria, including a wider range of taxa
224 than was enriched by protist inoculation.
225
226 *Rhizosphere-enriched bacterial genera may promote or suppress protist growth*
227 We lastly wondered why many rhizosphere colonizing bacteria are able to persist for long
228 periods in culture with diverse types of protists. We hypothesized that the bacteria are not a food
229 source for the protists, or alternatively, that they could provide a benefit to the protists. To test this, we
230 isolated four bacteria from the protist cultures having 16S rRNA gene similarity to rhizosphere-enriched
231 ASVs *Sphingomonas*-α (100% similarity), *Pedobacter*-β (98.4%), *Caulobacter* (98.0%), and
232 *Mucilaginibacter* (96%). These genera were all enriched in the rhizosphere after inoculation with *Allapsa*

233 (Fig. 3), which had a positive effect on root biomass. We measured the effects of these bacterial isolates
234 on antibiotic-treated cultures of *Allapsa* as well as two other isolates from phylum Cercozoa,
235 *Cercomonas* and *Thaumatomonas*, in which the four bacterial genera were detected (Fig. 1) but variably
236 enriched (Fig. 3). All protists grew when cultures were supplemented with *Caulobacter* or
237 *Sphingomonas,* but not when *Pedobacter, Mucilaginibacter,* or no bacteria were provided (Fig. S4); this
238 indicates that *Pedobacter* and *Mucilaginibacter* isolates were not a source of prey (15). To determine
239 whether the bacterial isolates suppress growth, we measured the effects of the bacteria on protists
240 supplemented with an excess of inert prey (heat-killed *E. coli*). At 3 days, *Sphingomonas* increased
241 growth of all three protists, increasing density of the cultures by 2.4 to 3.3-fold compared to *E. coli*
242 alone. *Caulobacter sp.* had a strong positive effect on growth of *Allapsa* (2.4x, $p<0.001$), but a smaller
243 effect on *Cercomonas* (1.5x, $p=0.066$), and no effect on *Thaumatomonas* (Fig. 6). Conversely, *Pedobacter*
244 and *Mucilaginibacter* were strongly antagonistic to *Allapsa* growth, had differing positive and negative
245 effects on *Cercomonas*, and had no significant effect on *Thaumatomonas*. Taken together, the results
246 show that protist-associated rhizosphere bacteria use differing strategies to persist under predation
247 pressure, supporting or inhibiting protist growth in broad or isolate-specific ways.
248

249 **Discussion**

250 Green algae in soil recruit bacterial communities that have strong structural and functional
251 parallels to plant root communities (16), and the algal microbiome is a proposed source of plant
252 beneficial bacteria (22). By surveying the microbiomes of diverse protist cultures and tracking their fates
253 in the rhizosphere, this study demonstrates that a wide diversity of rhizosphere microeukaryotes outside
254 the Chloroplastida have microbiomes largely comprised of plant-colonizing bacterial taxa. Diverse
255 rhizosphere bacteria persisted for years in active cultures of protists from SAR, Excavata, and Amoebozoa
256 supergroups, and maintained the capacity to colonize roots in a complex microbial environment. The
257 findings support the hypothesis that, in addition to their roles imparting selective and behavioral
258 advantages to rhizosphere bacteria (23, 24), predatory soil protists could recruit or promote a
259 microbiome that contributes to rhizosphere establishment. Understanding how these bacteria interact
260 with protists could help identify defense and signaling mechanisms important to bacterial survival, and
261 may ultimately help enable the design of improved microbial consortia to benefit agriculture.

262 Profiling the protist microbiomes revealed broad taxonomic overlap in among protists
263 independently isolated from different plants and field sites, indicating the broad prevalence of
264 Burkholderiales and Hyphomicrobiales. They also included groups previously documented to survive

265 protist internalization: members of *Sphingomonas*, *Burkholderia*, *Pseudomonas*, *Pseudoxanthomonas*,
266 *Pedobacter*, *Mesorhizobium, and Acidovorax* have been identified in protist cells (Karaś et al., 2015; Lin
267 et al., 2023; Morón et al., 2024; Zhang et al., 2023). *Variovorax* spp. are common epibionts and
268 endosymbionts of protists (28, 29), and *Bosea* and *Reyranella* sp. are free-living protist mutualists that
269 can be isolated using a strategy of protist co-culture (30, 31). While these studies support protist
270 selection of these taxa, it's also important to note that protist microbiome composition is likely shaped in
271 part by the local soil microbiome, stochastic events during isolation, culture conditions, and bacteria-
272 bacteria interactions. The microbiome communities identified here likely only represent a portion of true
273 protist interactors in soil, and culture conditions could enhance associations that are rare or weak in
274 nature. More work is needed to confirm which features of protist microbiomes represent replicable
275 selection patterns and true symbioses. Enriched bacteria included two ASVs associated with uncultured
276 Planctomyces species, indicating that protist inoculation could be a means for manipulation and
277 rhizosphere introduction of some unculturable bacteria on the plant. However, we did not observe
278 rhizosphere enrichment of the foodborne human pathogens hypothesized to be vectored by amoebae
279 on produce (32, 33).

280 Protist-associated bacteria were enriched in the maize rhizosphere after six weeks, persisting
281 even after the corresponding protists could no longer be detected. Some enriched genera identified here
282 were previously correlated with protist abundance in plot studies, indicating that protist-bacterial
283 network connections might reflect microbiome associations that are robust in the field. For example,
284 *Luteimonas*, *Brevundimonas*, *Pseudomonas* and *Pseudoxanthomonas* were positively correlated with a
285 *Colpoda* sp. in tomato rhizosphere (34). In this study the ASV *Sphingomonas-α* was enriched to high
286 levels by the widest spectrum of protists; we previously identified the same ASV as the largest protist
287 network hub on several field grown solanaceous crops (35). *Sphingomonas* abundance was previously
288 linked to increased plant growth following *Cercomonas* inoculation on cucumber seedlings, and
289 *Cercomonas* triggered biofilm formation of the bacteria *in vitro* (36). Our own observations that
290 *Sphingomonas-α* is correlated with protists in the field, found in microbiomes of diverse protist cultures,
291 enriched in the rhizosphere after protist inoculation, and broadly promotes protist growth *in vitro*,
292 suggest that *Sphingomonas* could be a partner in widespread synergistic associations with protists and
293 plants.

294 The effects of protist diversity on microbial communities is variable, and can shift dynamically
295 under different environmental and biotic stress conditions (37, 38). We observed that inoculation with a
296 mixture of protist cultures enriched a greater number and abundance of rhizosphere bacteria compared

297 with single protist inoculation, but only a few single protists affected biomass traits. The increase in
298 inoculum diversity may have affected the activity and distribution of bacteria through intermicrobial
299 interactions, resulting in decreased abundance of potentially beneficial bacteria such as *Sphingomonas*
300 and *Mucilaginibacter* (Fig. S1). Even when reducing inoculum diversity, changing community composition
301 greatly shapes protist effects on the microbiome. We previously found that an 18-protist mixture had a
302 biocontrol effect and greatly enriched the dominance of the genus *Duganella* in the rhizosphere after 3
303 weeks (19). The eight-protist mixture tested here did not enrich *Duganella*, which we later determined is
304 found specifically in the microbiome of an *Ochrophyta* sp. isolate that was excluded from this study
305 (UC29, Patel *et al*, in preparation). As we observed in this study, inclusion of a protist isolate hosting even
306 a few rhizosphere-colonizing bacteria can have a significant impact on the microbiome. The observation
307 of parallels among plant and protist microbiomes raises many questions: What is the mechanism and
308 nature of protist interactions with its microbiome? Do protists provide the bacteria a fitness or other
309 plant colonization advantage in the rhizosphere, or induce the bacteria to benefit the plant? The
310 interactions identified here provide a starting point for understanding the ecology and agricultural
311 significance of protist holobionts on plants.

312

313 **Methods**

314 *Culture preparation*

315 We selected protist cultures from a collection isolated from maize roots in 2020 (39). Protist
316 cultures were maintained in PAGE's amoebal saline (i.e., PAGE's solution; 0.12 g of NaCl, 0.004 g of
317 $MgSO_4 \cdot H_2O$, 0.004 g of $CaCl_2$, 0.142 g of Na_2HPO_4, and 0.136 g of KH_2PO_4 in 1 liter of dH_2O) amended
318 with heat-killed *E. coli* as previously described (19). Protists had been maintained with monthly
319 passaging for two years at the time of DNA extraction and inoculation onto plants. To prepare cultures
320 for plant inoculation, protists were transferred to sterile PAGE's solution and fed with heat-killed *E. coli*
321 weekly for one month, grown just until encystment, centrifuged for 30 minutes at 2000 x g, resuspended
322 in 4 mL PAGE's solution, and pelleted again for 15 minutes at 2,000 x g before resuspension in 500 uL
323 PAGE's solution. Protist concentration was determined by counting under a microscope, then protists
324 were diluted in PAGE's solution to a concentration of 1000 cells/mL. Cultures were mixed in equal
325 proportion to create the eight-protist mixture.

326 To generate protist-free soil bacteria, we collected 10 cm deep soil cores from five random
327 locations in maize fields from Lockwood Farm (Hamden, CT) and Griswold, CT. We suspended 10 g of soil

328 in 100 mL of sterile phosphate-buffered saline (PBS; pH 7.4; 8 g of NaCl, 1.44 g of Na_2HPO_4, 0.2 g of KCl,
329 and 0.24 g of KH_2PO_4 in 1 liter of dH_2O). Rhizosphere soil was collected from 5 plants of maize (*Zea mays*
330 L.) inbred line B73 (stage V5) collected from the Lockwood field on the same day as the bulk soil. Roots
331 were shaken to remove excess soil, and 5 roots per plant were agitated in sterile PBS to collect
332 rhizosphere soil. Soil samples were plated in serial dilutions on both tryptic soya agar and Reasoner's 2
333 agar plates (0.25 g of casein digest peptone, 0.25 g of peptic digest of animal tissue, 0.5 g of $MgS0_4 \cdot 7H_2O$,
334 0.3 g of $C_3H_3NaO_3$, 0.5 g of casein acid hydrolysate, 0.3 g of K_2HPO_4, 0.5 g yeast extract, 0.5 g soluble
335 starch, 0.5 g glucose, 15 g agar in 1 L of dH_2O), which were incubated at 28°C for 72 hours. After
336 incubation, the bacterial colonies from each plate were scraped and collected in PBS containing 20%
337 glycerol and mixed by vortexing. Suspensions were stored at -80°C until use. One aliquot was used for
338 colony enumeration. Bacterial inoculum for plant experiments was then prepared by thawing the tubes
339 on ice and resuspending to 10^6 cells/mL in sterile PBS.
340
341 *Plant inoculation and growth*
342 We prepared a soil mixture containing 53% sand, 37% vermiculite, and 10% field soil from
343 Lockwood farm, Hamden CT, USA, which is the same field from which all but two the protists were
344 originally isolated. The mixture was wetted and autoclaved twice in 12 cm pots. Seeds of B73 maize were
345 surface sterilized in 3% NaOCL for 1 min, then washed 10 times in sterilized dH_2O. The seeds were
346 germinated on sterile filter paper in the dark at 28°C for 2 days. In all but 20 pots, germinated seeds
347 were placed into the middle of the pots at a depth of 4 cm and inoculated with 1 mL of protist inoculum
348 (at a concentration of 10^3 cells/mL) or sterile PAGE's solution, then with 1 mL of bacteria (10^6 cells/mL) or
349 sterile phosphate buffered saline (pH 7.4), depending on the experimental treatment. Germinated seeds
350 were inoculated by slowly pipetting the solution directly onto the emerging radicle before covering with
351 the soil mixture. No germinated seeds were placed in the remaining 20 pots; instead, soil was removed
352 to 4 cm in the center of the pot, and the soil in this location was inoculated with 1 mL of bulk soil
353 bacteria and 1 mL of mixed protist inoculum (10 pots), or 1 mL of bulk soil bacteria alone (10 pots),
354 before being covered by soil. Pots were arranged in a randomized block design and maintained in the
355 greenhouse for six weeks during June and July, 2021 (16h days, temperatures 25-30°C). Plants were
356 watered with autoclaved distilled water when pot weights decreased. At collection, roots were shaken
357 vigorously to remove excess soil, and then placed in plastic bags containing 35 mL PBS. Roots were
358 agitated for 30 seconds to remove attached soil, after which the rhizosphere suspensions were
359 transferred to 50 mL tubes and stored at -80°C until DNA extraction. Root and shoot biomass were

360 recorded after drying for one week at 50°C. For the plant-free replicates, soil was removed to a depth of
361 4 cm, and ~1 g of soil was removed and transferred to a 50 mL tube containing 35 mL PBS.
362
363 *16S and 18S rRNA gene library preparation*
364 DNA extractions, PCR amplifications and sequencing was conducted following previously
365 published protocols (19). The Illumina sequences can be found in NCBI GenBank under BioSample
366 PRJNA950058.
367
368 *Read assembly and classification*
369 We assembled the reads using mothur v. 1.44.0 (40). 16S contigs > 300 bp and 18S contigs < 90
370 bp or > 250 bp were removed, as were contigs containing ambiguous bases, or homopolymers greater
371 than eight bases. Chimeras were removed in mothur using VSEARCH (41). 16S amplicon sequence
372 variants (ASVs) were classified using the SILVA v. 132 database, while 18S ASVs were classified using the
373 Protist Ribosomal Reference (PR^2) v. 4.12 database (42). Both classifications used the naïve Bayesian
374 classifier implemented in mothur. Libraries with fewer than 2000 reads were excluded from further
375 analyses.
376 The sterilized PAGE's solution sample was analyzed as a negative control. For the 16S analyses
377 the control run totaled 7.9% reads of the next smallest library, while for the 18S analyses the control run
378 totaled 7.7% reads of the next smallest library.
379
380 *Diversity and enrichment analyses*
381 We calculated diversity metrics of inoculum samples and rhizosphere communities after
382 subsampling to the smallest read number among inoculum samples or rhizosphere sample datasets
383 using the phyloseq v. 1.38.0 package (43) implemented in R v. 4.1.2. The 16S communities were
384 subsampled to 2195 reads for the protist inocula, 21547 for the bacterial inocula, and 2218 for the
385 rhizosphere samples, while the 18S communities were subsampled to 14666 reads for the rhizosphere
386 samples. Heatmap generation was performed using the MicEco v. 0.9.14 package (44), after removing
387 ASVs with fewer than 5 reads in the dataset. Bacterial ASV enrichment between rhizosphere samples was
388 performed using the DESeq2 v. 1.34.0 package (45) implemented in R. Adjusted P values for DESeq2
389 were calculated, and false positives were estimated using the Benjamini-Hochberg method.
390
391 *Bacterial isolation and identification*

392 Protist culture solutions were diluted onto plates of TSA agar and incubated at 28° C for 3 days.
393 96 colonies were selected and cultured at random. Bacterial genomic DNA was extracted and amplified
394 using the primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGTTACCTTGTTACGACTT 3') (46).
395 Partial 16S sequences were sequenced using Sanger sequencing at the Keck DNA Sequencing Core at
396 Yale University, New Haven, USA, and identified using Blast search in NCBI database. Strains identified as
397 *Mucilaginibacter*, *Caulobacter*, *Sphingomonas*, and *Pedobacter* were respectively isolated from cultures
398 of UC70, UC70, UC122, and UC225 and chosen for study. Sequence was deposited as Genbank
399 accessions PQ741793-PQ741796.
400
401 *Protist growth assay*
402 Cultures of *Allapsa* isolate UC225, *Cercomonas* isolate UC19, and *Thaumatomonas* isolate UC70
403 were treated with antibiotics to reduce endogenous bacteria as previously described (19). Protist
404 cultures were grown in PAGE's solution amended with heat-killed *E. coli* (O.D. 0.1) until the cells were
405 freshly encysted. Cysts were centrifuged twice at 4000 g and resuspended in fresh PAGE's solution to
406 remove excess remaining killed *E. coli* cells, then enumerated and resuspended in PAGE's solution to a
407 concentration of 1000 cysts/mL. Bacterial cultures were grown for 72 h in Reasoner's 2 broth media at
408 28°C.
409 Assays were conducted in 24-well cell culture plates. Protist cells were diluted to 100 cells in 1
410 mL of sterile PAGE's solution, while bacteria were diluted to an O.D. of 0.07. In addition, wells were
411 amended with heat-killed *E. coli* to an O.D. of 0.07. Three replicates each were set up of all protist-
412 bacteria pairwise combinations, while three replicates each were set up of protists grown without added
413 bacteria as controls. After the plates were set up, cells were visualized at 200 x magnification using a
414 Zeiss ID02 Invertoscope inverted Microscope and photographed using an Axiocam 305 color camera.
415 Three photos were taken of each well in a triangular pattern. Cells were photographed every 24 hours
416 for the following 6 days. Cells were enumerated using ImageJ, after which the number of cells in 1 mL
417 were calculated for each well and timepoint.
418
419 *Statistical Analysis*
420 Data were graphed using ggplot2 v. 3.4.0 (47), implemented in R version 4.3.0. Statistical
421 differences between treatments and controls were determined using ANOVA followed by Dunnett's
422 multiple comparison test using the DescTools package in R. Cell count data were analyzed in R using a
423 Dunnett Test to compare with the controls.

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558
559

560 **Tables**

561

562 **Table 1**. Protist culture microbiome bacterial ASVs that were enriched or abundant in the rhizosphere

563 after protist inoculation.

ASV[a]	Genus	Protist culture ID	Enriched in rhizosphere[b]	Abundant in rhizosphere[c]
1	*Azospirillum* sp.	UC19, UC48, UC74, UC122		X
2	*Bosea* sp.	UC74		X
3	*Bosea* sp.	UC19, UC22, UC74	X	X
4	*Bradyrhizobium* sp.	UC19, UC22, UC48, UC70, UC74, UC122		X
5	*Brevundimonas* sp.	none	X	
6	*Brevundimonas sp.*	UC242		X
7	Burholderiaceae uncl.	UC22		X
8	Burkholderia uncl.	UC74		X
9	Burkholderiaceae uncl.	UC122	X	X
10	*Caulobacter* sp.	UC225, UC242	X	X
11	*Caulobacter* sp.	UC19, UC225		X
12	*Cupriavidus* sp.	UC225		X
13	Enterobacteriaceae uncl.	UC48		X
14	*Luteimonas* sp.	UC70, UC122	X	X
15	*Mucilaginibacter* sp.	UC19, UC22		X
16	*Mucilaginibacter* sp.	none	X	
17	*Paenarthrobacter sp.*	UC242		X
18	*Pedobacter* sp.-α	UC22, UC70	X	X
19	*Pedobacter* sp.-β	UC225	X	X
20	*Pseudomonas* sp.	UC19, UC48, UC74		X
21	*Pseudoxanthomonas* sp.	UC122, UC19		X
22	*Reyranella* sp.	UC225, UC242	X	X
23	*Reyranella* sp.	none		X

24	*Rhodococcus* sp.	UC19, UC22, UC48, UC74, UC122, UC242		X
25	*Sphingomonas* sp.-α	UC22, UC48, UC70, UC122	X	X
26	*Sphingomonas* sp.-β	none	X	
27	uncl. Planctomycetales	UC122	X	X
28	uncl. Xanthobacteraceae	UC48, UC70, UC122, UC225		X
29	uncultured gen. SH-PL14	none	X	
30	*Variovorax* sp.	UC19, UC22, UC48, UC70, UC74, UC122, UC242		X

a. ASV identifiers are found in Table S5.

b. Significantly enriched in maize rhizosphere 42 days after single protist inoculation in greenhouse, relative to uninoculated plants

c. Among the 100 most abundant bacterial ASVs in the rhizosphere 42 days after protist inoculation, regardless of enrichment.

d. X indicates that a matching ASV was enriched in maize rhizosphere samples 21 days after inoculation with 18-member protist consortium in the growth chamber. (g) indicates that other ASVs from the same genus were enriched in the growth chamber.

Figure captions:

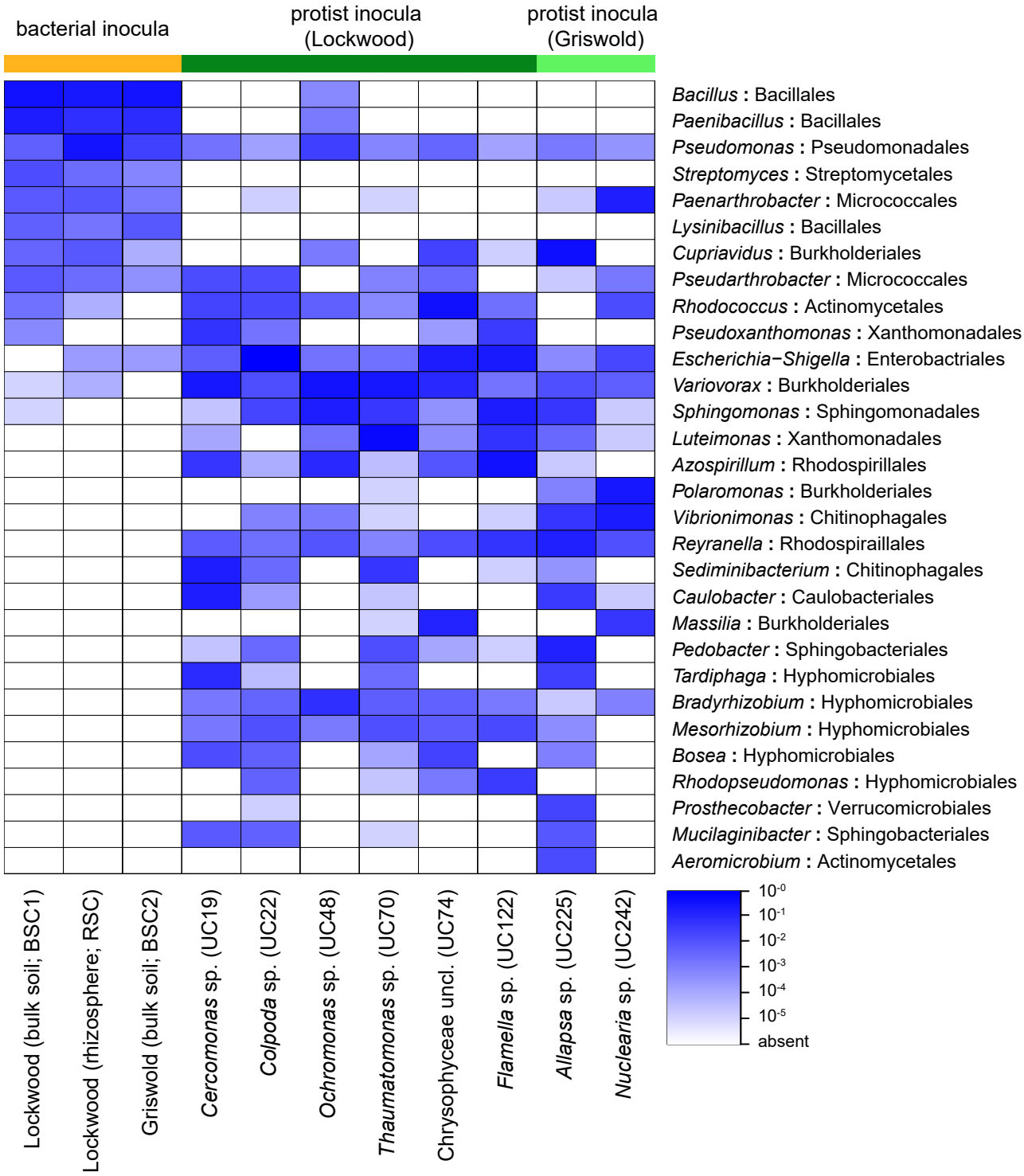
Figure 1: Bacterial microbiome composition of the protist-free bacterial inocula and the eight protist isolates from the maize rhizosphere. Genera having the highest mean abundance across samples are shown. Bacterial order is indicated after the colon. Shade corresponds to proportional abundance in the culture sample.

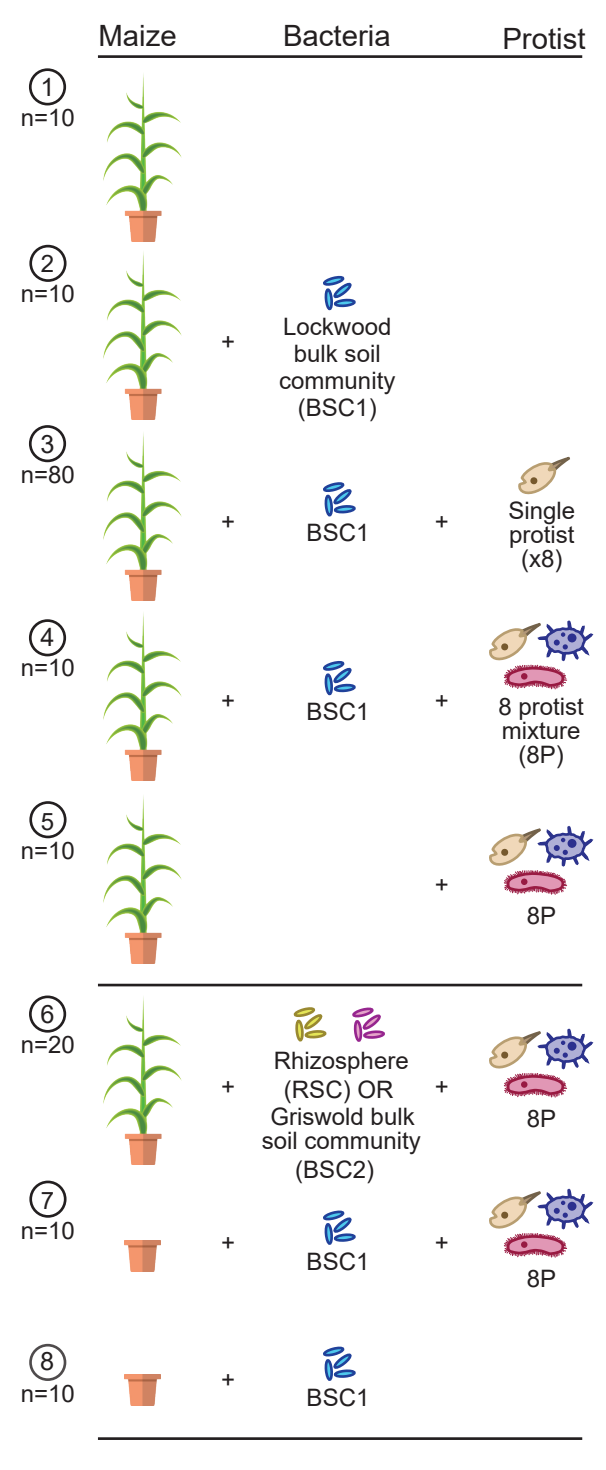
Figure 2: Experimental design showing eight combinations of protist and bacterial treatments. Numbers of planted replicates are listed under each circled number. Six plants failed to grow and eleven 16S rhizosphere libraries had fewer than 2000 reads, so these samples were removed from the study.

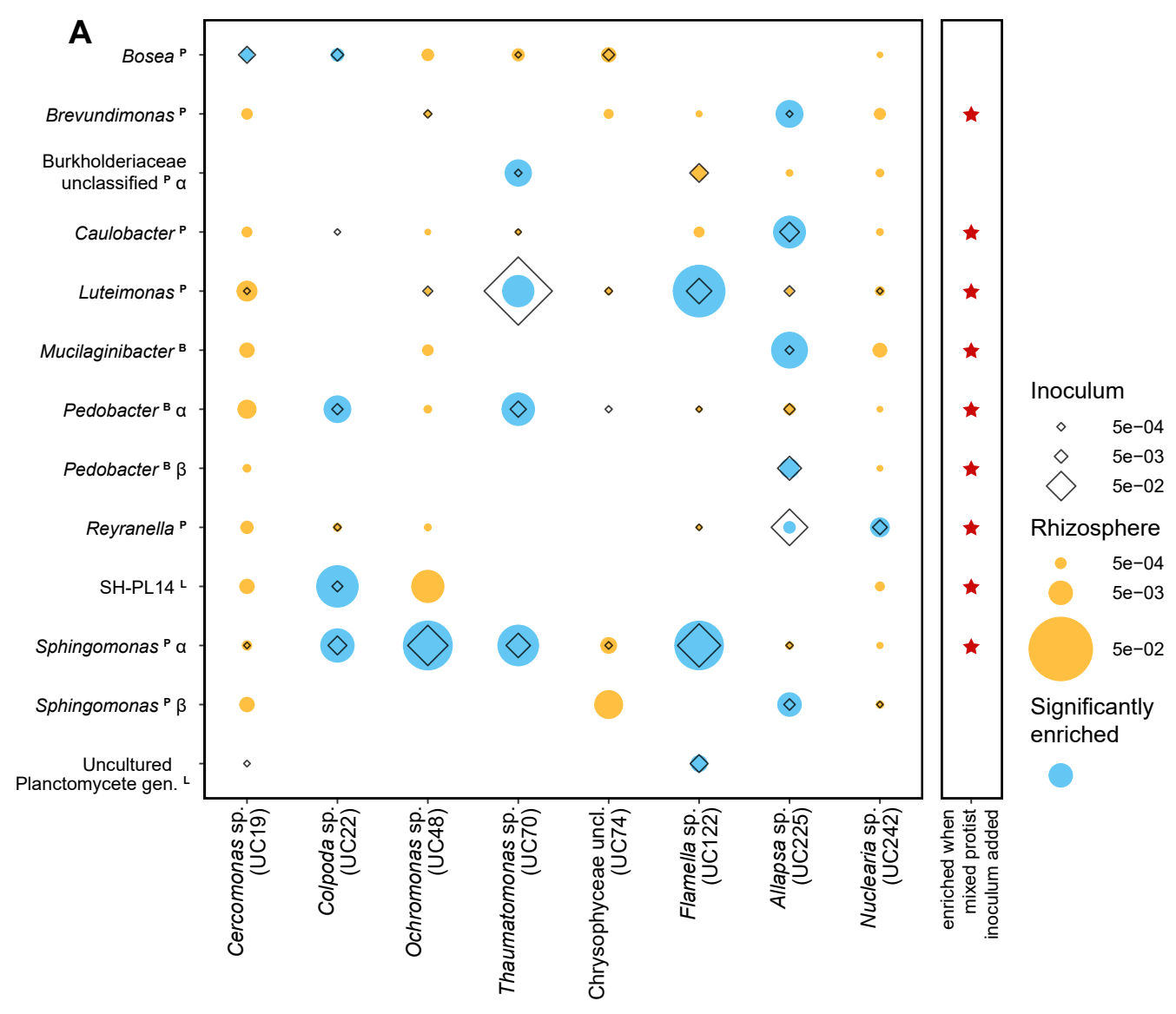
Figure 3: Enrichment of bacterial ASVs in the maize rhizosphere six weeks after protist inoculation onto germinated seeds. **A.** Enrichment by single protists. Diameter of diamonds and dots indicates the mean relative abundance of ASVs in the protist culture microbiome (n = 1) and the rhizosphere samples (n = 9 or 10), respectively. Blue color indicates statistical enrichment in the protist treatment relative to protist-uninoculated controls, as determined by DeSeq2. Red stars indicate that the bacteria were enriched when the eight protists were inoculated as a consortium. **B.** The proportion of rhizosphere reads representing protist-enriched ASVs after plants were inoculated with single protist cultures or mixed

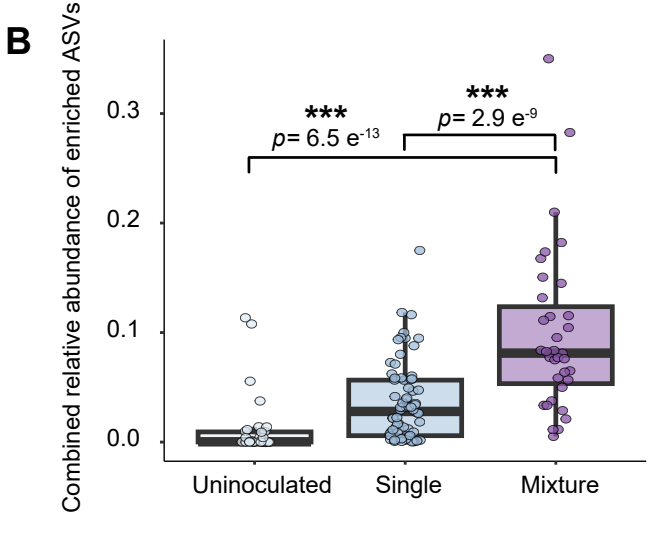
590 protists, or uninoculated. Asterisks indicate significant differences from the mixed protist treatment
591 using Dunnett's Multiple Comparison test (*** = p<0.0005).
592
593 **Figure 4:** Biomass traits of maize plants inoculated with single protists and an eight-member consortium
594 in a greenhouse study. **A-B.** Root biomass (**A**) and root-shoot mass ratio (**B**) six weeks after inoculation
595 with zero (n = 29), one (n = 78), or a mixture of eight protists (n = 29) onto germinated maize seeds.
596 Filled asterisks indicate significant differences from the single protist treatment at p < 0.05. **C-D.** Root
597 biomass (**C**) and root-shoot mass ratio (**D**) after individual protist inoculation in the presence of protist-
598 free soil bacteria (n = 10 for all treatments except UC22 and UC74, which had one plant fail to grow).
599 Asterisks indicate significant differences from the no-protist treatment at p < 0.05.
600
601 **Figure 5:** Effect of bacterial source (**A**) and rhizosphere environment (**B**) on protist enrichment of soil
602 bacteria. **A.** The eight-protist consortium was co-inoculated with bulk soil bacteria from Lockwood Farm
603 (BSC1), Griswold Farm (BSC2), maize rhizosphere soil bacteria from Lockwood Farm (RSC), or no bacteria.
604 **B.** Enrichment after the eight-protist consortium was inoculated into the center of unplanted pots. Dot
605 diameter indicates the mean relative abundance of the ASV (n = 9 or 10). Blue color indicates statistical
606 enrichment in the protist mixture treatment relative to protist-uninoculated controls within each
607 column.
608
609 **Figure 6:** Cell counts of three protist taxa (*Allapsa*, *Cercomonas*, and *Thaumatomonas*) after being fed
610 with heat-killed *E. coli* alone, or with heat-killed *E. coli* in addition to one of four bacterial cultures
611 isolated from protist cultures (n = 3 for each treatment). Cells were counted at 0, 3 and 6 days after the
612 cells were initially fed. Black asterisks indicate significantly more protists than those fed with heat-killed
613 *E. coli* alone, while red asterisks indicate significantly fewer (p < 0.05).
614
615 **Supplementary figure captions:**
616 **Figure S1:** Effect of bacterial treatment on (**A**) the β-diversity of rhizosphere bacterial communities and
617 (**B**) the biomass of maize plants. **A.** NMDS plot showing the clustering of 16S communities of maize
618 rhizospheres after inoculation with bulk soil bacteria from Lockwood Farm (BSC1), Griswold Farm (BSC2),
619 maize rhizosphere soil bacteria from Lockwood Farm (RSC), or no bacteria, and either no protists or the
620 eight-protist consortium. Dashed lines represent 95% confidence intervals. **B.** Root biomass of maize

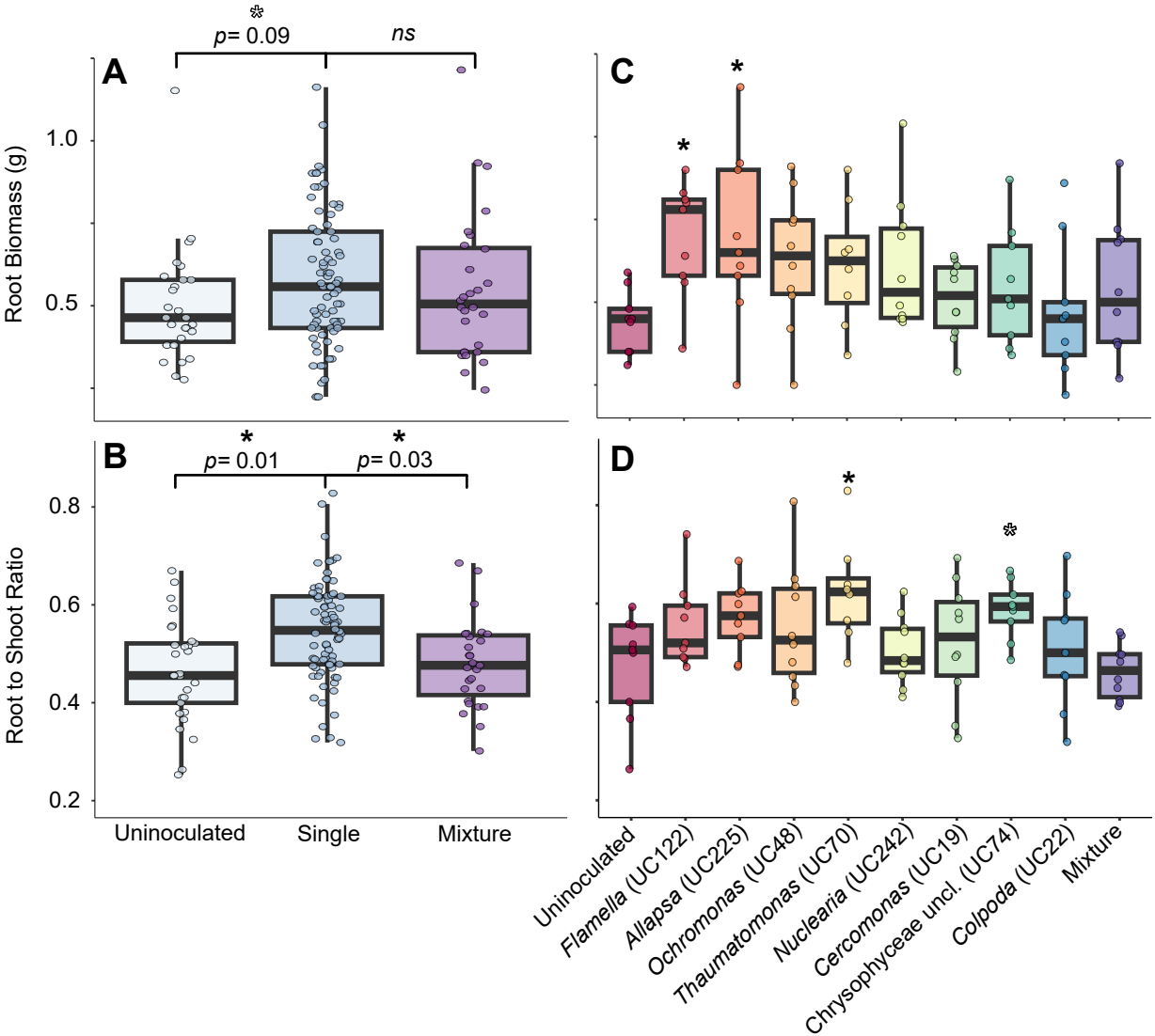
621 plants six weeks after inoculation with the different bacterial treatments, with or without the eight-
622 protist mixture.
623
624 **Figure S2:** Relative abundances of the 13 enriched bacteria in maize rhizospheres six weeks after
625 inoculation with zero, one, or a mixture of eight protists onto germinated maize seeds. Asterisks indicate
626 that relative abundances were significantly greater ($p < 0.05$) than in the mixture-inoculated plants
627 based on a Dunnett's Multiple Comparison Test.
628
629 **Figure S3:** Effect of rhizosphere on bacterial enrichment and abundance. **A.** Enrichment of five bacterial
630 ASVs in maize rhizospheres inoculated with bulk soil bacteria from Lockwood Farm (BSC1) alone or maize
631 rhizosphere soil bacteria from Lockwood Farm (RSC) alone compared with uninoculated controls. Circle
632 diameters indicate mean relative abundances of bacteria. **B.** Proportion of reads of the 13 enriched
633 bacterial ASVs in the plants inoculated with no protists, but different bacterial inocula. Asterisks indicate
634 that the read proportions are significantly greater when plants were inoculated with protists compared
635 with the plants that were not inoculated with protists at $p < 0.005$ (**) and $p < 0.001$ (***). **C.** Relative
636 abundance of the bacterial ASV Burkholderiaceae unclassified β in soil inoculated with the protist
637 mixture or no protist, with or without the plant.
638
639 **Figure S4:** Cell counts of three protist taxa (*Allapsa*, *Cercomonas*, and *Thaumatomonas*) after being fed
640 with no bacteria, or one of four bacterial isolates from protist cultures ($n = 3$ for each treatment). Cells
641 were counted at 0, 3 and 6 days after the cells were initially fed. Black asterisks indicate significantly
642 more protists than those fed with no bacteria ($p < 0.05$) based on a Dunnett's Multiple Comparison Test.
643

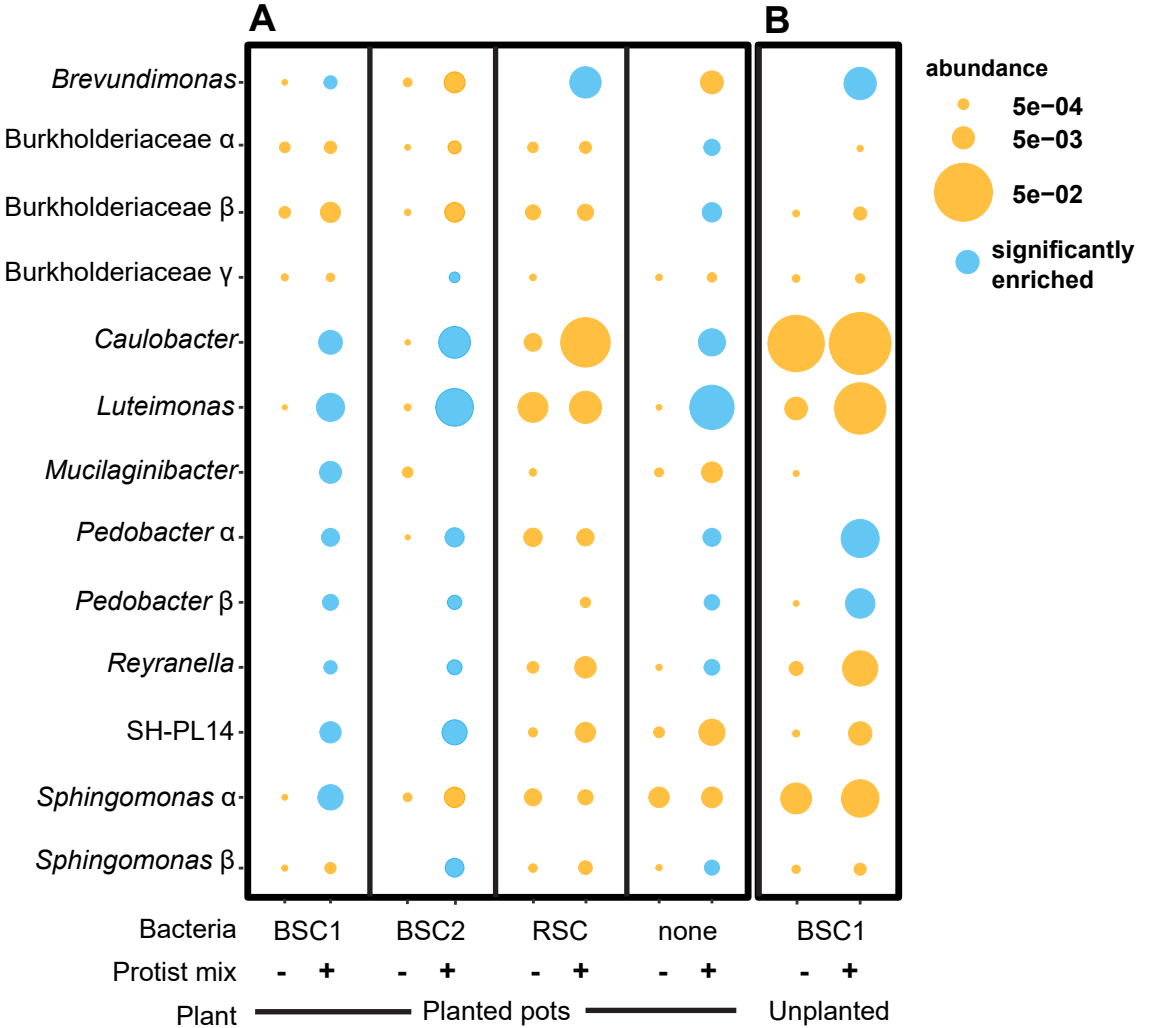

bacterial inocula
protist inocula (Lockwood)
protist inocula (Griswold)
Bacillus : Bacillales
Paenibacillus : Bacillales
Pseudomonas : Pseudomonadales
Streptomyces : Streptomycetales
Paenarthrobacter : Micrococcales
Lysinibacillus : Bacillales
Cupriavidus : Burkholderiales
Pseudarthrobacter : Micrococcales
Rhodococcus : Actinomycetales
Pseudoxanthomonas : Xanthomonadales
Escherichia−Shigella : Enterobactriales
Variovorax : Burkholderiales
Sphingomonas : Sphingomonadales
Luteimonas : Xanthomonadales
Azospirillum : Rhodospirillales
Polaromonas : Burkholderiales
Vibrionimonas : Chitinophagales
Reyranella : Rhodospiraillales
Sediminibacterium : Chitinophagales
Caulobacter : Caulobacteriales
Massilia : Burkholderiales
Pedobacter : Sphingobacteriales
Tardiphaga : Hyphomicrobiales
Bradyrhizobium : Hyphomicrobiales
Mesorhizobium : Hyphomicrobiales
Bosea : Hyphomicrobiales
Rhodopseudomonas : Hyphomicrobiales
Prosthecobacter : Verrucomicrobiales
Mucilaginibacter : Sphingobacteriales
Aeromicrobium : Actinomycetales
Lockwood (bulk soil; BSC1)
Lockwood (rhizosphere; RSC)
Griswold (bulk soil; BSC2)
Cercomonas sp. (UC19)
Colpoda sp. (UC22)
Ochromonas sp. (UC48)
Thaumatomonas sp. (UC70)
Chrysophyceae uncl. (UC74)
Flamella sp. (UC122)
Allapsa sp. (UC225)
Nuclearia sp. (UC242)
10-0
10-1
10-2
10-3
10-4
10-5
absent

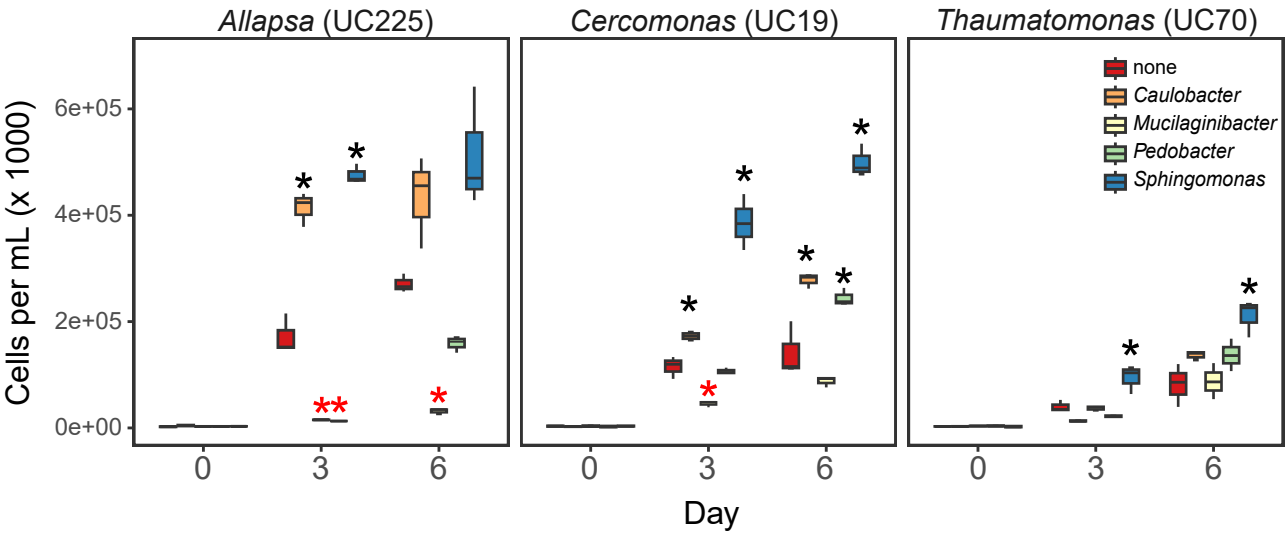

Maize
Bacteria
Protist
1
n=10
2
n=10
+
Lockwood bulk soil community (BSC1)
3
n=80
+
BSC1
+
Single protist (x8)
4
n=10
+
BSC1
+
8 protist mixture (8P)
5
n=10
+
8P
6
n=20
+
Rhizosphere (RSC) OR Griswold bulk soil community (BSC2)
+
8P
7
n=10
+
BSC1
+
8P
8
n=10
+
BSC1


A
Bosea P
Brevundimonas P
Burkholderiaceae unclassified P α
Caulobacter P
Luteimonas P
Mucilaginibacter B
Pedobacter B α
Pedobacter B β
Reyranella P
SH-PL14 L
Sphingomonas P α
Sphingomonas P β
Uncultured Planctomycete gen. L
Cercomonas sp. (UC19)
Colpoda sp. (UC22)
Ochromonas sp. (UC48)
Thaumatomonas sp. (UC70)
Chrysophyceae uncl. (UC74)
Flamella sp. (UC122)
Allapsa sp. (UC225)
Nuclearia sp. (UC242)
enriched when mixed protist inoculum added
Inoculum
5e−04
5e−03
5e−02
Rhizosphere
5e−04
5e−03
5e−02
Significantly enriched


B
Combined relative abundance of enriched ASVs
0.0
0.1
0.2
0.3
***
p= 6.5 e-13
***
p= 2.9 e-9
Uninoculated
Single
Mixture


A
B
C
D
Root Biomass (g)
Root to Shoot Ratio
1.0
0.5
0.8
0.6
0.4
0.2
p= 0.09
ns
p= 0.01
p= 0.03
Uninoculated
Single
Mixture
Uninoculated
Flamella (UC122)
Allapsa (UC225)
Ochromonas (UC48)
Thaumatomonas (UC70)
Nuclearia (UC242)
Cercomonas (UC19)
Chrysophyceae uncl. (UC74)
Colpoda (UC22)
Mixture


A
B
Brevundimonas
Burkholderiaceae α
Burkholderiaceae β
Burkholderiaceae γ
Caulobacter
Luteimonas
Mucilaginibacter
Pedobacter α
Pedobacter β
Reyranella
SH-PL14
Sphingomonas α
Sphingomonas β
abundance
5e−04
5e−03
5e−02
significantly enriched
Bacteria
BSC1
BSC2
RSC
none
BSC1
Protist mix
- +
- +
- +
- +
- +
Plant
Planted pots
Unplanted


Allapsa (UC225)
Cercomonas (UC19)
Thaumatomonas (UC70)
Cells per mL (x 1000)
6e+05
4e+05
2e+05
0e+00
0
3
6
Day
none
Caulobacter
Mucilaginibacter
Pedobacter
Sphingomonas