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1 **Soybean and cotton spermosphere soil microbiome shows dominance of soil-borne**
2 **copiotrophs**
3
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24 **Abstract**

25 The spermosphere is the transient, immediate zone of soil around imbibing and
26 germinating seeds, rich in microbial activity. It represents a habitat where there is contact
27 between seed-associated microbes and soil microbes, but is studied less compared to other plant
28 habitats. Previous studies on spermosphere microbiology were primarily culture-based or did not
29 sample the spermosphere soil as initially defined in space and time. Thus, the objectives of this
30 study were to develop an efficient strategy to collect spermosphere soils around imbibing
31 soybean and cotton in non-sterile soil and investigate factors contributing to changes in microbial
32 communities. The method employed sufficiently collected spermosphere soil as initially defined
33 in space (3-10 mm soil around a seed) by constraining the soil sampled with an cork borer and
34 confining the soil to a 12-well microtiter plate. Spermosphere prokaryote composition changed
35 over time and depended on the crop within six hours after seeds were sown. By 12 to 18 hours,
36 crops had unique microbial communities in spermosphere soils. Prokaryote evenness dropped
37 following seed imbibition and the proliferation of copiotroph soil bacteria in the phyla
38 Proteobacteria and Firmicutes. Due to their long history of plant growth promotion, prokaryote
39 OTUs in *Bacillus*, *Paenibacillus*, *Burkholderia*, *Massilia*, *Azospirillum*, and *Pseudomonas* were
40 notable genera enriched in the cotton and soybean spermosphere. There was no consistent
41 evidence that fungal communities changed like prokaryotes. However, fungi and prokaryotes
42 were hub taxa in cotton and soybean spermosphere networks. Additionally, the enriched taxa
43 were not hubs in networks, suggesting other taxa besides the copiotrophic may be important for
44 spermosphere communities. Overall, this study advances knowledge in the assembly of the plant
45 microbiome early in a plant's life, which may have plant health implications in more mature
46 plant growth stages.

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47

48 **Introduction**

49 When a seed is sown, it imbibes water and releases nutrient-rich seed exudates that fuel
50 interactions between soil and seed-associated microbes in a plant habitat called the spermosphere
51 (Nelson 2004; Nelson 2018; Schiltz et al. 2015; Shade et al. 2017; Windstam and Nelson
52 2008). Seed exudates have long been recognized to stimulate microbial growth, including a
53 direct link to facilitating pathogen growth chemotactically towards seeds (Nelson 2004; Short
54 1976; Slykhuis 1947; Stanghellini 1971). The spermosphere or “spermatosphere” was first
55 described by Slykhuis (1947), who observed the inhibition of a fungal pathogen by three fungal
56 species around a germinating seed. Verona (1958) defined the same habitat as a “zone of
57 elevated microbial activity” around a germinating seed. Nelson (Nelson 1986, 1988; 2004; van
58 Dijk and Nelson 2000; Windstam and Nelson 2008) more formally defined the spermosphere as
59 “the short-lived, rapidly-changing and microbiologically dynamic five-to-ten-millimeter zone of
60 soil around a germinating seed”, which is the definition we adhere to in this study. Despite its
61 importance for plant health outcomes, the spermosphere is less studied than other plant-
62 associated habitats such as the rhizosphere or phyllosphere (Aziz et al. 2022; Schiltz et al. 2015;
63 Shade et al. 2017).

64 Seed germination occurs in three distinct phases. Phase I is a physical process
65 characterized by seed imbibition and fast carbon-rich exudate release into the soil hours after
66 seeds are sown. The highest levels of exudate release are completed in as little as six hours
67 (Lynch 1978; Nelson 2004; Simon and Raja Harun 1972). The initial phase of exudate release
68 is followed by a plateau characterizing phase II, then radical emergence, which begins the
69 formation of the rhizosphere, and more exudate release in phase III (Nelson 2004; Schiltz et al.

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70 2015).

71 The spermosphere represents a critical zone for establishing vertically inherited seed
72 microbes, and horizontal interactions between soil and seed-associated microbes (Chesneau et al.
73 2022; Chesneau et al. 2020; Rochefort et al. 2021; Shade et al. 2017; Simonin et al. 2022). The
74 outcome of these interactions can affect the life or death of the plant soon after sowing seeds
75 (McKellar and Nelson 2003; Windstam and Nelson 2008). For example, soil-borne *Pythium* can
76 fully colonize and kill germinating seeds of various crop species within 12-24 hours (Hayman
77 1969; Nelson 1986, 1988; Stanghellini 1973).

78 Spermosphere pathogens still cause millions of dollars in crop loss yearly (Allen et al.
79 2017; Bradley et al. 2021; Mueller et al. 2020). Because of enhanced genetics and other factors,
80 seed ranks first or second in operating costs borne by soybean and cotton farmers each year
81 (USDA-ERS 2022). Additionally, trends toward earlier planting dates, increased frequency of
82 heavy pulsed rain events, and variable temperature conditions experienced by farmers at planting
83 can create soil moisture and temperature conditions that stress the germinating seed (Morris et al.
84 2021; Munkvold 1999; Roth et al. 2020). Conservation tillage (low or no-till) can also lead to
85 harboring plant pathogens on plant debris left in the field from the previous growing season
86 (Bockus and Shroyer 1998). Consequently, the protection of seeds from pathogens that specialize
87 in spermosphere colonization is vital to improved crop productivity.

88 Seed and seedling pathogens are primarily managed with chemical seed coatings
89 containing fungicides and oomicides. However, improved knowledge on spermosphere
90 microbiology and ecology would support the successful inclusion of alternative strategies to
91 chemical seed treatments. For example, biocontrol of *Pythium* from seed-applied *Enterobacter*
92 *cloaceae* could be achieved by metabolizing long-chain fatty acids, which otherwise stimulated

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93 the germination of *Pythium* sporangium (Kageyama and Nelson 2003; van Dijk and Nelson
94 2000; Windstam and Nelson 2008). Studies on the spermosphere have either been culture-based
95 or have more recently focused on the contribution of the indigenous seed microbiome by using
96 sterile or soilless growth conditions or pre-imbibed or pre-germinated seeds, which do not
97 sample the spatial and temporal properties of the spermosphere soil (Barret et al. 2015;
98 Johnston-Monje et al. 2016; Moroenyane et al. 2021; Rochefort et al. 2021). Indeed, natural
99 seed-associated epiphytes and endophytes compete with pathogens (Smith et al. 1999; Torres-
100 Cortes et al. 2019). While commendable, these studies largely ignore the influence of the initial
101 seed exudate release on the spermosphere soil microbes. Therefore, a mechanistic understanding
102 of the complex interactions in spermosphere soil will aid in novel treatments for seed and
103 seedling pathogens and help our understanding of plant microbiome assembly.
104 However, one major challenge in studying spermosphere soil using high-throughput
105 culture-independent techniques may be a lack of a quick and efficient method of collecting
106 spermosphere soil (Schiltz et al. 2015). Here, we aimed to capture changes in microbial diversity
107 in the spermosphere soils as soybean and cotton seeds underwent phases I and II of seed
108 germination (i.e., pre radical emergence). We sampled the spermosphere soil of cotton and
109 soybean by constraining the soil zone within wells of a 12-well plate and sampling precisely
110 three to six millimeters of soil around an imbibing seed with an appropriately sized cork borer,
111 extracted DNA, and sequenced the 16S and ITS from cotton and soybean spermosphere soil. We
112 hypothesized that seeds would imbibe water rapidly and follow previously established phases of
113 exudate release, which would alter microbial diversity and co-occurrence patterns. We also
114 hypothesized that spermosphere soils would be distinct based on crop species. Therefore, the
115 objectives of this study were twofold: 1) characterize the bacterial and fungal microbial

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116 communities associated with cotton and soybean spermosphere soil compared to control soil and
117 2) determine how microbial diversity and co-occurrences change over time as a seed imbibes
118 water.
119

120 Materials and Methods

121 **Soil collection and preparation.** The soil used in this study was collected from a field used for
122 cotton, soybean, and corn rotation from Prattville Agricultural Research Unit in Prattville,
123 Alabama (32.42533, -86.4452), since this soil showed consistent emergence of both cotton and
124 soybean in preliminary experiments (*data not shown*), and was not known to contain a high
125 abundance of any specific seedling pathogen. Approximately three liters of soil from the top ten
126 centimeters were collected and transported to the lab. The soil was sieved to eliminate stones and
127 pebbles and air-dried for 24 hours to ensure homogeneity in water content. The soil was used
128 immediately after air drying. Six to seven grams (6 ml) of soil was transferred to each well of the
129 12-well microtiter plates (VWR American cat no.:10861-556, USA), containing three one-
130 millimeter holes in the bottom of all wells for drainage. Each well in the 12-well microtiter plates
131 measured a total volume of 6.8 ml, depth of each well of a 12-well plate was 15 mm with a width
132 of 23 mm. Each well containing soil was watered with 1.5 ml of sterile water (25% soil
133 moisture), and the water was allowed to circulate for one hour before the seeds were sown.

134 Nontreated Williams-82 soybean or nontreated delinted Delta Pine 1646 B2XF cotton
135 were used in this study and were sorted to discard discolored seeds or seeds with cracked seed
136 coats (Nelson 1986). The weight of individual dry seeds was recorded before use and after
137 imbibition to record how much water was imbibed. The initial weight of soybean seeds was
138 between 170 and 250 mg, and cotton seeds weighed between 60 and 110 mg. The average size of

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139 soybeans used was between five to eight millimeters in diameter and spherical. Cotton seeds
140 were more oblong, three to four millimeters in diameter and ten millimeters long. Seeds were
141 surface-sterilized by soaking in 6% bleach solution for 10 minutes in a sterile petri-dish and
142 washed three times with sterile distilled water. Seeds were surface sterilized to maximize the
143 effect of seed exudates on the growth of microbes from the soil. Six replicate seeds were sown
144 into the center of individual wells, halfway into the 15 mm depth of the well, using flamed
145 forceps. Wells containing only soil without a cotton or soybean seed were used as a control. The
146 12-well microtiter plates were placed in a planting tray covered with a lid to keep the soil from
147 drying. Planting trays containing 12-well microtiter plates were placed inside a growth chamber
148 at 25°C.
149
150 **Collection of spermosphere**. Spermosphere soil samples were collected at 0, 6, 12, and 18 hours
151 after sowing. Wells containing control soil were sampled as a control, and are hereafter referred
152 to as control soil. Spermosphere soil and control soil samples were collected using an 11 mm
153 cork borer cleaned of soil with 70% ethanol and flame sterilized between samples. The 11 mm
154 cork borer was specifically used since the spermosphere is defined as the first 5-10 mm of soil
155 around a germinating seed (Nelson 2004) and allowed soil collection within this range based on
156 the seed sizes stated previously. Therefore, given the size of the well, the volume of soil used,
157 and the seed sizes, the spermosphere soil sampled consisted of three to six millimeters on either
158 side of a soybean seed and seven to ten millimeters above and below a soybean. Similarly, the
159 spermosphere soil sampled for cotton consisted of seven to eight millimeters on either side and
160 five to ten millimeters above and below the seed. A diagram of the sampling procedure for
161 soybean is shown in Figure 1. In preliminary experiments, bacterial populations in spermosphere

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162 soils sampled with this method increased significantly by 1.15 log in soybean and about 0.8 log
163 in cotton compared to control soil (Supplemental Figure 1).
164 Spermosphere soil containing the seed inside the core within the cork borer was
165 transferred into sterile envelopes, and 0.25 ml was immediately transferred to 2 ml disruptor
166 tubes (Omega Bio-Tek E.Z.N.A Soil DNA; Norcross GA), then stored at -80ºC until DNA
167 extraction. The remaining soil clinging to the seed was washed off, the seed blotted dry of excess
168 water, and the weight of the seed was recorded after sample collection and compared to the
169 initial individual seed weight to determine the water imbibed by each seed.
170
171 **DNA extraction, amplification, and sequencing**. The total DNA was extracted from the
172 spermosphere and control soils following the manufacturer's recommendation. Amplification
173 and library construction of 16S or ITS rDNA was performed with a three-step Polymerase Chain
174 Reaction (PCR) (Lundberg et al. 2013; Longley et al. 2020). Briefly, the 16S region of the
175 ribosomal DNA (rDNA) was amplified using the forward and reverse primers 515F and 806R
176 (Caporaso et al. 2011). Amplification of ITS used the primers ITS1F and ITS4. Following the
177 amplification of the respective rDNA regions, the amplicons were linked to variants of
178 frameshift primers, and then a 10 bp barcode was added for sample identification. Library
179 negative controls consisted of DNA extraction without soil and no template PCR water controls.
180 The ZymoBIOMICS microbial community DNA standard (Zymo Research, Irvine, CA) was
181 used as a positive control mock community. A fungal synthetic mock community was used as a
182 positive control for fungi (Palmer et al. 2018). DNA amplification was confirmed with gel
183 electrophoresis, and successfully amplified libraries were normalized using SequalPrep™
184 Normalization Plate Kit (Thermo Fisher, USA). Normalized amplicons were then pooled and

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185 concentrated 20:1 using the 50K Dalton Millipore filters (Sigma-Aldrich, USA). The pooled
186 library was cleaned using AMPure XP beads at a ratio of 0.7X (Beckman Coulter, USA).
187 Cleaned amplicon pools were verified by gel electrophoresis, quantified using a Qubit
188 fluorometer (Thermo Fisher, USA), and sequenced on an Illumina MiSeq 2x300 bp using the v3
189 600 cycles kit at SeqCenter LLC (Pittsburgh, PA). Primers and cycling parameters to construct
190 libraries were the same as in Noel (2022).
191
192 **Read processing.** The quality of demultiplexed reads was assessed using the FastQC, and primer
193 sequences were removed using cutadapt 4.0 (Martin 2011). Prokaryote 16S V4 sequences were
194 merged using VSEARCH 2.21.1 (Rognes et al. 2016). Only forward fungal ITS1 reads were
195 used since reverse reads did not overlap. Fungal reads were trimmed to remove the conserved
196 18S regions. Reads were then truncated to equal length (fungi 200 bp, prokaryote 300 bp) and
197 quality filtered using VSEARCH 2.21.1 with an expected error threshold of 1.0. Singletons were
198 removed and reads *de novo* clustered based on 97% identity into prokaryote OTUs (pOTUs) or
199 fungal OTUs (fOTUs) using USEARCH v11.0.667, which includes a chimera detection and
200 removal step (Edgar 2010; Edgar et al. 2011). The resulting pOTUs were aligned using MAFFT
201 v7.505 (Nakamura et al. 2018), and a phylogenetic tree was estimated using FastTree v2.1.20
202 (Price et al. 2010), then midpoint rooted with FigTree v1.4.4 (Rambaut 2018). Taxonomy was
203 assigned to resulting pOTUs using the SINTAX algorithm (Edgar 2016) against the SILVA
204 138.1 database (Quast et al. 2013). Fungal taxonomy was assigned using the ribosomal database
205 project's Naïve Bayesian Classifier algorithm against the UNITE fungal ITS database version
206 9.0 (Nilsson et al. 2019).
207

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208 **Data analysis.** Data were primarily analyzed using phyloseq v.1.34.0 (McMurdie and Holmes
209 2013) and vegan v2.5-7 (Oksanen et al. 2022) of the statistical software R v.4.2.2. All plots were
210 generated using the data visualization package 'ggplot2 v.3.3.5' (Wickham 2016). Contaminant
211 OTUs detected in the negative controls were removed with decontam v1.10.0 (Davis et al. 2018).
212 Samples with less than 10,000 were discarded. Fungal samples with less than 1000 reads were
213 discarded due to low sequencing coverage.

214 Richness, Pielou's evenness (Pielou 1966), and Faith's Phylogenetic Diversity (Faith
215 1992) were used to determine within sample diversity differences in diversity using Kruskal-
216 Wallis one-way analysis of variance. Read counts were then normalized using the cumulative
217 sum scaling with metagenomeSeq v1.32.0 (Paulson et al. 2013) and subjected to principal
218 coordinate analysis based on Bray-Curtis distances for fungi and prokaryotes or Weighted
219 Unifrac distances for prokaryotes only. This analysis was followed by a Permutational Analysis
220 of Variance (PERMANOVA) implemented with the 'adonis2' function to determine the
221 differences in centroids of the prokaryote or fungal communities across time points and soil
222 versus spermosphere. Differences in multivariate dispersion were also evaluated using the
223 'betadisper' function.

224 Differential abundance analysis was conducted with Analysis of Compositions of
225 Microbiomes with Bias Correction version 2 (ANCOM-BC2) (Lin and Peddada 2020).
226 Significantly different OTUs were detected based on Holm-Bonferonii corrected p-values. Then,
227 microbial co-occurrence networks with prokaryotes and fungi were constructed using SpiecEasi
228 v1.1.2 (Kurtz et al. 2015) and compared between soybean spermosphere soil, cotton
229 spermosphere soils, and control soil using NetCoMi v.1.1.0 (Peschel et al. 2021). For network
230 construction, spermosphere soil samples and soil samples without seeds at 12 and 18 hours were

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231 filtered to a common set of taxa with a relative abundance above 0.001% and occupancy above
232 90%. Co-occurrence association matrices were estimated using the Meinshausen and Bühlmann
233 algorithm with the 'nlambda' set to 100, sampled 100 times, and the 'lambda.min.ratio' set to 10^{-1}
234 . All resulting networks contained stability values of 0.048 or above, close to the 0.05 StARS
235 algorithm stability target. Association matrices for spermosphere soils or control soil were
236 compared using the 'netAnalyse' function from NetCoMi. Hub taxa were identified based on
237 eigenvector centrality values above the 95% quantile of a fitted log-normal distribution.
238 Comparison of the hub taxa composition was based on the Jaccard similarity index.
239 The Data files and scripts used for this analysis are available on GitHub
240 (https://github.com/Noel-Lab-Auburn/SpermosphereMicrobiome2022). Raw sequence reads
241 were deposited to the sequence read archive with the accession number PRJNA925866.
242
243 **Results**
244 **Sequencing outputs.** Mock OTUs for the fungi and prokaryotes made up 99.9% of the
245 composition of the positive controls, indicating minimal cross-contamination. Nine prokaryotic
246 OTUs were filtered after detection in negative control samples, resulting in 2,090,814 16S V4
247 reads of 8088 OTUs across 71 samples with a median read depth of 29,237 reads per sample.
248 Nineteen fungal OTUs were detected in negative controls and taken out, resulting in 2,534,301
249 ITS1 reads with 1904 fungal OTUs across 69 samples and a median read depth of 37,933 reads
250 per sample. Rarefaction curves indicate that much of the diversity was adequately captured
251 (Supplemental Figure 2).
252
253 **Prokaryote community dominance correlates with water imbibition.** The individual

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254 measurement of seed weight for soybean and cotton seeds before and after spermosphere
255 collection indicate that water was imbibed from the surrounding soil (Fig. 2a). Overall, soybean
256 seeds imbibed more (250-300 mg) than cotton seeds (50-80 mg) and both seeds increased in seed
257 weight within the first six hours indicating imbibition within this timeframe, then a plateau after
258 six hours.

259 We observed a reduction in prokaryote evenness (or increase in dominance) over time in
260 spermosphere soils but not in control soil (Fig. 2b). At time-point 0, there was no significant
261 difference in the evenness of prokaryote taxa ($P = 0.18$), as expected. At 6 hours, we observed a
262 reduction in the evenness of prokaryote taxa in soybean spermosphere soils, compared to cotton
263 spermosphere ($P = 0.004$) or control soil ($P = 0.002$). At 12 and 18 hours, both cotton and
264 soybean spermosphere samples had significantly reduced evenness compared to control soil and
265 each other ($P \leq 0.009$). Prokaryote evenness was significantly negatively correlated with water
266 imbibition, meaning that as seeds imbibed water and released exudates, prokaryote communities
267 became more dominant (cotton r = -0.58, $P < 0.001$; soybean r = -0.57, $P = 0.004$) (Fig. 2c).
268 However, the crop did not alter prokaryote richness or phylogenetic diversity compared to
269 control soil. Prokaryote richness and phylogenetic diversity dropped significantly over time
270 regardless of habitat ($P < 0.001$). Additionally, there was no consistent evidence that fungal
271 richness or evenness was altered in spermosphere samples compared to control soil. Still, a few
272 samples of soybean spermosphere soil and control soil dropped in evenness after 18 hours
273 (Supplemental Figure 3), becoming more dominant in one fOTU2 *Fusarium* (Supplemental
274 Figure 4).

275

276 **Spermosphere prokaryote composition depends on the crop**. Spermosphere soils had

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277 different prokaryote community compositions than control soil. A visualization of the change in
278 the most abundant prokaryote composition over time is shown in Figure 3. Prokaryote
279 communities were driven by habitat ($P < 0.001$), time since sowing ($P < 0.001$), and interaction
280 of both factors ($P < 0.001$) (Supplemental Table 1). The interaction prompted a closer look into
281 the differences observed between crops by splitting the data by time-point (Figure 4; Table 1). At
282 0 hours, no significant difference in prokaryote communities existed between bulk soil compared
283 to soybean and cotton spermosphere, as expected (Bray-Curtis, $P = 0.452$; Weighted Unifrac, P
284 $= 0.192$). However, as early as 6 hours, we observed significant differences between control soil
285 and spermosphere soil samples (Bray-Curtis, $P < 0.001$; Weighted Unifrac $P < 0.001$).
286 Differences were extended through 12 hours (Bray-Curtis, $P < 0.001$; Weighted Unifrac $P <$
287 0.001) and 18 hours (Bray-Curtis, $P < 0.001$; Weighted Unifrac $P < 0.001$), where it was clear
288 the spermosphere formed unique community compositions within soybean or cotton. Further,
289 differences in multivariate dispersions were not observed supporting true differences in centroids
290 rather than group dispersions (Table 1). This same trend was not observed with fungi. Time did
291 alter fungal community composition ($P = 0.01$), but there was no evidence that soybean or cotton
292 altered fungal community composition compared to control soils ($P = 0.09$).
293

294 **Enriched prokaryotes in the spermosphere have unique and shared taxa among crops.**

295 Differential abundance analysis determined sets of pOTUs significantly enriched in the
296 spermosphere of cotton and soybean compared to control soil (Figure 5a; Supplemental Table 2.
297 Ninety-four percent of the enriched taxa belonged to Proteobacteria (57%, n = 27) and
298 Firmicutes (36%, n = 17). The remaining three belonged to the Actinobacteria. Within the
299 Proteobacteria, the enriched taxa were spread across ten prokaryote families, with the most

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300 enriched taxa in the Oxalobacteraceae (41%, n = 11). The majority of the enriched Proteobacteria
301 were unidentified at the genus level (n = 12) but included *Massilia* (n = 3), *Noviherbaspirillum*
302 (n = 2), *Burkholderia/Paraburkholdaria* (n = 2), *Aquabacterium* (n = 1), *Pseudomonas* (n = 2),
303 *Cupriavidus* (n = 1), *Pantoea* (n = 1), *Paucimonas* (n = 1), *Rubellimicrobium* (n = 1), and
304 *Azospirillum* (n = 1) (Figure 5b). Within the Firmicutes, all but one pOTU belonged to the
305 Bacilli class with the genera *Paenibacillus* (n = 8), *Bacillus* (n = 4), *Brevibacillus* (n = 1),
306 *Exiguobacterium* (n = 1), and *Tumebacillus* (n = 1). Many enriched taxa were shared between
307 cotton and soybean (n = 18), indicating that similar taxa take advantage of releasing exudates
308 from seeds (Figure 5b). All the enriched pOTUs were present in control soil samples meaning it
309 was unlikely they originated from the seed but were present in the soil and proliferated upon
310 exudate release from the seeds.
311
312 **Cotton and soybean spermosphere networks are more connected and have distinct**
313 **microbial hub taxa**. Cotton and soybean spermosphere networks were compared to each other
314 and to the control soil to determine if they contained different topologies, different sets of
315 network hubs, and the centrality of spermosphere-enriched taxa. Overall, network topology
316 parameters were similar between networks except for the number of separate components. In
317 other words, the spermosphere soil networks mainly consisted of one more prominent
318 component and fewer disconnected sub-networks than the control soil network (Figure 6). For
319 example, the control soil network contained 30 components and 80 nodes within the largest
320 component. Soybean and cotton spermosphere soil networks had more nodes within the largest
321 component (cotton = 136, soybean = 121). However, control soil and a slightly higher positive
322 edge percentage (61% without a seed, 58% cotton spermosphere, 52% soybean spermosphere)

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323 (Supplemental Table 3).

324 Comparison between central nodes was significantly different, indicating that the hub
325 taxa were different between networks given the same sets of taxa used to construct the networks
326 (Table 2). Hub taxa for cotton consisted of six prokaryote OTUs and two fungal OTUs. Cotton
327 prokaryote hubs consisted of three Archaea OTUs in the Nitrososphaeraceae family (pOTU1009,
328 pOTU19, and pOTU12), two OTUs from the Gaiellales (pOTU46, pOTU119), and pOTU29
329 *Massilia* (Figure 6a; Table 3). Fungal cotton hubs were fOTU56 *Fusarium chlamydosporum*, and
330 a yeast fOTU36 *Hannaella oryzae* (Figure 6a; Table 3), whereas the soybean network had three
331 fungal hub taxa: fOTU64 *Helicoma*, fOTU10 *Bartalinia pondoensis*, and an unidentified Fungus
332 fOTU36 (Figure 6b; Table 3). Prokaryote hub taxa in the soybean spermosphere network
333 contained pOTU11 *Sphingomonas*, pOTU132 Nocardioides, pOTU1559 Chloroflexi TK10,
334 pOTU36 Angustibacter, and pOTU349 *Methylobacterium*/*Methylorubrum* (Figure 6b; Table 3).
335 The network from the control soil contained only fungal hubs, different than the identities of
336 spermosphere fungal hubs except for fOTU36 *Hannaella oryzae*. Spermosphere-enriched taxa
337 included in the network analysis were not hub taxa indicating that although enriched in a
338 spermosphere, other microbial taxa besides the enriched taxa play an essential role in
339 maintaining spermosphere network structure (Figure 6).

340

341 **Discussion**

342 To our knowledge, this is the first study to use culture-independent sequencing to study
343 soybean and cotton spermosphere soil microbiomes during the first phases of seed germination.
344 The advancement that allowed this was the method that constrained non-sterile soil to wells
345 within a 12-well plate and sampled around an imbibing seed with a cork borer. The technique

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346 enabled the precise and efficient collection of spermosphere soils as defined in space and time
347 (Nelson 2004), which we believe this represents a more realistic spermosphere habitat. The focus
348 on spermosphere soil in the first phases of seed germination differs from other studies that pre-
349 imbibe or pre-germinate seeds in axenic conditions. We hypothesized and observed a rapid
350 increase in water imbibition followed by a plateau characterizing phase I and phase II of seed
351 germination. Prokaryote community structure changed in as little as six hours for soybean and
352 twelve hours for cotton. We did observe that crops had unique prokaryote community structures
353 in the spermosphere that were distinct from the control soil, typified by differences in network
354 hub taxa and network topologies. The differing hub taxa demonstrate that others besides the
355 enriched taxa are integral to each crop's spermosphere community structure. However, despite
356 the differences in composition and hub taxa, among the most important observations was the
357 commonality in the enriched copiotrophic taxa with a long history of benefiting plant growth,
358 such as *Bacillus*, *Paenibacillus*, *Burkholderia*, *Massilia*, *Azospirillum*, and *Pseudomonas*.

359 In this study, we further define the development of the spermosphere of cotton and
360 soybean at six to twelve hours after sowing, which aligns with previous studies of increased
361 spore germination and full colonization of cotton seeds by *Pythium ultimum* twelve hours after
362 sowing (Nelson 1986, 1988). We observed an increase in water imbibed by both cotton and
363 soybean seeds in the first six hours, which is consistent with previous reports that documented
364 increased water imbibition and exudation within the first few hours after sowing (Simon and
365 Raja Harun 1972). Imbibition ceased representing phase II of germination, indicating the
366 saturation of nutrient reserves and synthesis of products required for the extending radicle
367 (Nelson 2004).

368 Similar to several other studies, we observed the soil microbes respond to seed exudates

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369 and dominated the spermosphere microbiomes (Buyer et al. 1999; Hardoim et al. 2012; Ofek et
370 al. 2011). We observed changes in phylogenetic dissimilarity between crops, and since
371 phylogenetically similar species are more likely to share ecological characteristics and functional
372 traits (Morrissey et al. 2016), it may be expected that the spermosphere communities in our study
373 changed in a functional capacity as in Buyer et al. (1999). However, we observed varying
374 spermosphere prokaryote composition in different plant species, which may highlight the
375 importance of sample collection at the initial stages of seed germination and imbibition rather
376 than at later hours potentially after radicle emergence. Additionally, as in Buyer et al. (1999),
377 future studies should also include other soils with inherently different communities to understand
378 the contribution of different soil microbial pools to forming the spermosphere.

379 The difference between crops may have also been due to differences in the amount of
380 water imbibed. We noticed that soybean imbibed more than cotton seeds, likely due to seed size
381 (Soldan et al. 2021; Vančura 1972). Vančura and Hanzlíková (1972) demonstrated increased
382 quantities of seed exudates as seed size increased. Different varieties of common bean have been
383 shown to differ in the amount of seed exudates, with larger seeded varieties releasing more
384 exudates (Kato et al. 1997). Thus, we speculate that the greater and faster turnover in microbial
385 communities of the soybean spermosphere compared to cotton may be due to the larger size of
386 soybean seeds and increased exudation, which potentially supported a larger habitat for the
387 microbes to occupy. It also leaves the question if microbial communities would have converged
388 on similar compositions if a later sampling point was included.

389 Regardless, as a result of water imbibition and seed exudation, we observed a change in
390 dominance in the spermosphere microbiome over time with both crops. Ota et al. (1991) showed
391 specific nitrogen-fixing bacteria had increased dominance in the spermosphere of cocklebur

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392 seeds but not in soil. Upon revealing enriched taxa in soybean and cotton spermosphere soils, we
393 found some commonalities. Importantly, Bacilli was enriched in both crop's spermosphere soils.
394 Since these *Bacilli,* including *Tumebacillus*, *Paenibacillus,* and *Bacillus,* have historically been
395 associated with plant growth promotion and disease protection and have commercial potential,
396 they were notable. It indicates their ability to utilize seed exudates quickly for growth. Seed
397 exudates have been reported to induce chemotaxis, seed colonization, and biofilm formation of
398 *B. amyloliquefaciens* (*velezensis*) by enhancing active cell division (Yaryura et al. 2008).
399 *Paenibacillus polymyxa* isolated from wheat and peanut rhizosphere increased the survival of
400 *Arabidopsis thaliana* in the presence of the oomycete pathogen *Pythium aphanidermatum* when
401 applied as root treatment (Timmusk et al. 2009). Identifying these enriched taxa is important for
402 prioritizing future work on a mechanistic understanding of the spermosphere microbial
403 interactions that will improve the development of efficacious biologically based disease solutions
404 (Nelson 2004; Weller 2007).

405 In terms of seed versus soil origin, there were OTUs with a low relative abundance and
406 low occupancy that only occurred in cotton or soybean spermosphere samples and were absent
407 from the soil. However, we hesitate to conclude they originated from the seed without directly
408 identifying seed epiphytes and endophytes since it was impossible to know if the unique
409 microbes were seed associates colonizing the spermosphere or if rare members of the soil only
410 present spermosphere samples by chance. Furthermore, surface disinfecting seeds used in this
411 study likely reduced the number of seed epiphytes that would colonize the spermosphere. The
412 implications of surface disinfecting seeds have been argued elsewhere (Barret et al. 2015;
413 Schiltz et al. 2015; Shade et al. 2017). Another limitation of our approach that limited our ability
414 to identify seed-associated microbes may be the use of OTUs rather than amplicon sequence

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415 variants (ASVs) or zero radius OTUs (zOTUs). A finer clustering method may be better suited to
416 studying the transmission of seed-associated microbes into the spermosphere since genotypes
417 originating from the seed may be grouped within 97% OTUs originating from the soil. We
418 recognize microbes originating from the seed can colonize seedlings and other plant organs,
419 which can alter plant health (Bintarti et al. 2022b; Bintarti et al. 2022a; Chesneau et al. 2022;
420 Chesneau et al. 2020; Johnston-Monje et al. 2016; Mitter et al. 2017; Rochefort et al. 2021;
421 Shade et al. 2017; Simonin et al. 2022). For example, it was recently demonstrated that crop
422 flowers sprayed with a beneficial bacterium can colonize endosperm and transmit to germinating
423 seeds (Mitter et al. 2017). While the importance of seed-associated microbes on plant health is
424 evident, little is known about seed endophytes and interactions with horizontally acquired soil
425 organisms, which tend to contribute a large portion of the microbial diversity to the seedling
426 microbiome (Buyer et al. 1999; Nelson 2018; Rochefort et al. 2021).

427 In terms of the microbial networks, we observed different hubs and different topologies
428 given the same set of taxa used for network construction. While fungal diversity was not altered
429 in this study, fungal OTUs were identified as hubs, potentially demonstrating meaningful
430 interactions with in a spermosphere soil. Of most interest was the yeast *Hannella* since these
431 fungi are commonly found in soils, the phyllosphere, and as part of the core seed and
432 phyllosphere microbiome (Noel et al. 2022; Simonin et al. 2022; Yurkov 2018). *Dioszegia*, in
433 the same family as *Hannaella*, was identified as a network hub in the phyllosphere (Agler et al.
434 2016), and the closely related yeast *Bullera* has been a network hub of the soybean phyllosphere
435 (Longley et al. 2020). These yeasts are generally non-pathogenic, but their ecological role is
436 poorly understood (Gouka et al. 2022). The prokaryote hubs were also intriguing because cotton
437 contained several Nitrososphaeraceae pOTUs, which likely are involved with ammonia-

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438 oxidizdation in soils (Reyes et al. 2020). Cotton spermosphere hubs also had a *Massilia* pOTU.
439 *Massilia* is known for below-ground associations and the ability to solubilize phosphate (Silva et
440 al. 2017), but has also been found as a hub in above-ground plant tissues (Longley et al. 2020).
441 For soybean, *Sphingomonas* and *Methylobacterium*/*Methylorubrum* pOTUs were notable
442 network hubs since these genera have been demonstrated to be abundant in the phyllosphere and
443 core seed microbiome and produce plant growth-promoting hormones and UVA-absorbing
444 compounds (Kwak et al. 2014; Yoshida et al. 2017). The difference in hub taxa between crops
445 demonstrates that soybean and cotton construct unique microbial communities early in life,
446 which may have plant health consequences at or beyond the spermosphere stage.
447 However, spermosphere-enriched pOTUs were not identified as network hubs; instead,
448 they were located more peripheral in the networks, indicating they may be copiotrophs
449 responding quickly to the availability of carbon-rich exudates from the seeds (Torres-Cortes et
450 al. 2018). Spermosphere networks were more connected with larger components than the soil
451 network. Increased soil network complexity was associated with increased microbiome function
452 (Wagg et al. 2019). Therefore, it may be hypothesized that seed exudates help stimulate
453 associations between organisms or sub-communities and form more connected or stable
454 communities. However, further research is needed to determine how topological features of
455 networks are associated with plant health and why hub taxa connect to other taxa and help
456 assemble plant microbiomes.
457 The technique used in this study enabled quick and efficient collection of spermosphere
458 soil within phase I and II of seed germination and showed the enrichment of beneficial
459 copiotrophic taxa. However, these copiotrophic taxa were not central to microbial networks. This
460 technique could easily be applied to other sequencing methods like metagenomics or

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461 metatranscriptomics for a better understanding of spermosphere soil microbiome functions.
462 Coupled with sequencing the seed microbiomes will be powerful to study interactions between
463 seed and seedling pathogens, chemical or biological seed treatments, and interactions with
464 pathogens in the spermosphere – thereby improving knowledge of spermosphere ecology, which
465 will lead to improved understanding of the plant microbiome.
466
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705 **Table and Figures**

706 **Table 1**. Permutational Analysis of Variance (PERMANOVA) and dispersion for prokaryote
707 communities separated by time post sowing based on Bray-Curtis and weighted Uni-frac
708 distances

709

710 **Table 2**. Comparison of centrality network parameters

711

712 **Table 3**. Hub taxa identified for each habitat

713

714 **Supplemental Table 1**. Permutational Analysis of Variance (PERMANOVA) for prkaryote and
715 fungal communities

716

717 **Supplemental Table 2**. Differentially abundant bacterial taxa in soybean and cotton
718 spermosphere soils

719

720 **Supplemental Table 3**. Topological properties of networks

721

722 **Figure 1. Diagram depicting the sampling technique used for sampling spermosphere soils.**
723 The spermosphere is defined as the 5-10 mm of soil directly surrounding a seed. (a) Photo
724 demonstrating the sampling technique of spermosphere soil contained within an 11 mm cork
725 borer. (b) Sampling with an 11 mm cork borer inside the confining space within wells of a 12-
726 well plate allowed direct and controlled sampling of the spermosphere around single seeds.

727

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728 **Figure 2. Prokaryote spermosphere evenness follows water imbibition.** (a) Water imbibition
729 over time for cotton and soybean seeds (n = 6). (b) Prokaryote evenness over time in control soil,
730 soybean spermosphere soil, or cotton spermosphere soil. Soybean had significantly lower
731 evenness (higher dominance) after 6, 12, and 18 hours compared to control soil. Cotton followed
732 the same trend but was significantly less even after 12 and 18 hours. Comparisons were made
733 with Wilcox ranked sign test ($\alpha = 0.05$; n = 6) (c) Spearman correlation between prokaryote
734 evenness and water imbibition.
735
736 **Figure 3. Composition of the most abundant prokaryote OTUs changes over time.** Relative
737 abundance of the top twenty most abundant prokaryote OTUs shifts over time within soybean
738 spermosphere soil, cotton spermosphere soil, or control soil.
739
740 **Figure 4. Prokaryote spermosphere composition changes over time and crop.** (a-d) Principal
741 coordinate analysis based on Weighted Unifrac distances. (e-h) Principal coordinate analysis
742 based on Bray-Curtis distances. Reported significance values above each plot are the result of a
743 permutational test of variance ($\alpha = 0.05$; n = 6). Accompanying dispersion tests are shown in
744 Table 1.
745
746 **Figure 5. Differential abundance of prokaryote OTUs shows enrichment of specific taxa**
747 **within the spermosphere.** (a) Points represent individual OTUS. Positive values on the x-axis
748 indicate the OTU was numerically more abundant in a soybean spermosphere compared to soil
749 without a soybean seed. Similarly, positive y-axis values indicate the OTU was numerically
750 more abundant in a cotton spermosphere compared to soil without a cotton seed. Colored points

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751 are pOTUs detected as significantly different in soybean or cotton. Grey circle points are non-
752 significant. Point shape indicates significance in one crop or both. Points are colored by the
753 prokaryote order. Significance was determined within the ANCOM-BC2 algorithm with a Holm-
754 Bonferroni correction (α-0.05). (b) Composition of significantly enriched pOTUs colored by
755 genera.
756
757 **Figure 6. Spermosphere soils have different topological properties and different sets of hub**
758 **taxa compared to control soil.** Networks for (a) cotton spermosphere soil and (b) soybean
759 spermosphere soil and (c) control soil were constructed with the same set of taxa. Nodes with
760 different shape and colors indicate the prokaryote or fungal class. Less transparent nodes are
761 significant hubs based on eigenvector centrality above the 95% quantile. Nodes with red outlines
762 were significantly enriched in a spermosphere as detected in Fig.4.
763
764 **Supplemental Figure 1**. Preliminary experiment conducted to demonstrate the effectiveness of
765 the sampling procedure shown in Figure 1. Bacterial populations within spermosphere were
766 greater in soybean or cotton spermosphere soils compared to control soil.
767
768 **Supplemental Figure 2**. Sequencing outputs for prokaryotes (16S) and fungi (ITS). (a)
769 Composition and taxonomic output of the mock community samples. (b) rarefaction curves for
770 prokaryotes and fungi. Dashed lines indicate the median read depth. (c) contaminant filtering
771 based on OTU prevalence in negative control samples. (d) Histogram of read depth per sample.
772

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773 **Supplemental Figure 3**. Within sample diversity measurements for soybean or cotton
774 spermosphere samples compared to control soil. (a) prokaryote richness significantly dropped
775 over time ($P < 0.001$) consistently across habitats (spermosphere or soil without seed). (b)
776 Similarly, Faith's phylogenetic distance also followed a similar pattern. No consistent
777 differences were observed over time or between habitats for fungal (c) richness or (d) evenness.
778
779 **Supplemental Figure 4**. Fungal composition of the top 20 most abundant Fungal OTUs in
780 cotton spermosphere soil, soybean spermosphere soil, or control soil.
781

a

spermosphere

soil

log fold change cotton - soil

log fold change soybean - soil

Order
Alicyclobacillales
Azospirillales
Bacillales
Brevibacillales
Burkholderiales
Clostridiales
Enterobacterales
Exiguobacterales
Micrococcales
Paenibacillales
Pseudomonadales
Rhodobacterales
Streptomycetales

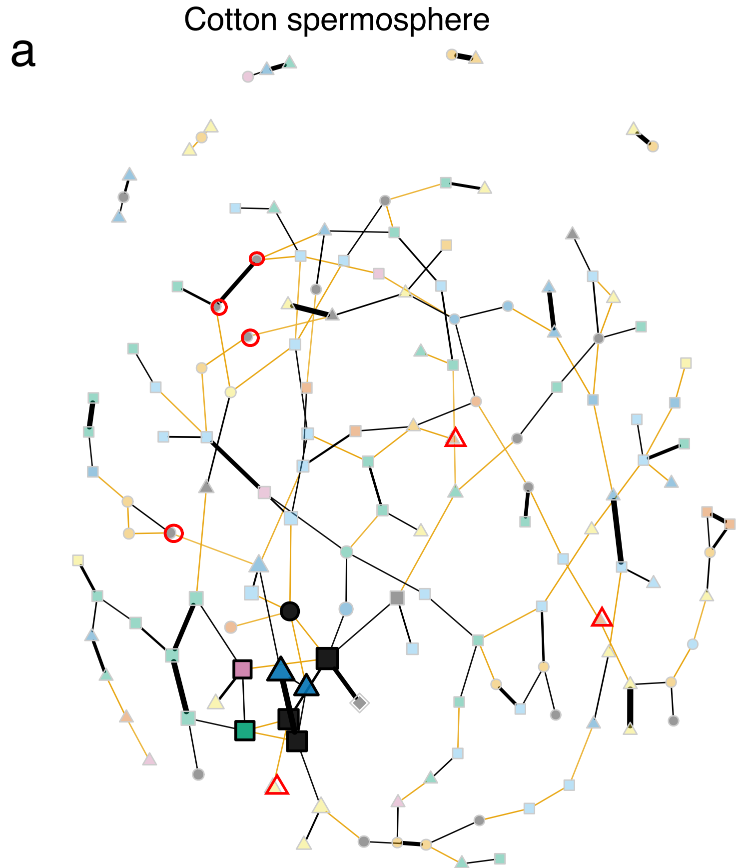
Significance
sig. cotton only
sig. soybean only
sig. cotton and soybean
not significant

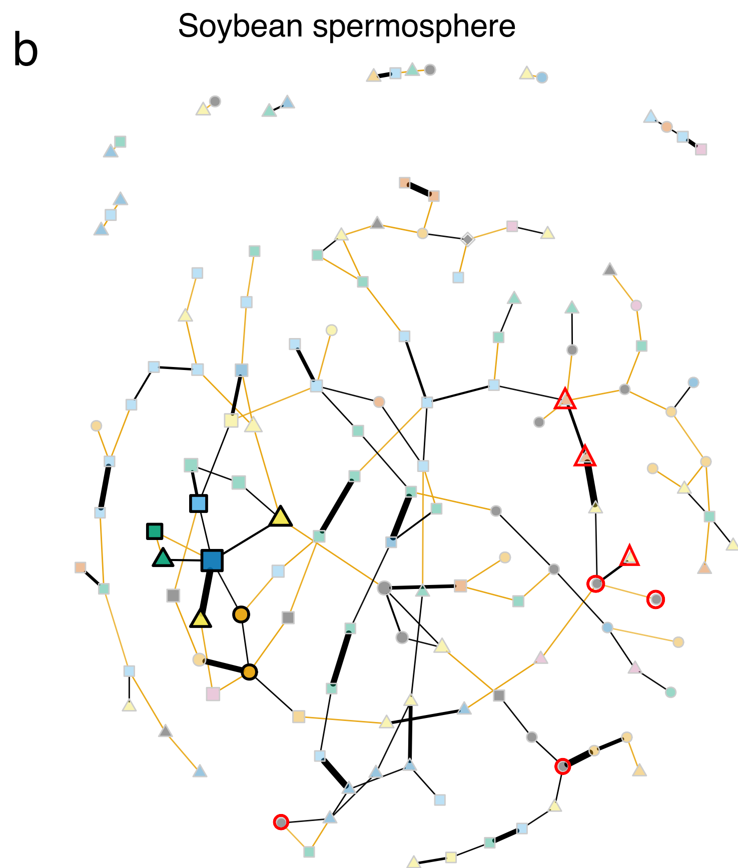
b

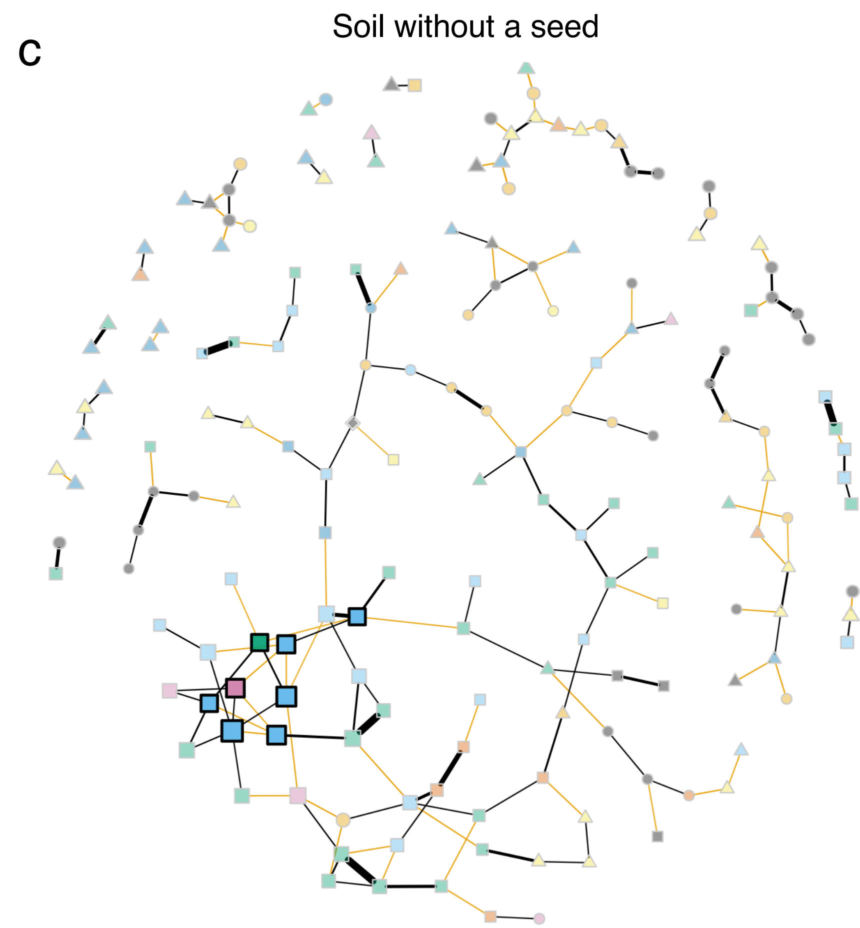
Count

Cotton only
Soybean and Cotton
Soybean only

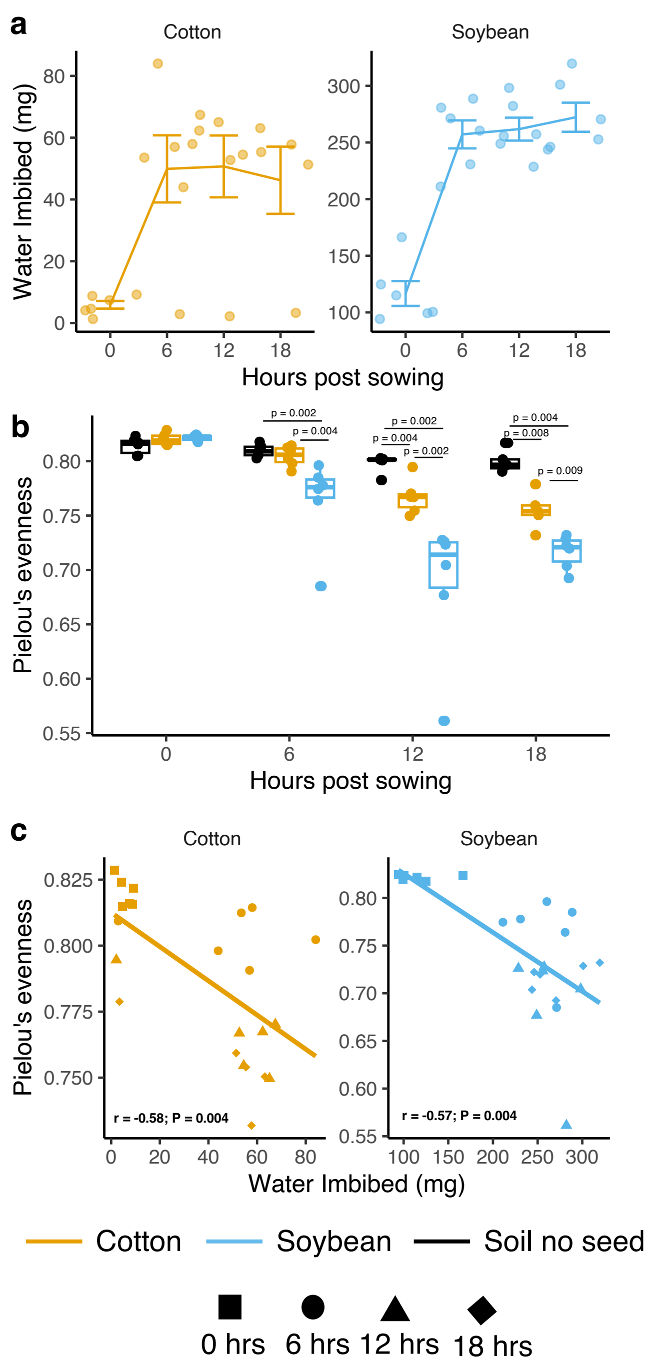
Aquabacterium
Azospirillum
Bacillus
Brevibacillus
Burkholderia-Paraburkholderia
Clostridium
Cupriavidus
Exiguobacterium
Massilia
Noviherbaspirillum
Paenarthrobacter
Paenibacillus
Pantoea
Paucimonas
Pseudomonas
Rubellimicrobium
Tumebacillus
unidentified

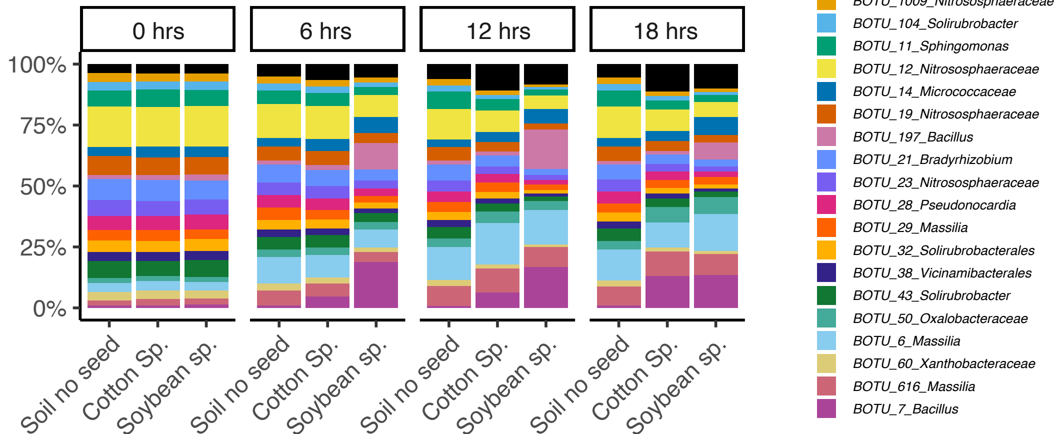

a
Cotton spermosphere

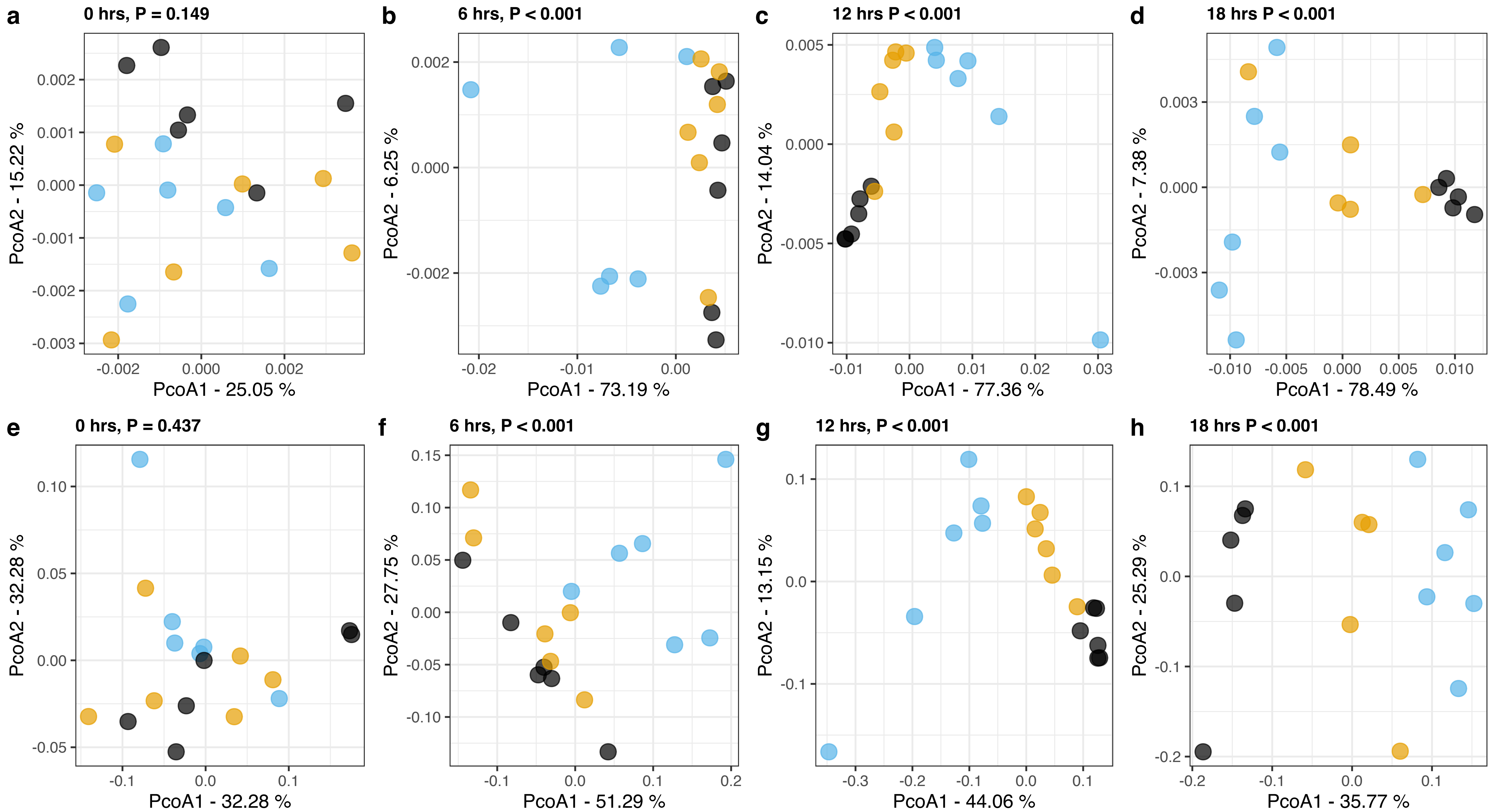

b
Soybean spermosphere


c
Soil without a seed

N components = 9
N components excluding isolated nodes = 6
N nodes in largest component = 136
N components = 20
N components excluding isolated nodes = 8
N nodes in largest component = 121
N components = 30
N components excluding isolated nodes = 21
N nodes in largest component = 80
Gammaproteobacteria
Alphaproteobacteria
Nitrospiria
Myxococcia
bacteriap25
Verrucomicrobiae
Methylomirabilia
Entotheonellia
Vicinamibacteria
Acidobacteriae
Chloroflexia
TK10
Actinobacteria
Thermoleophilia
Bacilli
Gemmatimonadetes
Nitrososphaeria
Blastocatellia
Dothideomycetes
Sordariomycetes
Leotiomycetes
unidentified Fungi
Mortierellomycetes
Tremellomycetes
Eurotiomycetes
Negative co-association
Positive co-association


a
Cotton
Soybean
Water Imbibed (mg)
Hours post sowing
b
Pielou's evenness
Hours post sowing
p = 0.002
p = 0.004
p = 0.002
p = 0.004
p = 0.002
p = 0.004
p = 0.008
p = 0.009
c
Cotton
Soybean
Pielou's evenness
r = -0.58; P = 0.004
r = -0.57; P = 0.004
Water Imbibed (mg)
Cotton
Soybean
Soil no seed
0 hrs
6 hrs
12 hrs
18 hrs


Prokaryote
0 hrs
6 hrs
12 hrs
18 hrs
Relative abundance (%)
100%
75%
50%
25%
0%
Soil no seed
Cotton Sp.
Soybean sp.
BOTU_10_Comamonadaceae
BOTU_1009_Nitrososphaeraceae
BOTU_104_Solirubrobacter
BOTU_11_Sphingomonas
BOTU_12_Nitrososphaeraceae
BOTU_14_Micrococcaceae
BOTU_19_Nitrososphaeraceae
BOTU_197_Bacillus
BOTU_21_Bradyrhizobium
BOTU_23_Nitrososphaeraceae
BOTU_28_Pseudonocardia
BOTU_29_Massilia
BOTU_32_Solirubrobacterales
BOTU_38_Vicinamibacterales
BOTU_43_Solirubrobacter
BOTU_50_Oxalobacteraceae
BOTU_6_Massilia
BOTU_60_Xanthobacteraceae
BOTU_616_Massilia
BOTU_7_Bacillus



Soil no seeds
Cotton spermosphere
Soybean spermosphere
a
0 hrs, P = 0.149
PcoA1 - 25.05 %
PcoA2 - 15.22 %
b
6 hrs, P < 0.001
PcoA1 - 73.19 %
PcoA2 - 6.25 %
c
12 hrs P < 0.001
PcoA1 - 77.36 %
PcoA2 - 14.04 %
d
18 hrs P < 0.001
PcoA1 - 78.49 %
PcoA2 - 7.38 %
e
0 hrs, P = 0.437
PcoA1 - 32.28 %
PcoA2 - 32.28 %
f
6 hrs, P < 0.001
PcoA1 - 51.29 %
PcoA2 - 27.75 %
g
12 hrs, P < 0.001
PcoA1 - 44.06 %
PcoA2 - 13.15 %
h
18 hrs P < 0.001
PcoA1 - 35.77 %
PcoA2 - 25.29 %

a

Spermosphere = 5-10 mm
soil around seed

b

Cork borer = 11 mm

7-10 mm

3-6 mm

15 mm

5-8 mm

Soil

23 mm

Table 3. Hub taxa identified for each habitat

Crop	OTU	Taxonomy
Cotton		
spermosphere soil	pOTU46	Gaiellales
	pOTU119	Gaiellales
	pOTU29	*Massilia*
	pOTU1009	Nitrososphaeraceae
	pOTU19	Nitrososphaeraceae
	pOTU12	Nitrososphaeraceae
	fOTU36	*Hannaella oryzae*
	fOTU56	*Fusarium chlamydosporum*
Soybean		
spermsphere soil	pOTU1559	Chloroflexi TK10
	pOTU132	Nocardioides
	pOTU36	Angustibacter
	pOTU11	*Sphingomonas*
	pOTU349	*Methylobacterium /Methylorubrum*
	fOTU64	*Helicoma*
	fOTU36	Fungi
	fOTU10	*Bartalinia pondoensis*
Soil without a seed	fOTU1	*Stagonosporopsis oculi-hominis*
	fOTU13	Teichosporaceae
	fOTU15	Cucurbitariaceae
	fOTU17	*Alternaria tenuissima*
	fOTU22	Neopestalotiopsis
	fOTU32	Pleosporales
	fOTU36	*Hannaella oryzae*
	fOTU4	*Cladosporium cladosporioides*

Table 2. Comparison of centrality network parameters

Centrality network parameter	Cotton vs. Soybean		Cotton vs. Control soil		Soybean vs. Control soil	
	Jaccard index[a]	P	Jaccard index[a]	P	Jaccard index[a]	P
degree	0.034	< 0.001	0.128	0.001	0.044	< 0.001
betweenness centrality	0.154	< 0.001	0.156	0.001	0.154	< 0.001
closeness centrality	0.152	< 0.001	0.246	0.092	0.206	0.019
eigenvector centrality	0.169	0.003	0.188	0.007	0.206	0.019
hub taxa	0.000	0.002	0.067	0.019	0.000	0.001

[a]Jaccard index of 1 indicates perfect agreement between soybean or cotton spermosphere networks

Table 1. Permutational Analysis of Variance (PERMANOVA) and dispersion for prokaryote communities separated by time post sowing based on Bray-Curtis and weighted Uni-frac distances

Dissimilarity Metric	Hours post sowing	df	PERMANOVA[a] R^2	PERMANOVA[a] F	PERMANOVA[a] P	Dispersion F	Dispersion P
Bray-Curtis	0	2	0.113	0.959	0.437	0.937	0.407
	6	2	0.273	2.815	<0.001	0.539	0.606
	12	2	0.424	5.517	<0.001	0.828	0.533
	18	2	0.417	4.648	<0.001	0.092	0.911
Weighted Unifrac	0	2	0.142	1.238	0.192	1.898	0.190
	6	2	0.491	7.245	<0.001	2.538	0.066
	12	2	0.609	11.675	<0.001	1.186	0.376
	18	2	0.696	14.863	<0.001	1.401	0.304

[a]Permanova and dispersion tests were performed on 999 permutations