1   **Experimental evidence pointing to rain as a reservoir of tomato phyllosphere microbiota**

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ABSTRACT

Plant microbiota play essential roles in plant health and crop productivity. Comparisons of community composition have suggested seeds, soil, and the atmosphere as reservoirs of phyllosphere microbiota. After finding that leaves of tomato (*Solanum lycopersicum*) plants exposed to rain carried a higher microbial population size than leaves of tomato plants not exposed to rain, we experimentally tested the hypothesis that rain is a so far neglected reservoir of phyllosphere microbiota. Rain microbiota were thus compared with phyllosphere microbiota of tomato plants either treated with concentrated rain microbiota, filter-sterilized rain, or sterile water. Based on 16S rRNA amplicon sequencing, one-hundred and four operational taxonomic units (OTUs) significantly increased in relative abundance after inoculation with concentrated rain microbiota but no OTU significantly increased after treatment with either sterile water or filter-sterilized rain. Some of the genera to which these 104 OTUs belonged were also found at higher relative abundance on tomatoes exposed to rain outdoors than on tomatoes grown protected from rain in a commercial greenhouse. Taken together, these results point to precipitation as a reservoir of phyllosphere microbiota and show the potential of controlled experiments to investigate the role of different reservoirs in the assembly of phyllosphere microbiota.

37 **INTRODUCTION**

38

39 Microbial communities associated with plants, often referred to as plant-associated microbiota and

40 as constituents of the plant microbiome, influence a remarkable number of processes of plant

41 biology and affect plant health and crop yield (Goh et al., 2013, Badri et al., 2013, Hacquard et al.,

42 2015, Lu et al., 2018, Torres-Cortés et al., 2018, Durán et al., 2018, Ritpitakphong et al., 2016,

43 Berg & Koskella, 2018). The phyllosphere, considered here as the plant compartment that extends

44 from the outside to the inside of the leaf (Morris, 2002, Vacher et al., 2016), harbors a high

45 diversity of microorganisms, with bacteria being the most abundant domain (Lindow & Brandl,

46 2003, Vorholt, 2012).

47　　　　Phyllosphere microbiota are exposed to fluctuating environmental stresses, including

48 changes in UV exposure, temperature, water availability, osmotic stress, and humidity (Hirano &

49 Upper, 2000, Jacobs & Sundin, 2001, Lindow & Brandl, 2003, Vacher et al., 2016). The core

50 bacterial phyla that can withstand these environmental stressors include Proteobacteria,

51 Actinobacteria, Bacteroidetes, and Firmicutes (Vorholt, 2012, Bulgarelli et al., 2013). However,

52 at lower taxonomic ranks, the phyllosphere microbiome greatly varies with changing biotic and

53 abiotic factors (Lindemann & Upper, 1985, Rastogi et al., 2012, Copeland et al., 2015, Wagner et

54 al., 2016).

55　　　　Culture-independent 16S rRNA amplicon analyses have expanded our knowledge of the

56 composition of the phyllosphere microbiome of several plant species (Knief et al., 2012, Williams

57 et al., 2013, Kembel et al., 2014, Copeland et al., 2015, Grady et al., 2019), including tomato

58 (*Solanum lycopersicum*) (Ottesen et al., 2013, Ottesen et al., 2016). The bacterial genera

59 *Pseudomonas*, *Erwinia*, *Sphingomonas, Janthinobacterium, Curtobacterium, Agrobacterium,*

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60   *Stenotrophomonas, Aurantimonas, Thermomonas, Buchnera, Enterococcus*, *Rubrobacter*,

61   *Methylobacterium*, *Deinococcus,* and *Acidovorax* have all been observed to be associated with the

62   phyllosphere of tomatoes grown in the field (Ottesen et al., 2013, Ottesen et al., 2016, Toju et al.,

63   2019).

64   Microbes constantly cycle across interconnected habitats maintaining healthy ecosystems

65   (van Bruggen et al., 2019). Importantly for this study, it has been shown that plants represent an

66   important source of microbes that are released as aerosols into the atmosphere (Lindemann et al.,

67   1982, Constantinidou, 1990, Lighthart & Shaffer, 1995, Šantl-Temkiv et al., 2013, Bowers et al.,

68   2011, Vaïtilingom et al., 2012). The atmosphere then serves as a vehicle for microbial dispersal

69   not only locally, but also globally (Bovallius et al., 1978, Brown & Hovmøller, 2002, Schmale &

70   Ross, 2015) and air-borne microbial communities are deposited back to earth surfaces as

71   precipitation. The atmosphere thus represents a crucial route for the dissemination of beneficial

72   and pathogenic species (Polymenakou, 2012, Monteil et al., 2014, Monteil et al., 2016). There is

73   even some evidence that air-borne microbes contribute to the formation of precipitation itself

74   (Christner et al., 2008, Morris et al., 2014, Amato et al., 2015, Amato et al., 2017, Failor et al.,

75   2017). Proteobacteria followed by Bacteroidetes, Actinobacteria, and Firmicutes have been

76   identified as the most common phyla both in the atmosphere (Peter et al., 2014, Hiraoka et al.,

77   2017, Cáliz et al., 2018) as in precipitation (Cáliz et al., 2018, Aho et al., 2020), notable largely

78   the same as the core phyla of the phyllosphere microbiome mentioned earlier.

79   Microbial community assembly is largely influenced by deterministic (selection) and

80   stochastic (dispersal) processes that determine the complex structure and function of microbiomes

81   (Powell et al., 2015, Zhou & Ning, 2017, Graham & Stegen, 2017). This assembly process has

82   been extensively studied in roots, which recruit microbial communities from the surrounding soil

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83   (Fitzpatrick et al., 2018, Pérez-Jaramillo et al., 2019). However, sources that drive the phyllosphere

84   microbiome assembly are still under debate. It has been suggested that soil is the major reservoir

85   of the phyllosphere microbiome (Zarraonaindia et al., 2015, Wagner et al., 2016, Grady et al.,

86   2019). In fact, it has been observed that the leaf microbiome reflects soil bacterial diversity at an

87   early stage of growth but significantly differs as plants grow and mature (Copeland et al., 2015).

88   On the contrary, (Maignien et al., 2014, Ottesen et al., 2016) found evidence that dry deposition

89   of air-borne microbes constitutes an important source of phyllosphere microbiota.

90   A few studies have also explored wet deposition of air-borne microbes in precipitation as

91   a potential reservoir of phyllosphere microbiota. For example, (Morris et al., 2008) concluded that

92   the plant pathogen *Pseudomonas syringae* is disseminated through the water cycle because of its

93   ubiquitous presence in compartments of the water cycle and plants. Using whole genome

94   sequencing, (Monteil et al., 2016) confirmed these conclusions by finding that *P. syringae* bacteria

95   isolated from diseased cantaloupe plants and *P. syringae* isolates from rain, snow, and irrigation

96   water were members of the same population. Recently, rain was also found to affect the overall

97   composition of plant phyllosphere microbiota but it was not determined if rain-borne bacteria were

98   the source of the observed shifts (Allard et al., 2020). Moreover, it is well known that fungal spores

99   are released from plants, travel long distances through the atmosphere, and can be deposited back

100   on plants with rain (Woo et al., 2018).

101   After finding that the phyllosphere of tomato plants exposed to rain contained a higher

102   bacterial population size than the phyllosphere of tomato plants not exposed to rain, we

103   hypothesized that rain-borne bacteria might contribute to the assembly of phyllosphere microbiota

104   beyond pathogenic bacteria and fungi. Putative rain-borne tomato phyllosphere colonizers were

105   identified in a series of controlled laboratory experiments and by comparing the composition of

106   tomato phyllosphere microbiota of plants naturally exposed to rain with those not exposed to rain.

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108   **MATERIALS AND METHODS**

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110   **Determination of the population size of tomato phyllosphere microbiota.** Store-bought

111   tomato seeds of the cultivar 'Rio Grande' were germinated in autoclaved soil (60 min/ fast cycle).

112   For growth under laboratory conditions, plants were kept for 4 weeks on shelves under 14 h of

113   light and 10 h of dark. For exposure to outdoor conditions, 4-week-old tomato plants were

114   transplanted into large plastic pots. Pots were then either transported to a research farm and placed

115   on gravel near a maintained lawn (Kentland Farm, Blacksburg, VA, USA) or put on the flat roof

116   of the 3-story Latham Hall research building at Virginia Tech (Blacksburg, VA, USA) several

117   meters above and away from soil and vegetation.

118   **Culture-dependent analysis of phyllosphere microbiota.** Leaf disks were aseptically

119   collected with a #1 cork borer and placed in a tube containing 200 µL of sterile 10 mM $MgSO_4$

120   solution and 3 glass beads. Tubes were placed in a mini bead beater (Biospec Products, Inc.,

121   Bartlesville, OK, USA) and shaken for 2 min to release bacterial cells. Serial dilutions were plated

122   on R2A plates supplemented with cycloheximide to inhibit fungal growth. Plates were incubated

123   at room temperature and colony-forming units were counted 4 days later.

124   **Rain collection.** Rain was collected as described in (Failor et al., 2017). In short,

125   autoclavable bags were wrapped in aluminum foil and autoclaved for 40 min/fast cycle. Trash cans

126   were arranged away from structures on the roof of the Latham Hall research building. Surfaces of

127   containers were sprayed with 75% ethanol to prevent contamination. Sterile bags were placed in

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128   the cans and the lid placed back on top until the beginning of a rainfall event, at which point they

129   were removed. The lids of three cans were then removed but one can was kept closed during the

130   precipitation event as a negative control. After the end of precipitation events, 1 liter (L) of sterile

131   water was poured into the negative control can, simulating the precipitation event. After rainfall

132   events ended, bags containing rain water were removed and placed at $4°C$ until processing.

133   For DNA extraction, three L of rainwater were vacuum filtrated (reusable filter holders

134   from Thermo Scientific Nalgene, USA) through a 0.2 µm pore filter membrane (Supor$^®$ 200 PES

135   membrane Disc Filter, PALL, USA). Filters were removed using sterile tweezers, placed into a 15

136   mL Eppendorf tube and stored at -80°C until processing.

137   **Rain as bacterial inoculum and plant treatments.** Tomato plants were grown in the

138   laboratory for four weeks as described above for the culture-dependent analysis of phyllosphere

139   microbiota. Two L of rainwater were vacuum-filtered as described under rain collection. To

140   concentrate the bacterial microbiota present in rain hundred fold, the filter membranes were

141   incubated for 10 min with shaking in 20 mL of sterile water, which was then used as inoculum

142   (referred to as concentrated rain microbiota or 100X-rain from now on). The rain that passed

143   through the filter was used as bacterial-free inoculum (referred to as filtered rain or filter-sterilized

144   rain). Autoclaved double-distilled water was used as sterile water treatment. Groups of four plants

145   placed together into 13 inch x 16 inch plastic bags were sprayed until run off. The bags were left

146   open for two hours to let the plants dry before the day 0 time point DNA extraction from two of

147   the four tomato plants. Bags were then closed for 2 days to create a high humidity environment

148   favorable to plant colonization after which they were kept open for 5 days until the day 7 time

149   point DNA extraction from the remaining two tomato plants.

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150   **Growth of outdoor and greenhouse tomatoes.** Tomato plants were purchased at Home

151   Depot at the beginning of June 2019 and transplanted into pots and placed on the flat roof of the

152   Latham Hall building on the Virginia tech campus by setting them on a large, clean sheet of plastic

153   to reduce contamination from the roof floor. Plants were regularly waters without touching leaves.

154   One set of plants were brought indoors before each rainfall event while the other set of plants were

155   kept outside to be exposed to rainfall. Each rainfall event was collected and DNA extracted as

156   described above and leaves from both sets of plants were harvested and DNA extracted as

157   described below.

158   **Harvesting plants leaves.** All plant leaves were removed and collected in a ziplock plastic

159   bag. Sterile distilled water was added (300 mL) and samples were sonicated for 10 minutes using

160   a 1510 BRANSON sonicator (Brandsonic, Mexico) (Ottesen et al., 2013). The leaf wash was

161   vacuum-filtered on to the same kind of 0.22 μm pore filter described above to collect microbial

162   cells dislodged from leaf tissue during sonication, as described above. Filter membranes were

163   placed into a 15 mL Eppendorf tube and stored at -80°C until processing.

164   Plant leaves were also collected using the same procedure inside a commercial greenhouse

165   where tomatoes were grown either hydroponically or in non-sterilized soil under an organic

166   production regiment.

167   **DNA extraction.** DNA extraction from all the 0.22 μm filter membranes was performed

168   using the Power Water DNA isolation kit (Qiagen, USA) according to the manufacturer's protocol

169   with minor modifications. DNA concentration and quality were assessed by UV

170   spectrophotometry (NanoDrop 1000, Thermo, USA) and visualized on a 1 % agarose gel.

171   **Library preparation and sequencing.** For the 16S rRNA amplicon sequencing we used

172   the barcoded primers a799wF (5'AMCVGGATTAGATACCCBG3') and new1193R

173   (5'ACGTCATCCCCACCTTCC3'). A 28 cycle PCR was performed using the HotStarTaq Plus

174   Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by

175   28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final

176   elongation step at 72°C for 5 minutes was performed. After amplification, PCR products obtained

177   from the various samples were mixed in equal concentrations and purified using Agencourt

178   Ampure beads (Agencourt Bioscience Corporation, Beverly, MA, USA). All steps from PCR to

179   paired-end (2×300 bp) amplicon sequencing on the Illumina MiSeq platform were performed at

180   Molecular Research LP (MR DNA™, Shallowater, TX, USA).

181   For metagenomic sequencing using the Illumina platform, total DNA was sequenced using

182   150 bp paired-end reads on the HiSeq 4000 Illumina platform, Duke University Sequencing and

183   Genomic Technologies Shared Resource, Durham, NC, USA. For metagenomic sequencing using

184   the Nanopore platform, DNA libraries were prepared following the '1D Native barcoding genomic

185   DNA protocols (SQK-LSK109 and EXP-NBD104) provided by Oxford Nanopore Technologies

186   (ONT).

187   **Bioinformatic analysis.** Raw 16S rRNA paired-end sequences were processed by

188   Molecular Research LP (MR DNA™, Shallowater, TX, USA) as follows: 1) reads were joined

189   together after q25 trimming of the ends and reoriented in the 5'-3' direction, 2) barcodes and

190   primer sequences were removed, and 3) sequences shorter than 200bp, sequences with ambiguous

191   base calls, and sequences with homopolymer runs exceeding 6 bp were removed. Operational

192   taxonomic units (OTU) were assigned using the open source Quantitative Insights into Microbial

193   Ecology (QIIME) version 1.9.1bioinformatic pipeline (Caporaso et al., 2010), using the open-

194   reference protocol at 97% sequence identity with UCLUST as the clustering tool and SILVA

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195 release 128 (Quast et al., 2013) as the database. All OTUs annotated as mitochondria, chloroplasts,

196 cyanobacteria, unassigned, and OTUs with fewer than five reads were removed from the dataset.

197   The QIIME-generated output file in the Biological Observation Matrix (BIOM) format was

198 used for downstream data analysis and visualization in R version 3.3 using the Vegan (Oksanen

199 J., 2020), Phyloseq 1.26.1 and ggplot2 3.3.2 packages (McMurdie & Holmes, 2013, Hadley, 2009).

200   Samples were rarefied to the lowest sample depth to compute diversity analysis. The rain

201 core microbiome analysis was performed with the microbiome R package (Lahti L., 2020) using

202 a detection threshold of 0.1% and 100% as a prevalence threshold. Alpha diversity was assessed

203 using observed OTUs, Shannon, and Simpson indices. Differences in alpha diversity were

204 determined by pairwise Wilcoxon rank sum test with the Holm correction method. Beta diversity

205 was analyzed based on unweighted UniFrac distance, weighted UniFrac distance, and Bray-Curtis

206 dissimilarity. Differences in beta diversity were determined using PERMANOVA as implemented

207 in adonis2 (from vegan 2.5-6 using models with 999 permutations, adonis2(dist.matrix

208 ~Treatment*TimePoint + DateExperiment, permutations = 999). Dissimilarity matrices were

209 visualized using the Principal Coordinates Analysis (PCoA) ordination method as implemented in

210 the Phyloseq package. DESeq2 (Love et al., 2014) was used to identify OTUs that were

211 differentially abundant across treatment groups and time points. OTUs were filtered using a False

212 Discovery Rate (FDR) cutoff of 0.01.

213   For metagenomic data analysis, raw 150 bp paired-end reads from Illumina were processed

214 to remove short and low quality reads using Trimmomatic (version 0.38) (Bolger et al., 2014).

215 Reads with an average per base quality below 30 and read length below 150bp were filtered out.

216 Filtered reads were then classified taxonomically using Centrifuge (version 1.0.4) (Kim et al.,

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217   2016) and Sourmash version 2.0.0 (Brown, 2016) only retaining species that were identified by

218   both classifiers.

219   For Oxford Nanopore Technologies sequencing, the fast5 files containing the raw reads

220   obtained from the MinION sequencer were base-called using Guppy (v3.3.2). The ONT workflow

221   What is in my pot (WIMP v2019.7.9) was used for bacterial identification and a classification

222   (Juul et al., 2015).

223

224   **RESULTS**

225

226   **The bacterial population size on tomato plants exposed to rain is larger than that of**

227   **tomato plants not exposed to rain.** Our investigation into the role of rain in shaping the

228   phyllosphere microbiome started by comparing the bacterial population size on tomato plants

229   grown indoors under controlled conditions with that of plants grown outside exposed to

230   environmental disturbances including rainfall. We observed that tomato plants grown over four

231   weeks outdoors in plastic pots at the Virginia Tech Kentland research farm harbored bacterial

232   populations of significantly larger size compared to plants grown under laboratory conditions for

233   the same period **(Figure 1A)**. Interestingly, even plants grown on the roof of a campus research

234   building, which minimized microbial dispersal from soil and plants compared to the farm

235   environment, had bacterial populations that were significantly larger compared to those of plants

236   grown indoors **(Figure 1B)**. These results suggest that air-borne and rain-borne bacteria through

237   dry and/or wet deposition had colonized the tomato plants grown outside.

238   To test the effect of rain on the bacterial population size in the tomato phyllosphere under

239   controlled conditions, we collected rain and used it as an inoculum to treat 4-week old tomato

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240   plants that had been grown under laboratory conditions. Seven days after plants were treated with

241   rain, they carried a significantly larger bacterial population count compared to plants that had been

242   treated with autoclaved rain **(Figure 1C)**. This result suggested that at least some rain-borne

243   bacteria are able to colonize plant leaves efficiently and may thus impact bacterial population

244   composition in the phyllosphere.

245   **Rain-borne microbiota in Blacksburg, Virginia, are highly variable.** As a first step

246   towards identifying which bacterial taxa present in rainfall may efficiently colonize the tomato

247   phyllosphere, we characterized the bacterial diversity associated with nine rainfall events during

248   2015 and 2016. Rainfall was collected in sterile plastic bags on the same roof of the research

249   building previously used to grow tomatoes, DNA was extracted, and the 16S rRNA gene was

250   amplified and sequenced **(Table 1)**. In total, 1,186,365 short reads were obtained. After 97% OTU

251   clustering and removal of all non-bacterial and unassigned reads, a total of 892,142 reads

252   remained. All samples were rarefied to 6419 reads per sample and 5958 OTUs overall were

253   identified. Rarefaction curves (**Supplementary Figure 1**) show that this is an underestimate of the

254   total number of OTUs since not all samples were sequenced to saturation. The number of OTUs

255   per rarefied sample ranged from 541 (June 2019) to 1782 (April 2016).

256   Taxonomic diversity analysis revealed Proteobacteria, Actinobacteria, Bacteroidetes,

257   Acidobacteria, and Firmicutes to be the dominant taxa at the phylum level. At the class level,

258   Alphaproteobacteria followed by Gammaproteobacteria, Actinobacteria, Acidobacteria, and

259   Bacilli were most abundant. However, there were considerable differences among samples. For

260   example, Chlamydiae represented the most abundant taxon in the rain sample collected in August

261   2015 at 36% relative abundance, represented only 0.4% in the sample collected in July 2016, and

262   were less than 0.1% in all other samples. Deltaproteobacteria were only present in August 2015,

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263 May 2016 and July 2016 at 12.79%, 1.42%, and 17.03% relative abundance, respectively.

264 Actinobacteria were most abundant in rain samples collected in April 2016 (25.56%) and

265 December 2016 (16.29%) while they only represented 2.18% of the October 2016 sample.

266 Gammaproteobacteria represented 80% of relative abundance in June 2019 but only averaged

267 28.41% in the other samples **(Figure 2A)**.

268   Even more dramatic differences in relative abundance between samples were observed at

269 the genus level **(Figure 2B)**. However, seventeen OTUs were identified across all nine samples

270 when setting a detection threshold of 0.1%. These OTUs belong to the following genera (listed in

271 order of decreasing relative abundance): *Acidiphilium*, *Bryocella*, Beijerinckiaceae 1174-901-12,

272 *Methylobacterium, Massilia,* Burkholderiaceae DQ787673.1.1527, *Pantoea*, *Pseudomonas* and

273 *Sphingomonas* (**Supplementary Figure 2**).

274   **Concentrated rain microbiota, filter-sterilized rain, and sterile water all affect the**

275 **phyllosphere of lab-grown tomato plants.** Next, we decided to determine if inoculation with rain

276 microbiota would not only increase the size of the tomato phyllosphere microbiota as seen in

277 **Figure 1** but also change its composition because of colonization of tomato leaves by rain-borne

278 bacteria. In six independent experiments, 100-fold concentrated rain microbiota (100X-rain)

279 derived from six out of the nine collected rainfall events described above (April 2015, August

280 2015, March 2016, April 2016, May 2016, July 2016) were used to inoculate tomato plants. In

281 parallel, a separate set of tomato plants were treated each time with filter-sterilized rain obtained

282 from the concentration step above. For each of the 2016 experiments, additional tomato plants

283 were treated with sterile water. 16S rRNA amplicons were prepared and sequenced from DNA

284 extracted from leaf washes of tomato plants two hours after treatments (day-0) and seven days

285 later (day-7). For the March 2016 and May 2016 experiments enough rain and enough plants were

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286 available so that each treatment was done in duplicate. For the April 2016 experiment, two day-7

287 100X-rain samples were taken. For the other three experiments, only one sample per treatment and

288 time point was processed.

289 In total, 3,291,016 reads were obtained from 45 phyllosphere samples (**Supplementary**

290 **Table 1**). After 97% OTU clustering and removing all non-bacterial and unassigned reads, a total

291 of 3,118,320 reads remained in the data set. Rarefaction curves show that most of the samples were

292 deeply sequenced **(Supplementary Figure 1)**.

293 After subsampling to 6,670 reads per sample, we identified a total of 9,923 OTUs and

294 measured the alpha diversity based on the total number of observed species and by Shannon and

295 Simpson diversity indices. **Figure 3** shows the alpha diversity values for rain compared to treated

296 plants at day 0 and day 7. Although alpha diversity of rain microbiota was highly variable, the

297 number of observed OTUs in rain was significantly higher than day-7 samples for all three

298 treatments (p-values of 0.020, 0.050, and 0.028 respectively as determined by the pairwise

299 Wilcoxon rank sum test with the Holm correction method) **(Supplementary Table 2)**. Also, the

300 number of observed OTUs significantly decreased in plants treated with 100X-rain from day 0 to

301 day 7 (p-value 0.027). Comparing Shannon's index, we observed a depletion in richness from day

302 0 to day 7 for plants treated with 100X-rain (p-value 0.049) as well as for plants treated with

303 filtered-rain (p-value 0.023). No significant differences in alpha diversity were observed between

304 day 0 and day 7 for plants treated with sterile-water.

305 Principal coordinate analysis (PCoA) derived from weighted and unweighted UniFrac

306 distance metrics and from Bray-Curtis dissimilarity **(Figure 4)** revealed that most rain samples

307 clustered together while phyllosphere samples did not. While PCoA derived from weighted

308 Unifrac distances only separated phyllosphere samples along the second coordinate, PCoA derived

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309   from unweighted Unifrac distances and Bray-Curtis dissimilarity separated phyllosphere samples

310   along both axes. Therefore, bacterial communities on lab-grown tomato plants were even more

311   dissimilar from each other than the dissimilarity between rain communities. A permutational

312   multivariate analysis of variance (PERMANOVA) for Bray-Curtis dissimilarity revealed that date

313   of experiment, day of sampling (day 0 versus day 7), and treatment (100X-rain, filter-sterilized

314   rain, and sterile water) were all significantly associated with bacterial community composition.

315   (**Supplementary Table 3**). In regard to the date of experiment, it can be seen in panels 4B and 4C

316   how samples clearly cluster by date of experiment along the X axis. Since this clustering is

317   independent of treatment and day of sampling, the starting tomato phyllosphere population appears

318   to have been different between experiment dates. Secondly, day 7 samples differed from day 0

319   samples on the Y axis independently of which treatment was applied revealing that inoculation

320   and incubation under high humidity of plants by itself shifted the composition of the tomato

321   phyllosphere population. Thirdly, although treatment was significant, there was interaction

322   between treatment and date of experiment. Therefore, the effect of 100X-rain was not significantly

323   different compared to the effect of filter-sterilized rain or sterile water. In other words, beta

324   diversity analysis was unable to reveal if rain-borne bacteria present in the 100X-rain treatments

325   affected the tomato phyllosphere community, possibly because of the differences in the

326   composition of the microbial rain and phyllosphere communities between experiments.

327   After finding that beta diversity analysis was inconclusive, we decided to compare the

328   actual taxonomic diversity between samples. We observed an enrichment in Proteobacteria in the

329   tomato phyllosphere on day 7 compared to the day 0 regardless of treatment. In contrast, relative

330   abundance of Actinobacteria and Firmicutes was dramatically reduced 7 days post-treatment in all

331   plants treated with 100X-rain but not after treatment with filter-sterilized rain or sterile water

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332 **(Figure 5)**. At the class level, Gammaproteobacteria were significantly enriched in all tomato

333 phyllosphere day 7 samples. Actinobacteria and Bacilli were reduced on day 7 in plants treated

334 with 100X rain compared with filter-sterilized rain or sterile water. Unexpectedly, no consistent

335 increase of any taxon at either phylum, class, or genus level was observed from day 0 to day 7 for

336 plants treated with 100X-rain alone.

337 **One-hundred and four rain-borne OTUs increased significantly in relative**

338 **abundance in the tomato phyllosphere exclusively after treatment with concentrated rain**

339 **microbiota.** Since we did not find any taxon between genus and phylum level that exclusively

340 increased from day 0 to day 7 on tomato plants treated with 100X-rain, we wanted to determine if

341 we could find any individual rain-borne OTUs that did so. To do this we used DESeq2 (Love et

342 al., 2014) as it is relatively robust to small and unequal sample sizes as in the present study..

343 First, we directly compared day-0 and day-7 phyllosphere microbiota treated with 100X-

344 rain and found that one-hundred and four OTUs (out of a total of 7,994 OTUs) significantly

345 increased (**Supplementary Table 4**). These OTUs belonged to the genera *Massilia* (27 OTUs),

346 *Pantoea* (18 OTUs), *Duganella* (13 OTUs)*, Pseudomonas* (11 OTUs), *Enterobacter* (5 OTUs),

347 *Flavobacterium* (3 OTUs), *Janthinobacterium* (2 OTUs), and *Curtobacterium* (1 OTUs). 16

348 unknown OTUs from the *Burkholderiaceae* and 5 unknown OTUs from the *Enterobacteriaceae*

349 families significantly increased in relative abundance as well **(Figure 6A)**. Importantly, not a

350 single OTU significantly differed in abundance between day 0 and day 7 on tomato plants treated

351 with either filtered-rain or dd-water. This suggests that the OTUs which increased in relative

352 abundance from day 0 to day 7 on tomatoes after treatment with 100X-rain originated from rain

353 and were not members of the bacterial community present in the tomato phyllosphere prior to

354 inoculation.

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355   We then complemented the previous comparison with a slightly different analysis

356   comparing the bacterial composition of rain microbiota with phyllosphere microbiota seven days

357   after treatment with 100X-rain. We observed that thirty-five rain-borne OTUs out of a total of

358   5,958 OTUs had a significantly higher relative abundance on tomatoes at day-7 compared to their

359   relative abundance in rain (**Figure 6B**). These OTUs (in order of decreasing differential

360   abundance) mostly belonged to the same genera as the genera identified in the day-0 to day-7

361   comparison : *Massilia, Pseudomonas*, *Pandoraea*, *Streptomyces*, and *Pantoea*. The genus *Pantoea*

362   was the genus that most consistently increased in relative abundance. In fact, *Pantoea* was detected

363   in all rain collections (**Figure 7A**) and reached a high relative abundance (between 4% - 44%) in

364   the tomato phyllosphere after 7 days each time its abundance in rain was above 1% (observed 4

365   out of 6 times). Genus-level abundance in rain and phyllosphere samples are also shown for

366   *Flavobacterium, Janthinobacterium*, *Pseudomonas, Methylobacterium,* and *Massilia*, which all

367   successfully colonized the tomato phyllosphere each time they were detected in rain (**Figure 7B-**

368   **E**).

369   In contrast, 61 OTUs found in rain samples significantly decreased in relative abundance

370   by day 7 suggesting that these rain-borne taxa were definitely not able to colonize tomato leaves.

371   These OTUs belonged to the following genera (list of the first 10 bacterial genera in order of

372   decreasing differential abundance): *Acidiphilium*, Beijerinckiaceae-1174-901-12, *Bryocella,*

373   *Actinomycetospora, Methylobacterium, Methylocella*, *Granulicella, Belnapia, Modestobacter,*

374   and *Blastococcus*. The genus *Acidiphilium* best exemplifies this group of taxa. It was detected at

375   high relative abundance in most rain samples, it was observed on plants treated with 100X-rain on

376   day 0, but it was never found on plants treated with 100X-rain on day 7 (**Figure 7G**). This clearly

377   shows that *Acidiphilium* is a common rain-borne bacterial genus that does not include any

378   members able to colonize the tomato phyllosphere.

379   Several other pairwise comparisons were made to gain additional insights into differences

380   in composition between microbiota at the OTU level. For example, we determined which OTUs

381   were present in significantly higher abundance in rain compared to tomatoes treated with sterile

382   water on day 0 to identify OTUs that are commonly present in rain but present in low abundance

383   (or not at all) on laboratory-grown tomato plants **(Supplementary Figure 3A)**. One hundred

384   seventy-four such OTUs (out of a total of 5,958 OTUs present in rain) were identified. Most of

385   these OTUs belonged to the following genera (list of the first 10 bacterial genera in order of

386   decreasing differential abundance in rain): *Caedibacter*, *Tumebacillus*, *Acidiphilium*,

387   *Methylobacterium, Rhodovastum, Belnapia, Sphingomonas, Methylocella, Bacillus and Pantoea.*

388   On the other hand, 109 OTUs had significantly higher relative abundance in laboratory-

389   grown tomatoes (tomatoes at day 0 after being treated with sterile water) than in rain

390   (**Supplementary Figure 3A**). These taxa are thus common inhabitants of tomatoes grown under

391   our laboratory conditions in the absence of rain. They mostly belonged to the following genera

392   (list of the first 10 bacterial genera in order of decreasing differential abundance):

393   *Hyphomicrobium*, *Rhodanobacter*, *Chryseobacterium*, *Burkholderia*, *Paenibacillus*,

394   *Nocardioides, Pandoraea*, *Bacillus*, *Novosphingobium,* and *Streptomyces*.

395   One genus that did not fall into any of the above categories was the genus *Bacillus*: *Bacillus*

396   OTUs were observed at high relative abundance in rain samples as well as in the tomato

397   phyllosphere at day 0 independent of treatment and on day 7 after sterile-rain and dd-water

398   treatments but not after 100X-rain treatments (**Figure 7H)**. This suggests that *Bacillus* OTUs were

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399   present in rain as well as on lab-grown tomatoes but were outcompeted by other rain-borne bacteria

400   added with the 100X-rain treatments.

401   To more precisely identify the OTUs that represented the most efficient tomato colonizers,

402   we used metagenome shotgun sequencing to re-sequence the microbiota associated with rain

403   samples and with plants treated with 100X-rain on day 0 and day 7. The metagenome shotgun

404   sequencing approach generated 260,035,170 short-reads. After quality control, 7,543,305 reads

405   remained, of which 98.26% were identified as bacterial reads. **Table 2** summarizes the results

406   listing the bacterial species present in rain that numerically increased in abundance from day 0 to

407   day 7 and ranked from high to low based on their relative abundance on day 7. Based on this

408   analysis, rain-borne species *Pantoea vagans* and *Pantoea agglomerans* were the most effective

409   tomato phyllosphere colonizers followed by *Pseudomonas citronellolis*, *Novosphingobium*

410   *resinovorum*, an unnamed *Buttiauxella* species, *Erwinia gerundensis*, *Pseudomonas fluorescens*,

411   *Cedecea neteri*, and an unnamed *Massilia* species. Additional *Pantoea*, *Massilia*, *Pseudomonas*,

412   *Janthinobacterium*, and *Enterobacter* species ranked highly as well. The metagenomic analysis

413   thus mostly confirmed and refined our 16S rRNA results of which rain-borne taxa are the most

414   effective colonizers of the tomato phyllosphere.

415   **There are consistent differences between phyllosphere microbiota of tomato plants**

416   **never exposed to rain and naturally exposed to rain outdoors.** After identifying bacterial taxa

417   present in rain that efficiently colonized tomato leaves under laboratory conditions, we tested the

418   hypothesis that these taxa would be abundant in tomato plants grown outdoors in pots containing

419   autoclaved soil on the roof of the same research building where we had collected rain samples

420   earlier (7 samples) but be missing, or at least be underrepresented, in phyllosphere microbiota of

421   greenhouse-grown tomatoes that had never been exposed to rain. This second set of plants included

422   tomato plants grown in a hydroponic system (29 samples) and tomato plants grown in soil (18

423   samples), both in a commercial greenhouse (Supplementary Table 5). In total, we obtained

424   4,166,519 reads. After 97% OTU clustering and removing all non-bacterial and unassigned reads,

425   a total of 3,080,204 reads remained. Samples were rarefied to 2,546 reads per sample and 10,525

426   OTUs overall were identified. Taxonomic diversity analysis revealed Proteobacteria, Firmicutes,

427   Actinobacteria and Bacteroidetes to be the dominant bacterial taxa **(Figure 8A)**, the same phyla

428   identified on the lab-grown tomatoes. Alpha diversity analysis supported by the total number of

429   observed species, and by the Shannon and Simpson diversity indices **(Figure 8B)** together with a

430   pairwise comparisons using the Wilcoxon rank sum test showed significant differences in the

431   number of OTUs between the hydroponic and the soil system (**Supplementary Table 6**).

432   To identify any OTUs present at significantly higher relative abundance in tomato plants

433   grown outside exposed to rain compared to the tomato plants grown in the greenhouse

434   hydroponically in the absence of rain, DESeq2 was used again. Forty OTUs from the genera

435   *Pandoraea*, *Curtobacterium*, *Massilia*, *Gemmatirosa*, *Kineococcus*, *Methylobacterium*,

436   *Sphingomonas*, *Buchnera*, *Alloiococcus*, *Pseudomonas,* and *Streptococcus* were found (**Figure**

437   **9A**). Similarly, 58 OTUs from the genera *Pantoea*, *Pelomonas*, *Kineococcus*, *Methylobacterium*,

438   *Massilia*, *Aureimonas*, *Buchnera*, *Alloiococcus*, *Pseudomonas*, *Streptococcus*, and *Sphingomonas*

439   were of significantly higher relative abundance on the tomato plants grown outside exposed to rain

440   compared to the tomato plants growing in soil never exposed to rain in the greenhouse (**Figure**

441   **9B**). Note that, as we hypothesized, OTUs in the genera *Massilia*, *Curtobacterium*, *Pseudomonas*,

442   and *Pantoea,* were among the same genera as the OTUs identified to significantly increase in

443   relative abundance in the phyllosphere of tomato plants treated with concentrated rain microbiota

444   7 days post inoculation. However, unexpectedly, the actual OTUs of these genera identified in this

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445   comparison were not the same as those identified in the controlled laboratory experiments (see

446   discussion section for possible explanations).

447   Finally, we performed another small experiment collecting rain on the same roof as in the

448   previous experiments and exposing one set of tomato plants to all rain events while taking another

449   set of plants inside before each rain event. From one rain event (June 10, 2019), phyllosphere

450   communities from a single tomato plant exposed to rain (collected on June 21, 2019), and another

451   tomato plant not exposed to rain (collected on the same date) were sequenced together with a

452   sample of the corresponding rainfall using ONT' MinION platform. A total of 194,591 long reads

453   were analyzed using the ONT taxonomic classifier WIMP. **Table 3** shows the most abundant

454   species present in the three samples ranked by relative abundance on the tomato plant exposed to

455   rain. Yet again, species of the genera *Massilia*, *Pantoea*, *Methylobacterium*, *Pseudomonas*, and

456   *Janthinobacterium* (and some additional species outside of these genera) were present in the rain

457   sample as well as in the tomato sample exposed to rain but absent (or almost absent) in the tomato

458   sample not exposed to rain. While this experiment was too small for any statistical analysis, the

459   result is consistent with the laboratory results as well as the comparison of tomatoes grown outside

460   exposed to rain with those grown in a greenhouse not exposed to rain.

461

462   **DISCUSSION**

463

464   While our general understanding of the plant microbiome has increased dramatically over the last

465   few years, the basic question of where the bacteria that colonize and inhabit the phyllosphere

466   originate from has remained unanswered. The main approach in trying to answer this question has

467   been to make comparisons of the composition of the phyllosphere microbiome with the

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468   microbiomes of putative reservoirs (Maignien et al., 2014, Zarraonaindia et al., 2015, Ottesen et

469   al., 2016, Wagner et al., 2016, Grady et al., 2019). To complement this approach, here we used

470   controlled laboratory experiments.

471   We decided to focus on rain since previous studies provided evidence that at least the

472   bacterial leaf pathogen, *Pseudomonas syringae*, may be disseminated by precipitation and

473   efficiently colonize the plant phyllosphere (Monteil et al., 2016, Morris et al., 2008). Moreover, at

474   least temporary shifts in the composition of phyllosphere communities after rain events were

475   observed (Allard et al., 2020). In a first step, we found that tomato plants naturally exposed to rain

476   outdoors, or simply sprayed with rainfall when grown in the lab, carried significantly higher

477   bacterial populations sizes compared to lab-grown plants that had not been treated with rainfall or

478   that were treated with sterilized rainfall. While plants grown outside may have also been colonized

479   by airborne bacteria through dry-deposition or by soil-borne bacteria after being splashed during

480   rainfall events, the observation that treating lab-grown plants with rain caused the bacterial

481   population size to increase more than spraying lab-grown plants with sterilized rain was a strong

482   indication that the observed increase in population size was due to the colonization and growth of

483   rain-borne bacteria.

484   To dig deeper into the possibility that rain harbors bacteria that can effectively colonize

485   and grow on tomato leaves, we decided to characterize the taxonomic composition of rainfall

486   events and then determine if any of the identified members of the rain microbiota would increase

487   in relative abundance on tomato plants treated with concentrated rain microbiota (100X-rain) but

488   not on tomato plants treated with filter-sterilized rain or sterile water. We decided on using

489   concentrated rain microbiota instead of rain since we had previously found that rain contained as

490   few as 4×10^3 CFU l^{-1} (Failor et al., 2017) and the maximum volume that we can spray on a tomato

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491   plant before water runs off the leaves is only 10 mL. Therefore, as few as 40 bacteria may get

492   inoculated on an entire plant using rain as inoculum in a lab experiment, which we deemed not to

493   be enough to lead to a bacterial population size that could be analyzed using 16S rRNA amplicon

494   sequencing. We thus used 100X-rain concentrate, acknowledging that we artificially aided rain-

495   borne bacteria in our experiment.

496   The most important observation from the characterization of the rainfall microbiota was

497   that each collected rainfall event harbored a very different bacterial community. This is in line with

498   other recent results on rainfall collected in the USA, Europe, and Asia (Aho et al., 2020, Cáliz et

499   al., 2018, Woo & Yamamoto, 2020) that showed that the taxonomic composition of microbiota in

500   rain changes with origin of air masses and season. This meant for our experiment that we could

501   not expect to find the same taxa to colonize and grow on tomatoes in each inoculation experiment.

502   It was also important to use appropriate controls when inoculating lab-grown tomato plants

503   with 100X-rain. Importantly, tomato plants were not grown in sterile conditions. Therefore, they

504   already carried microbiota at the time of inoculation and simply spraying these plants with water

505   and incubating them at high humidity (as we did to favor plant colonization) could be expected to

506   lead to changes in relative abundance of the pre-existing microbiota. Moreover, rain contains

507   nutrients and bacteriophages that are not removed by filtering and that will also affect pre-existing

508   microbiota. Therefore, we used both, filter-sterilized rain (including nutrients and possibly

509   bacteriophages but no bacteria) and autoclaved double-distilled water (expected to contain neither

510   nutrients nor bacteriophages nor bacteria) as controls.

511   Comparison of rain microbiota with phyllosphere microbiota on day-0 and day-7 for the

512   three different treatments yielded some results confirming our hypothesis that rain contains

513   effective colonizers of tomato leaves while other results were ambiguous. Firstly, the observed

514   drop in alpha diversity for phyllosphere microbiota from day-0 to day-7 after treatment with 100X-

515   rain is in agreement with the expected increase in relative abundance of some effective tomato leaf

516   colonizers accompanied by a decrease in relative abundance of many rain-borne bacteria that are

517   not adapted to tomato leaves and that are thus not effective tomato colonizers. The observed

518   decrease in alpha diversity from day-0 to day-7 for the sterile rain treatment may have been due to

519   growth of some pre-existing tomato phyllosphere members that thrived under the high humidity

520   conditions applied for two days after inoculation and the nutrients added with the rain water, which

521   helped them outcompete other members of the pre-existing microbiota. It is also possible that

522   bacteriophages present in the filter-sterilized rain reduced the relative abundance of some

523   phyllosphere members to below the detection limit.

524   In regard to beta diversity, we had expected the composition of the day 0 microbiota to be

525   similar to each other because tomato plants of the same cultivar were grown in autoclaved soil in

526   relatively stable laboratory conditions. Therefore, it was surprising to find phyllosphere samples

527   to differ more from each other (even on day-0 after sterile water treatments) compared to the

528   differences among rain samples. This was the case for weighted UniFrac distances, unweighted

529   UniFrac distances, and the Bray-Curtis dissimilarity static. A possible explanation is that the

530   composition of the phyllosphere microbiota of our lab-grown tomatoes was determined by

531   stochastic processes because of the low concentration of plant-associated bacteria present in the

532   indoor air and the autoclaved soil that was used for growing.

533   Another unexpected result was that beta diversity significantly changed from day-0 to day-

534   7 for all three treatments. Although the high humidity maintained for two days after inoculation

535   was expected to have some effect on the phyllosphere community, we still expected 100X-rain to

536   have a stronger effect on beta diversity than sterile water. Similarly unexpected, the taxonomic

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537   composition at the phylum, class, and genus level on day-0 and day-7 did not reveal any consistent

538   change unique to the treatment with 100X-rain compared to filter-sterilized rain or sterile water.

539   Taken together, these results suggested that, if they existed, tomato leaf colonizers present in rain

540   were individual species and changes in abundance of these individual species were not evident

541   from the overall comparison of beta diversity or taxonomic composition at higher taxonomic ranks.

542   Therefore, we decided to look at changes at the OTU level. To do this, we used DESeq2

543   (Love et al., 2014) a tool originally developed to identify significant changes in gene expression

544   in RNA-seq experiments, which have similar challenges to OTU tables, and which has been shown

545   to be effective for smaller OTU datasets like ours (Weiss et al., 2017). We made several

546   comparisons. Most importantly, not a single OTU significantly increased from day 0 to day 7 after

547   treatment with filter-sterilized rain or sterile water but 104 OTUs increased significantly after

548   treatment with 100X-rain. Since none of these OTUs significantly increased from day-0 to day-7

549   after treatment with filter-sterilized rain or sterile water, they were most likely rain-borne.

550   The 104 OTUs belong to 10 genera with one of them being the genus *Pantoea*. The DeSeq2

551   analysis identified 18 OTUs of this genus that significantly increased in relative abundance from

552   day 0 to day 7 after being treated with 100X-rain. Importantly, *Pantoea* OTUs were also present

553   in all rain samples (including the one sequenced with ONT's MinION). Moreover, the species *P.*

554   *agglomerans* and *P. vagans* were identified as the most abundant species in several of the tomato

555   phyllosphere day 7 samples after being treated with 100X-rain based on metagenomic sequencing.

556   Also, two *Pantoea* OTUs were present in significantly higher relative abundance in tomatoes

557   grown outside compared to tomato grown organically inside a commercial greenhouse, and *P.*

558   *agglomerans* and *P. vagans* were both identified in the rain metagenome as well as in the

559   metagenome of a tomato plant exposed to rain but not in a tomato plant not exposed to rain using

560   ONT MinION sequencing. Moreover, the genus *Pantoea* is well known to include plant pathogenic

561   bacteria and beneficial plant-associated bacteria (Coutnho & Venter, 2009, Walterson &

562   Stavrinides, 2015, Mechan Llontop et al., 2020), we previously identified 192 *Pantoea* isolates in

563   a culture-dependent study of precipitation samples (Failor et al., 2017), and *Pantoea* species were

564   recently identified both in rainfall before and after falling through a forest canopy, with higher

565   relative abundance in the throughfall samples (Ladin et al., 2021). Therefore, based on the results

566   obtained here and data in previous literature, members of the genus *Pantoea* are likely

567   phyllosphere inhabitants that originate from rainfall.

568   Another rain-borne genus that includes species that appear to successfully colonize tomato

569   plants is *Massilia*. Members of this genus were found in all rain samples (those analyzed by 16S

570   rRNA amplicon sequencing and the one sequenced with ONT's Minion). Twenty-seven OTUs of

571   this genus significantly increased between day-0 phyllosphere samples and day-7 samples for

572   100X-rain treated plants. Two *Massilia* species were also found among the species with the highest

573   relative abundance in the metagenomic sequences of the 100X-treated tomato samples on day 7.

574   One *Massilia* OTU each was more abundant in tomatoes grown outdoors compared to

575   hydroponically or organically grown tomatoes in the commercial greenhouse, respectively. Four

576   *Massilia* species were found in the rain sample and tomato sample grown outdoors but not in the

577   tomato plant not exposed to rain when using ONT MinION sequencing. As with *Pantoea*, *Massilia*

578   species were cultured out of precipitation by us previously (Failor et al., 2017) and were recently

579   found in rain and rain that had fallen through a forest canopy (Ladin et al., 2021). Finally, OTUs

580   and named species belonging to the genus *Massilia* have been found in plants, soil, and even

581   extreme environments (Ofek et al., 2012, Rastogi et al., 2012, Bodenhausen et al., 2013, Purahong

582   et al., 2018, Singh et al., 2019, Holochová et al., 2020). Therefore, members of the genus *Massilia*

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583   may cycle through multiple environments and some of them may be transported by rain to leaf

584   surfaces where they colonize the phyllosphere.

585   Other rain-borne genera likely to colonize the tomato phyllosphere based on our data

586   include *Janthinobacterium*, which, as the genus *Massilia*, is a member of the Burkholderiaceae

587   family. Four *Janthinobacterium* species were also found in rain and in the rain-exposed tomato

588   plant but not in the tomato plant protected from rain in the ONT MinION experiment. However,

589   *Janthinobacterium* was not found at significantly higher relative abundance in tomato plants

590   grown outside compared to greenhouse-grown tomatoes. Its inconsistent presence in rain may

591   explain this result.

592   Finally, we found evidence for OTUs and named species of the genus *Pseudomonas* to

593   colonize tomato plants. Unexpectedly though, we did not find a single member of the

594   *Pseudomonas syringae* species complex (*P. syringae sensu lato*), which includes many plant

595   pathogenic and commensal lineages (Vinatzer et al., 2016, Monteil et al., 2016). We do not have

596   any good explanation why we neither found *P. syringae* at relatively high abundance in the

597   analyzed rain samples nor in our phyllosphere samples treated with 100X-rain although we had

598   previously cultured *P. syringae* from rain (Failor et al., 2017) and from plants in our geographic

599   area (Clarke et al., 2010). We conclude that while *P. syringae* pathogens are present in rain and

600   disseminated by precipitation, they may not be a major component of precipitation microbiota, at

601   least not in the geographic area where the experiments here described were performed.

602   Importantly though, while our data and the literature suggest that members of some

603   common phyllosphere genera are rain-borne, the fact that we found different enrichment of OTUs

604   from these genera across experimental conditions, lab versus greenhouse, precluded the

605   identification of likely rain-borne tomato phyllosphere colonizers at the species level. One possible

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606   explanation for this is that each rain event harbored such different taxa and this increased variation

607   coupled with low replication meant that OTUs with higher relative abundance on tomatoes grown

608   outside were simply missed in the lab experiments. Conversely, the small number of analyzed

609   tomato plants grown outside exposed to rain made it difficult to find significant differences

610   compared to the tomatoes grown inside not exposed to rain. Finally, laboratory conditions may

611   not have allowed some of the OTUs found outside to effectively grow on tomato plants inside the

612   laboratory. On the other hand, artificial light and almost constant temperature and humidity may

613   have favored tomato colonization of related, but different, species compared to the most effective

614   colonizers of tomato plants grown outside, where plants are exposed to natural sunlight, including

615   UV radiation and dramatic temperature and humidity changes. These differences in environmental

616   conditions may explain why members of the genera *Sphingomonas* and *Methylobacterium* were

617   consistently found in rain and in significantly higher abundance on tomato plants grown outside

618   than in greenhouse-grown tomatoes but OTUs of these genera did not significantly increase in our

619   controlled laboratory experiments after 100X-rain treatments. Finally, the use of concentrated rain

620   microbiota instead of rain may have led to increased competition between rain-borne bacteria and

621   suppressed the colonization efficiency and growth of some while favoring the growth of others.

622   To follow up on the results reported here and to gain further insight into the relative

623   importance of seeds, soil, the atmosphere, and precipitation as reservoirs of phyllosphere

624   microbiota, we propose to expand the kind of controlled experiments we performed here but

625   growing tomatoes outdoors from either sterilized seeds or non-sterilized seeds, either not limiting

626   exposure to precipitation or limiting exposure to precipitation (for example, through the use of

627   mobile rain-out shelters), and growing plants in native versus sterile soil. Moreover, strain-level

628   metagenomics (Olm et al., 2021) of all reservoir microbiota and phyllosphere microbiota could

629   provide the necessary strain-level resolution to identify which strains from which reservoirs are

630   the most important colonizers of the phyllosphere.

631

632   **DATA DEPOSITION**

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634   Sequences and metadata are being deposited at NCBI under BioProject DPRJNA719680. All

635   read processing steps, bioinformatic workflows, and scripts used in this research are available on

636   GitHub (https://github.com/marcoeml/VinatzerLab-Mechan-rain-phyllosphere-microbiota-2020).

637

638   **FUNDING**

639

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865

866 **TABLES**

867 **Table 1.** Metadata for analyzed rain samples.
868

Rain collection ID	Month	Year	# raw reads	used for plant inoculations
VTP049	April	2015	7,133	Yes
VTP050-1	August	2015	75,555	Yes
VTP050-2	August	2015	68,612	Yes
VTP061	March	2016	75,166	Yes
VTP062	April	2016	7,533	Yes
VTP063	May	2016	27,729	Yes
VTP064	July	2016	142,736	Yes
VTP065	October	2016	35,216	No
VTP066	December	2016	32,866	No
VTP2019	June	2019	419,596	No

869
870

871   **Table 2.** Species with highest relative abundance on tomato plants on day-7 after treatment with
872   100X-rain based on metagenomic sequencing.
873

species	# positive day 7 samples[1]	average abundance in rain[2]	average abundance on day 0[2]	average abundance on day 7[2]	highest abundance on day 7
Pantoea vagans	4	0.09	0.01	2.86	8.69
Pantoea agglomerans	3	0.03	0.01	2.12	4.25
Novosphingobium resinovorum	1	0.00	0.00	1.54	1.54
Pseudomonas citronellolis	2	0.01	0.08	1.21	1.98
Buttiauxella sp.	2	0.01	0.03	0.79	1.49
Janthinobacterium sp.	1	0.01	0.01	0.77	0.77
Pseudomonas fluorescens	4	0.03	0.02	0.75	1.16
Erwinia gerundensis	2	0.01	0.01	0.75	1.31
Massilia putida	3	0.01	0.01	0.63	0.76
Janthinobacterium agaricidamnosum	1	0.01	0.00	0.62	0.62
Cedecea neteri	2	0.01	0.02	0.53	1.00
Pseudomonas orientalis	2	0.00	0.00	0.53	0.79
Massilia sp.	4	0.01	0.02	0.45	0.90
Flavobacterium sp. HYN0086	1	0.06	0.02	0.41	0.41
Flavobacterium anhuiense	1	0.08	0.04	0.40	0.40
Flavobacterium sp.	1	0.04	0.02	0.33	0.33
Pseudomonas azotoformans	4	0.01	0.00	0.31	0.48
Pseudomonas sp.	2	0.00	0.00	0.27	0.33
Pseudomonas putida	1	0.02	0.01	0.27	0.27
Enterobacter sp.	1	0.00	0.01	0.26	0.26
Klebsiella michiganensis	1	0.00	0.01	0.25	0.25
Pseudomonas protegens	1	0.00	0.02	0.25	0.25
Pantoea ananatis	1	0.00	0.00	0.24	0.24
Pseudomonas trivialis	1	0.00	0.00	0.24	0.24
Pseudomonas rhizosphaerae	2	0.01	0.01	0.23	0.27
Brevundimonas sp.	1	0.01	0.00	0.23	0.23
Pseudomonas veronii	1	0.00	0.00	0.23	0.23
Pseudomonas syringae	1	0.01	0.02	0.22	0.22
Enterobacter cloacae	2	0.02	0.01	0.15	0.24
Escherichia coli	3	0.14	0.12	0.15	0.22
Sphingobium sp.	1	0.00	0.00	0.15	0.15
Hyphomicrobium sp.	1	0.01	0.00	0.13	0.13
Paraburkholderia fungorum	1	0.00	0.00	0.11	0.11

Staphylococcus aureus	1	0.11	0.10	0.11	0.11
Pseudomonas sp.	1	0.00	0.00	0.10	0.10
Pseudomonas sp.	1	0.01	0.00	0.10	0.10

[1] out of 5
[2] only considering experiments
for which the species was
present on day 7

874

875

Table 3. Taxonomic classification of ONT MinION reads using WIMP of a rain sample, a tomato plant exposed to rain, and a tomato plant not exposed to rain.

Taxon[1]	rain	tomato exposed to rain	tomato not exposed to rain
Massilia putida	2,461	165	0
Massilia **sp. WG5**	2,794	119	0
Deinococcus gobiensis	0	67	1
Deinococcus actinosclerus	0	56	2
Roseateles depolymerans	33	55	0
Pantoea agglomerans	659	45	1
Methylobacterium **sp. PR1016A**	37	32	0
Acidovorax **sp. KKS102**	17	32	0
Pseudomonas oryzihabitans	25	25	2
Variovorax paradoxus	98	22	0
Phycomyces blakesleeanus	15	22	29
Mitsuaria **sp. 7**	50	19	1
Pantoea vagans	18	18	1
Methylobacterium phyllosphaerae	75	18	1
Methylobacterium aquaticum	42	17	0
Microbacterium testaceum	0	16	0
Deinococcus soli Cha et al. 2016	0	15	0
Rubrivivax gelatinosus	47	15	0
Massilia **sp. B2**	826	14	1
Rhizobacter gummiphilus	53	13	0
Microbacterium **sp. BH-3-3-3**	12	12	1
Methylobacterium extorquens	1,102	11	2
Pantoea sp. PSNIH1	0	10	1
Massilia **sp. NR 4-1**	550	9	1
Janthinobacterium **sp. 1_2014MBL_MicDiv**	384	9	0
Janthinobacterium **sp. LM6**	350	9	1
Ralstonia solanacearum	150	9	0
Janthinobacterium agaricidamnosum	338	8	0
Sphingomonas taxi	627	7	0
Janthinobacterium svalbardensis	265	6	0
Modestobacter marinus	162	5	3
Herbaspirillum frisingense	199	4	0
Pseudomonas syringae	165	4	0
Pseudomonas fluorescens	10,173	3	0
Geodermatophilus obscurus	163	3	0

43

Pseudomonas protegens	906	2	1
Pseudomonas putida	289	2	1

879   [1]Only species with at least two reads in either the tomato plant exposed to rain or the tomato
880   plant not exposed to rain are listed ranked from high to low by the number of reads in the tomato
881   plant exposed to rain. Species with a higher number of reads in the tomato plant exposed to rain
882   are in bold.

883

884

885   **FIGURE LEGENDS**

886

887   **Figure 1.** Bacterial population size in the phyllosphere of tomato plants A) grown exposed to rain

888   in plastic pots at the Virginia Tech Kentland Farm compared to plants grown inside the laboratory

889   B) grown on the roof of the Latham Hall research building exposed to rain compared to plants

890   grown under laboratory conditions, and C) grown inside the laboratory seven days after being

891   either treated with rain or autoclaved rain. T-test, $P < 0.001$.

892

893   **Figure 2.** Relative abundance (RA) of bacterial taxa in rainfall collected in Blacksburg, VA, on 9

894   different days in 2015, 2016, and 2019. Samples were rarefied to 6,419 sequences. A) RA at the

895   class level (only classes with a RA above 1% are shown), B) RA at the genus level (only generated

896   with RA above 2% are shown). For August 15, results are based on two technical replicates (see

897   also Table 1).

898

899   **Figure 3.** Alpha diversity measurements for rain compared to treated plants at day-0 vs day-7.

900   Three measures of alpha diversity (observed OTUs, Shannon diversity index, and Simpson

901   diversity index) were used. For rain, only samples used for plant inoculations were included.

902

903   **Figure 4.** Principal coordinates analysis (PCoA) derived from weighted UniFrac distances (A),

904   unweighted UniFrac distances (B), and the dissimilarity matrix of Bray-Curtis (C). Only rain

905   samples used for plant inoculation were included. For experiments for which replicates were

906   available, the replicate samples are labeled as "monthyear.1" and "monthyear.2".

907

908   **Figure 5.** Relative abundance (RA) of bacterial taxa of plants treated with either 100X-rain,

909   filtered rain, or sterile water, at day-0 and day-7. Experiments are listed by dates from left to

910   right (April '15, August '15, March '16, April '16, May '16, and July '16). A) RA at the phylum

911   level (abundance > 1%), B) . RA at the genus level (abundance > 3%). For experiments for

912   which replicates were available relative abundance is based on all replicates.

913

914   **Figure 6:** Differential abundance analysis at the level of Operational Taxonomic Unit (OTU) using

915   DESeq2 (Love et al., 2014). The fold change is shown on the X axis and genera are listed on the

916   Y axis. Each colored dot represents a separate OTU. A) Comparison of phyllosphere microbiota

917   of plants treated with 100X-rain between day-0 and day-7, B) Comparison between rain microbiota

918   and phyllosphere microbiota 7 days after treatment with the respective 100X-rain sample.

919

920   **Figure 7.** Relative abundance of a representative selection of rain-borne genera that either failed

921   or succeeded in colonizing the tomato phyllosphere. Dates of experiments are listed on the X axis

922   of panels G and H. Relative abundance is shown on the Y axis for rain, tomato plants on day-0 and

923   day-7 after being treated with 100X-rain (Rain), filtered rain, or sterile water (see panel B for

924   figure legend). A) *Pantoea*, B) *Flavobacterium*, C) *Janthinobacterium*, D) *Pseudomonas*, E)

925   *Massilia*, F) *Methylobacterium*, G) *Acidiphilium*, and H) *Bacillus*.

926

927   **Figure 8.** Taxonomic composition and Alpha diversity of phyllosphere microbiota of tomato

928   plants grown hydroponically or in soil (both in a commercial greenhouse) and of tomato plants

929   grown outside (on the roof of the Latham Hall research building). A) Relative abundance at the

930   phylum level (only phyla with a RA above 1% are shown), B) Alpha diversity (observed OTUs,

931   Shannon diversity index, and Simpson diversity index).

932

933   **Figure 9.** Differential abundance analysis at the level of Operational Taxonomic Unit (OTU)

934   using DESeq2 (Love et al., 2014). The fold change is shown on the X axis and genera are listed

935   on the Y axis. Each colored dot represents a separate OTU. A) Plants grown hydroponically in a

936   greenhouse compared with plants grown on the roof of the Latham Hall research building, B)

937   Plants grown in soil in a greenhouse compared with plants grown on the roof of the Latham Hall

938   research building.

939

940   **Supplementary Figures**

941

942   **Supplementary Figure 1.** Rarefaction curves of rain microbiota and tomato phyllosphere

943   microbiota treated with 100X-rain, filtered rain, or sterile water on day-0 and day-7.

944

945   **Supplementary Figure 2.** The core rain microbiome. Rain-associated OTUs at a detection

946   threshold of 0.1% and 100% as a prevalence threshold.

947

948   **Supplementary Figure 3.** Differential abundance analysis at the OTU level usingDESeq2 (Love

949   et al., 2014). A) Comparison of rain microbiota with the tomato phyllosphere treated with sterile

950   water at day-0 during the same experiment, B) Comparison of rain microbiota with the tomato

951   phyllosphere microbiota on the day there were treated with 100X-rain (day-0).

952
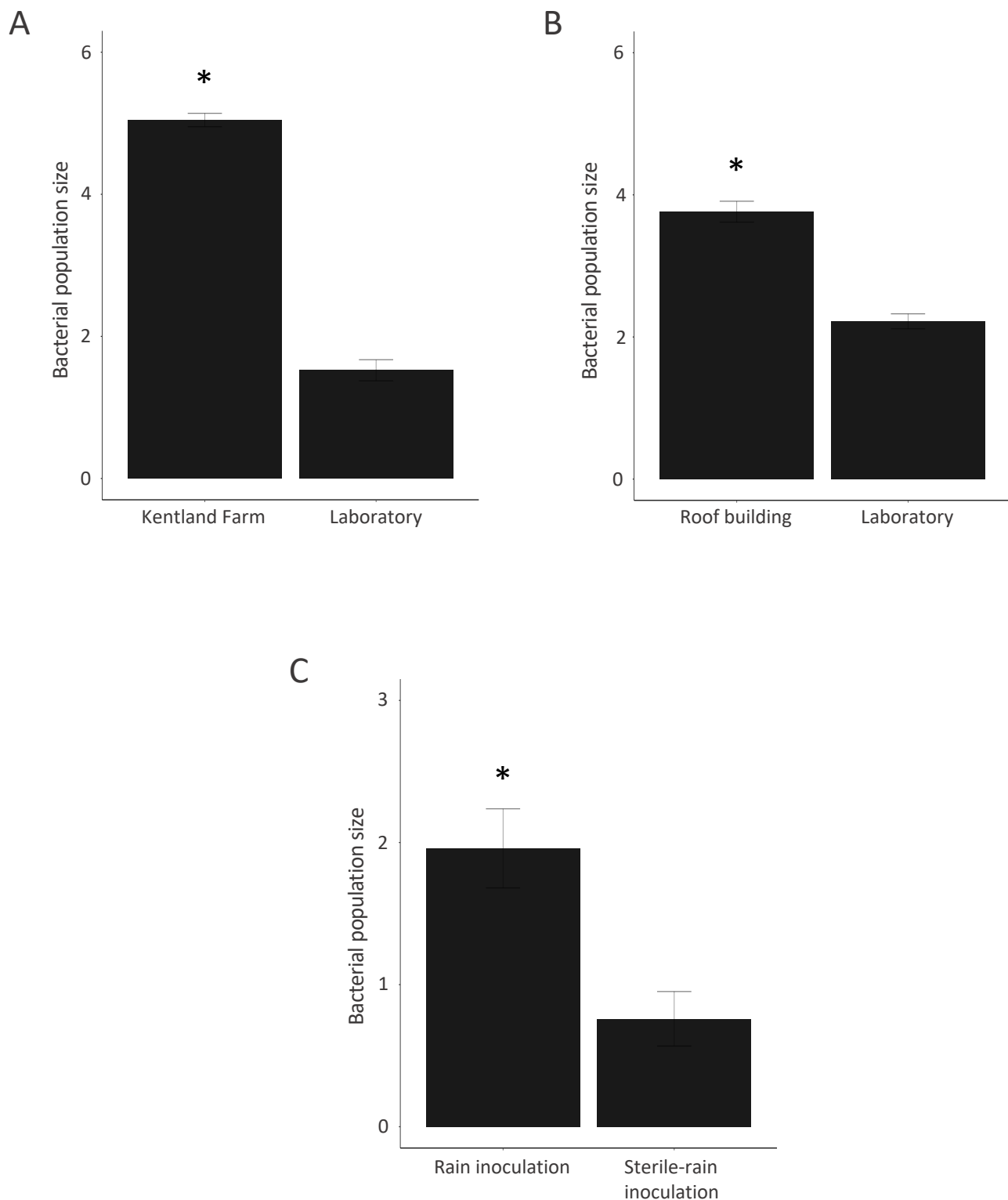
Figure 1. Bacterial population size in the phyllosphere of tomato plants A) grown exposed to rain in plastic pots at the Virginia Tech Kentland Farm compared to plants grown inside the laboratory B) grown on the roof of the Latham Hall research building exposed to rain compared to plants grown under laboratory conditions, and C) grown inside the laboratory seven days after being either treated with rain or autoclaved rain. T-test, $P < 0.001$.
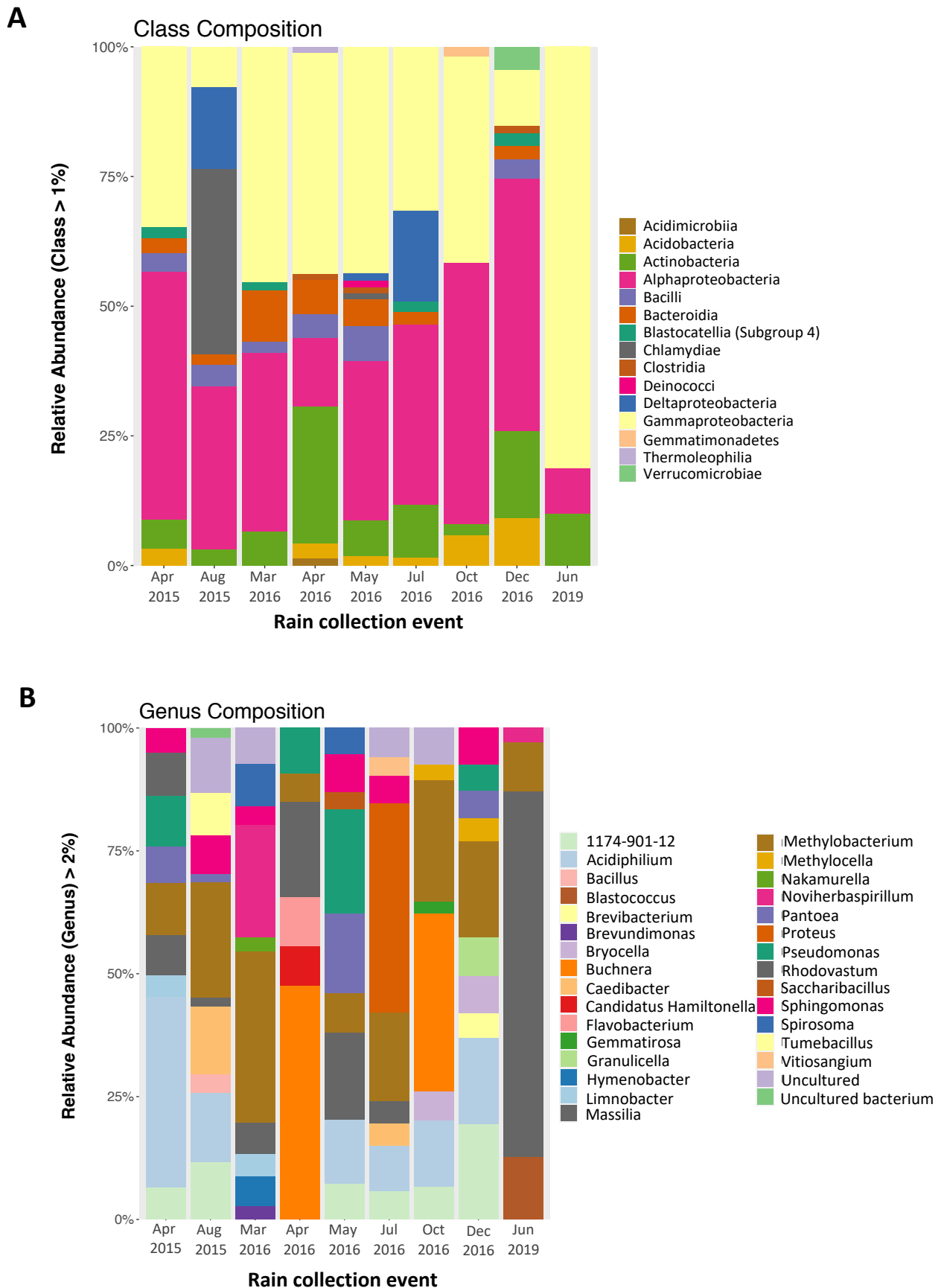
A

B

Figure 2. Relative abundance (RA) of bacterial taxa in rainfall collected in Blacksburg, VA, on 9 different days in 2015, 2016, and 2019. Samples were rarefied to 6,419 sequences. A) RA at the class level (only classes with a RA above 1% are shown), B) RA at the genus level (only generated with RA above 2% are shown). For August 15, results are based on two technical replicates (see also Table 1).
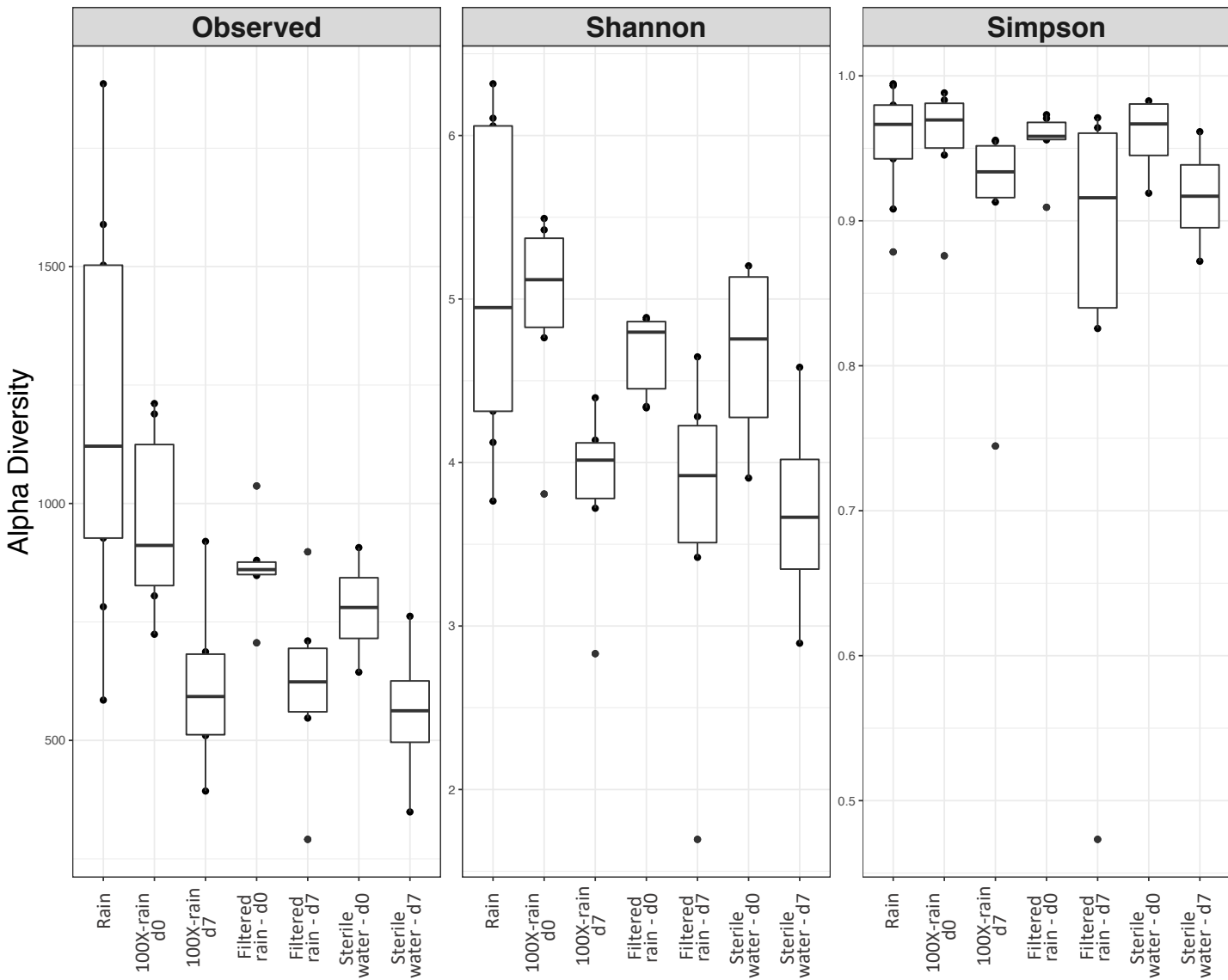
Figure 3. Alpha diversity measurements for rain compared to treated plants at day-0 vs day-7. Three measures of alpha diversity (observed OTUs, Shannon diversity index, and Simpson diversity index) were used. For rain, only samples used for plant inoculations were included.
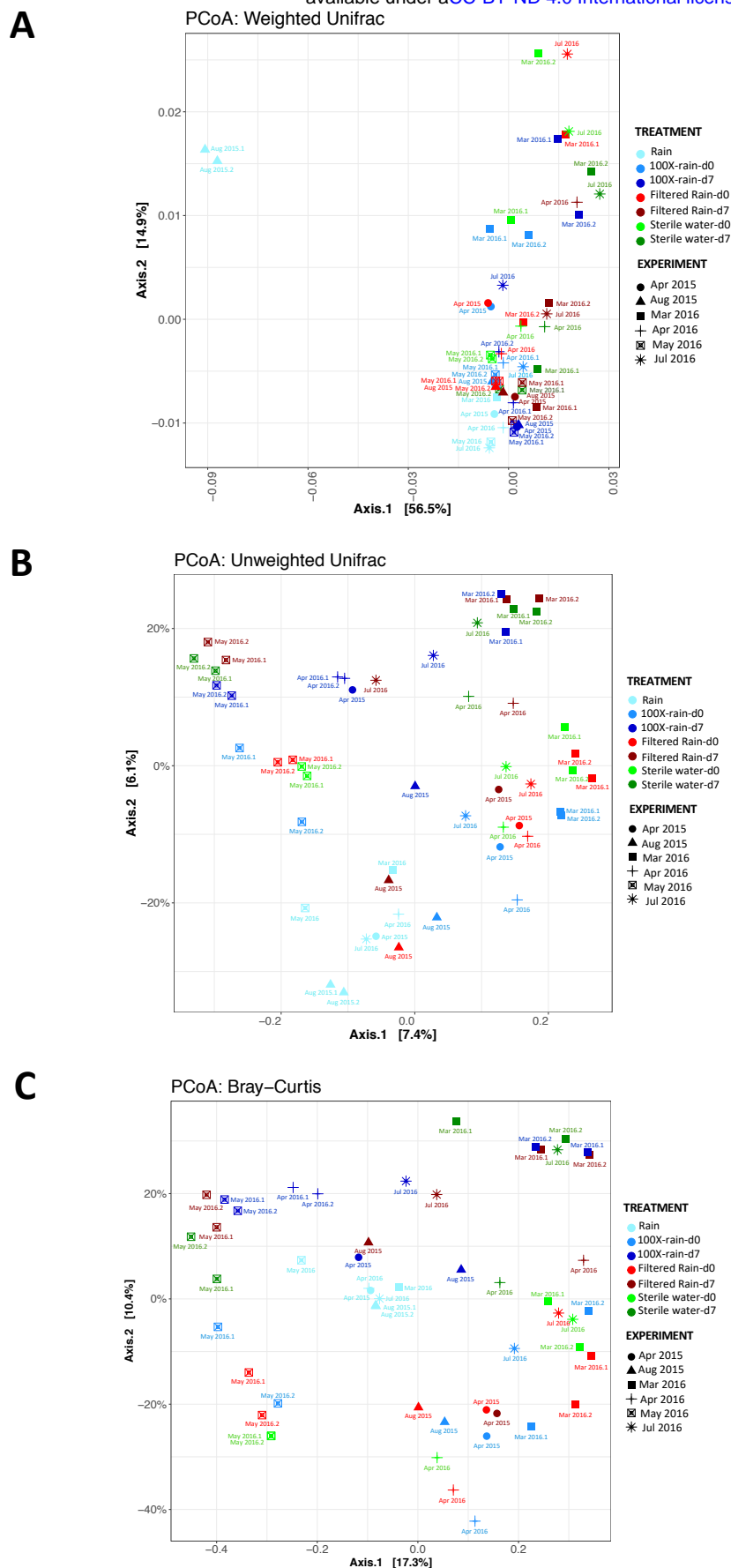
Figure 4. Principal coordinates analysis (PCoA) derived from weighted UniFrac distances (A), unweighted UniFrac distances (B), and the dissimilarity matrix of Bray-Curtis (C). Only rain samples used for plant inoculation were included. For experiments for which replicates were available, the replicate samples are labeled as "monthyear.1" and "monthyear.2".
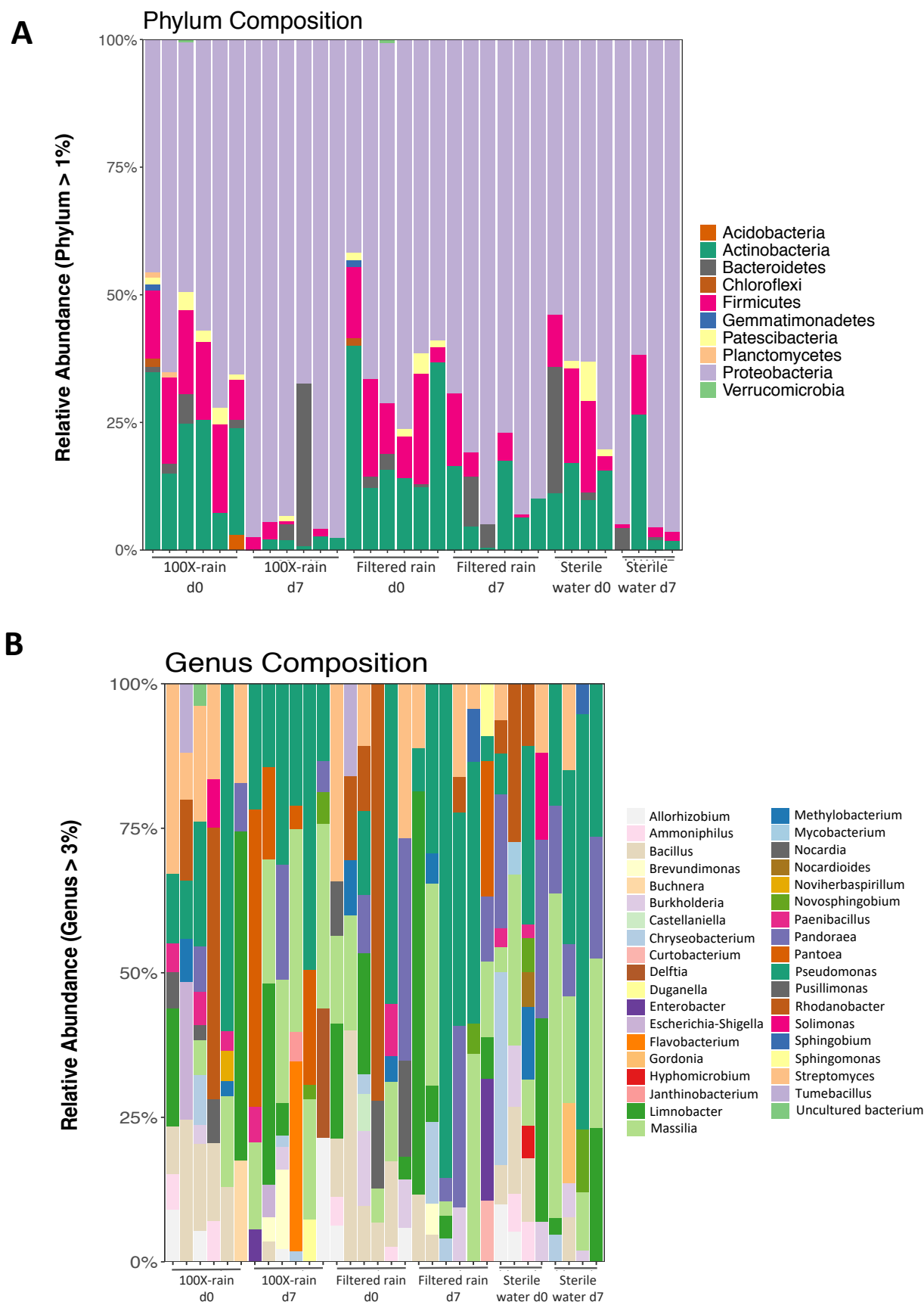
Figure 5. Relative abundance (RA) of bacterial taxa of plants treated with either 100X-rain, filtered rain, or sterile water, at day-0 and day-7. Experiments are listed by dates from left to right (April '15, August '15, March '16, April '16, May '16, and July '16). A) RA at the phylum level (abundance > 1%), B) . RA at the genus level (abundance > 3%). For experiments for which replicates were available relative abundance is based on all replicates.
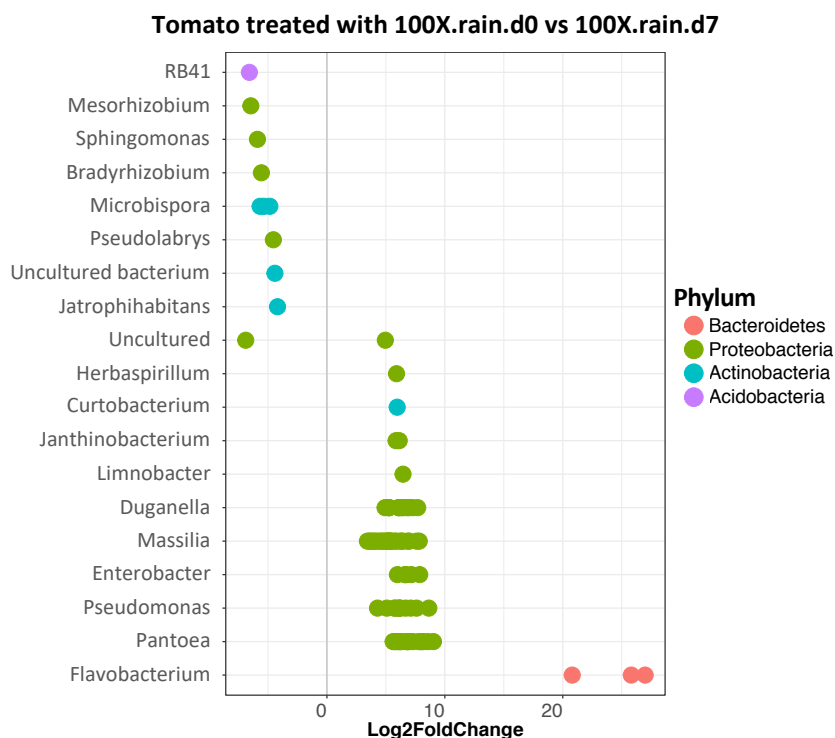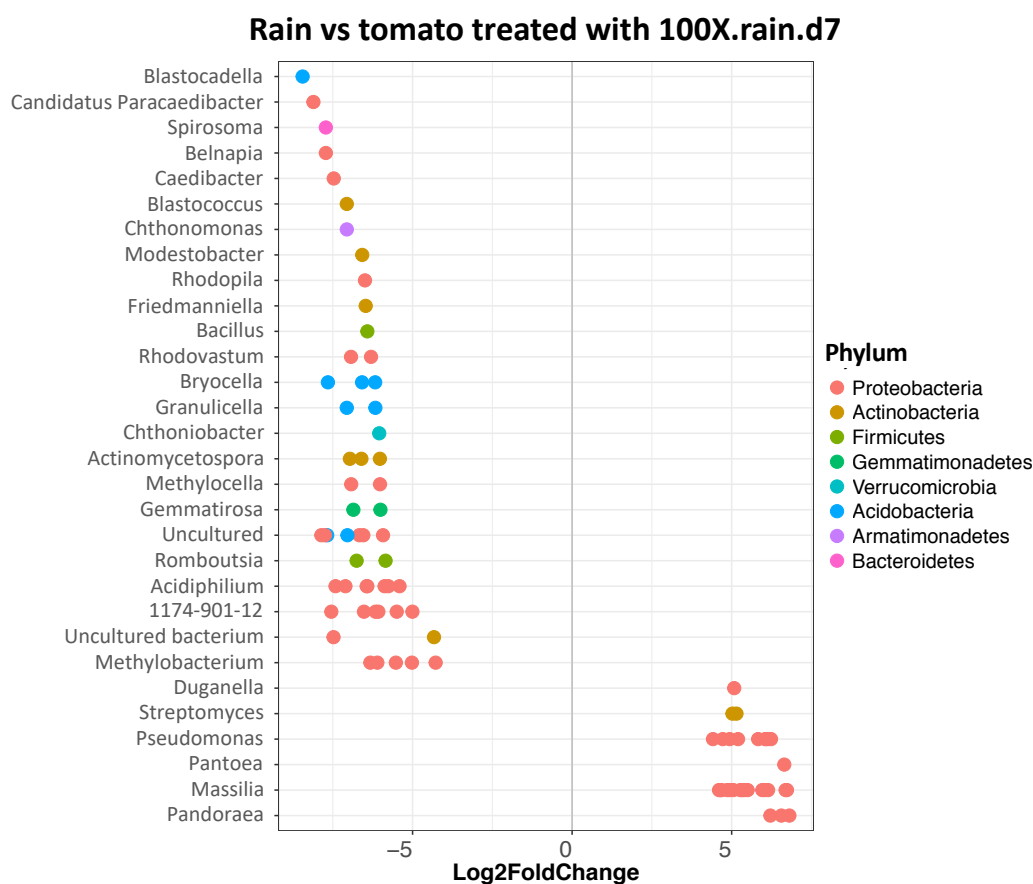
Figure 6: Differential abundance analysis at the level of Operational Taxonomic Unit (OTU) using DESeq2 (Love et al., 2014). The fold change is shown on the X axis and genera are listed on the Y axis. Each colored dot represents a separate OTU. A) Comparison of phyllosphere microbiota of plants treated with 100X-rain between day-0 and day-7, B) Comparison between rain microbiota and phyllosphere microbiota 7 days after treatment with the respective 100X-rain sample.
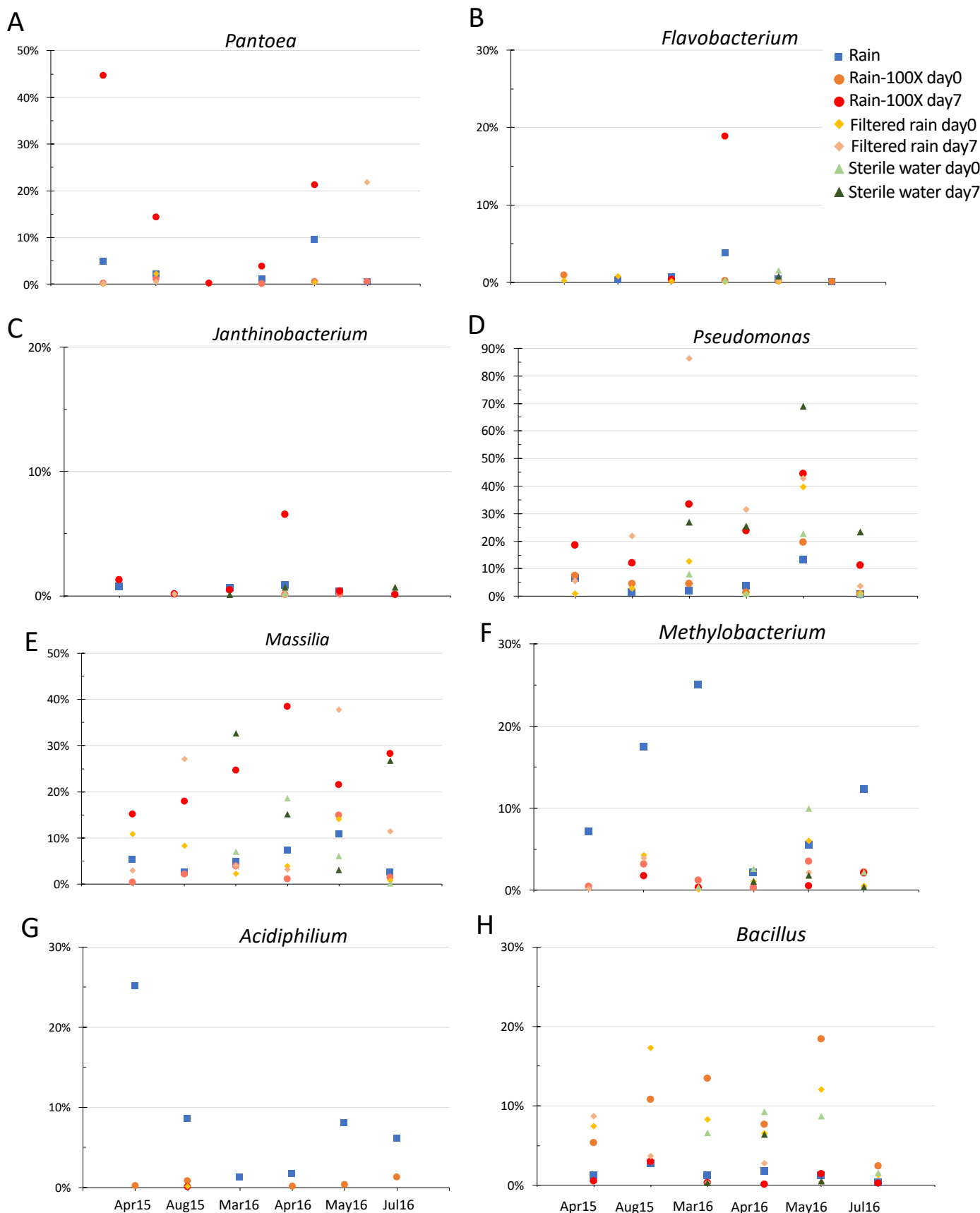
Figure 7. Relative abundance of a representative selection of rain-borne genera that either failed or succeeded in colonizing the tomato phyllosphere. Dates of experiments are listed on the X axis of panels G and H. Relative abundance is shown on the Y axis for rain, tomato plants on day-0 and day-7 after being treated with 100X-rain, filtered rain, or sterile water (see panel B for figure legend). A) *Pantoea*, B) *Flavobacterium*, C) *Janthinobacterium*, D) *Pseudomonas*, E) *Massilia*, F) *Methylobacterium*, G) *Acidiphilium*, and H) *Bacillus*.
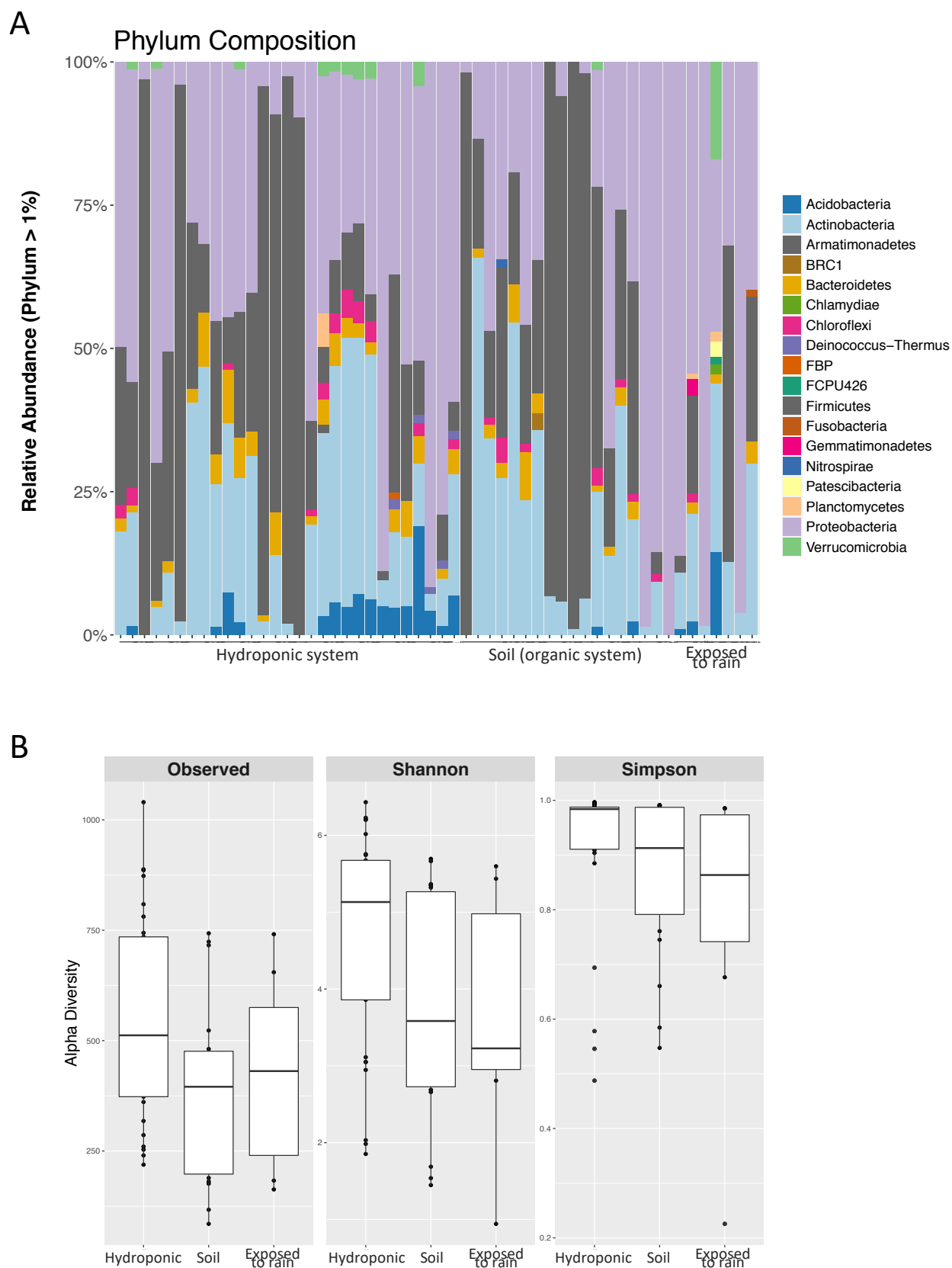
Figure 8. Taxonomic composition and Alpha diversity of phyllosphere microbiota of tomato plants grown hydroponically or in soil (both in a commercial greenhouse) and of tomato plants grown outside (on the roof of the Latham Hall research building). A) Relative abundance at the phylum level (only phyla with a RA above 1% are shown), B) Alpha diversity (observed OTUs, Shannon diversity index, and Simpson diversity index).
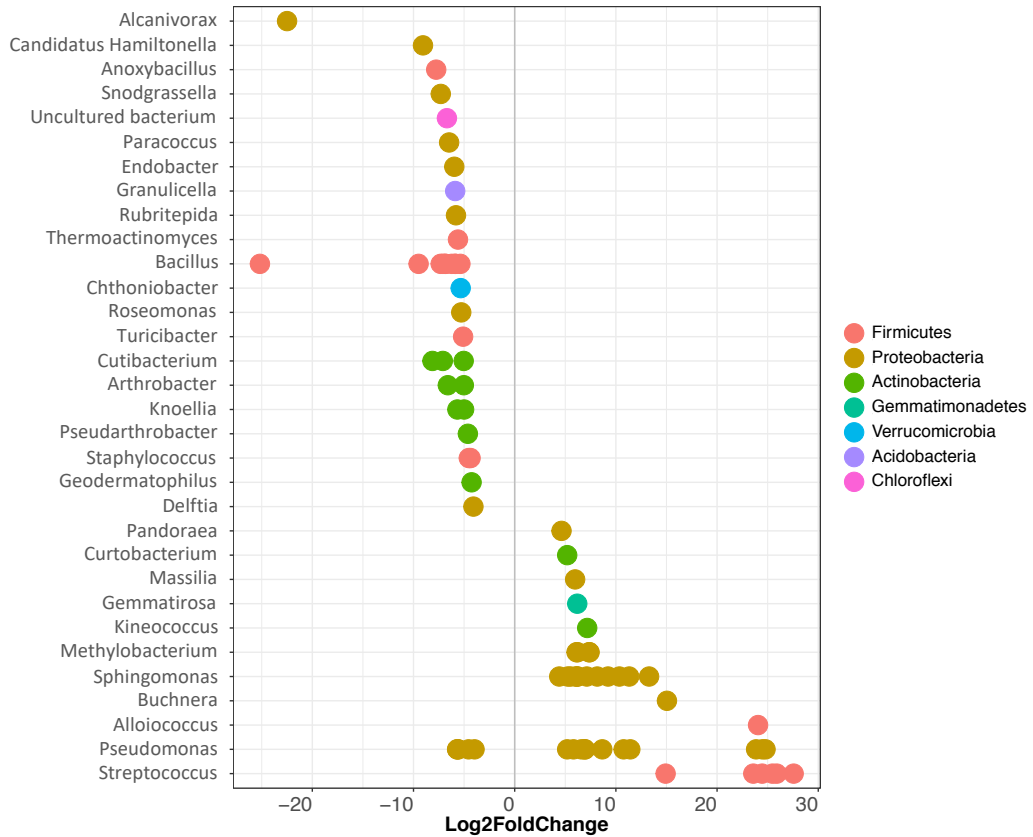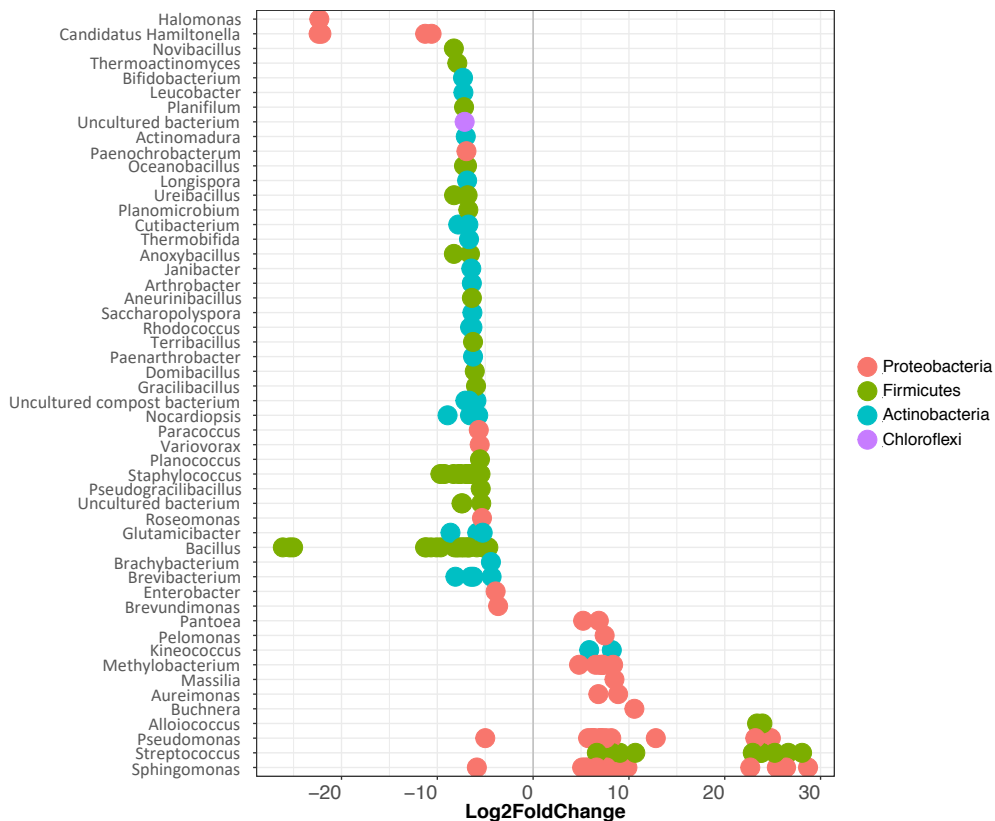
Figure 9. Differential abundance analysis at the level of Operational Taxonomic Unit (OTU) using DESeq2 (Love et al., 2014). The fold change is shown on the X axis and genera are listed on the Y axis. Each colored dot represents a separate OTU. A) Plants grown hydroponically in a greenhouse compared with plants grown on the roof of the Latham Hall research building, B) Plants grown in soil in a greenhouse compared with plants grown on the roof of the Latham Hall research building.
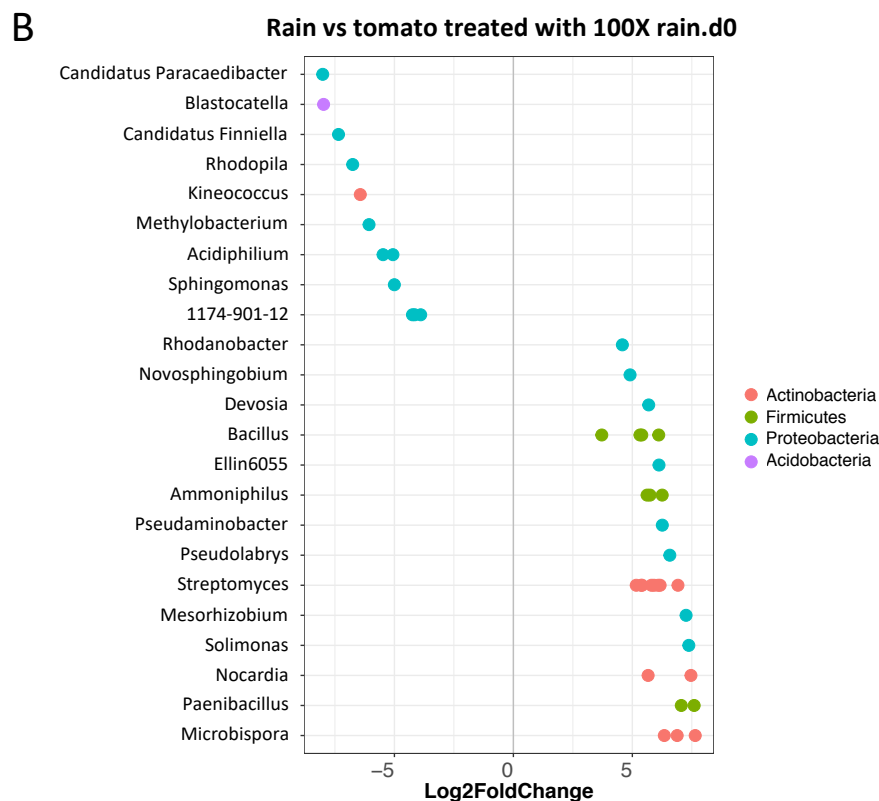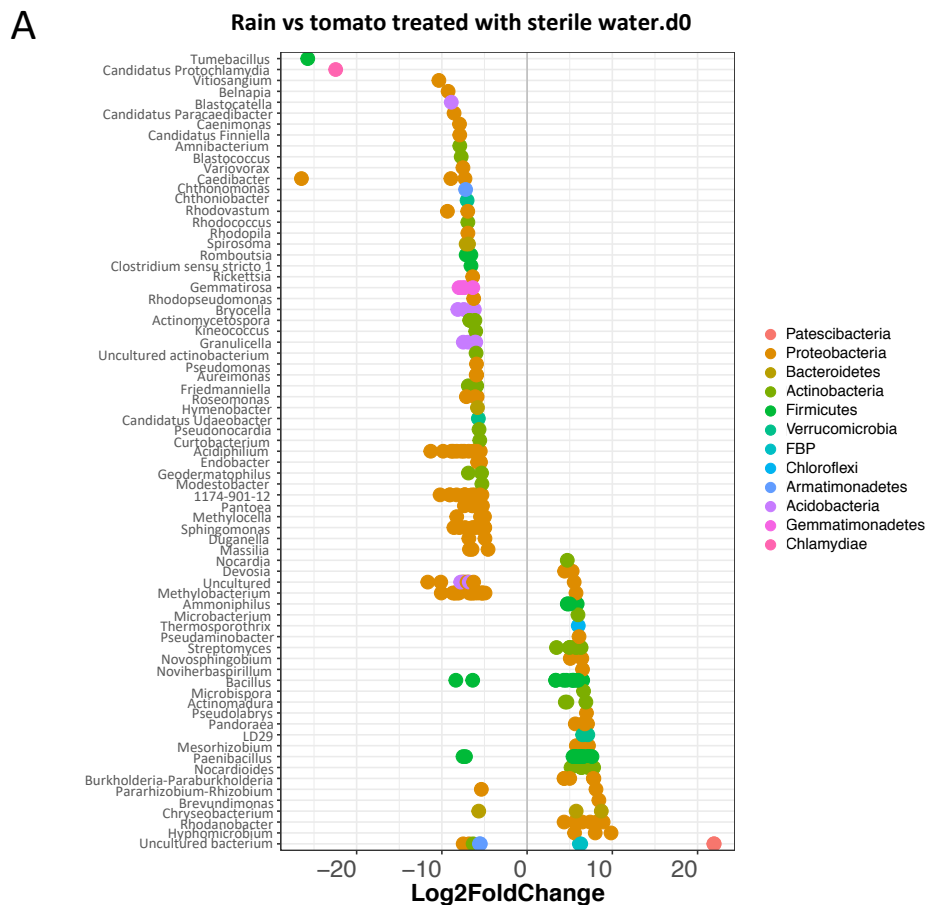
Supplementary Figure 1. Rarefaction curves of rain microbiota and tomato phyllosphere microbiota treated with 100X-rain, filtered rain, or sterile water on day-0 and day-7.

Rain core microbiome

Detection threshold (Relative abundance %)
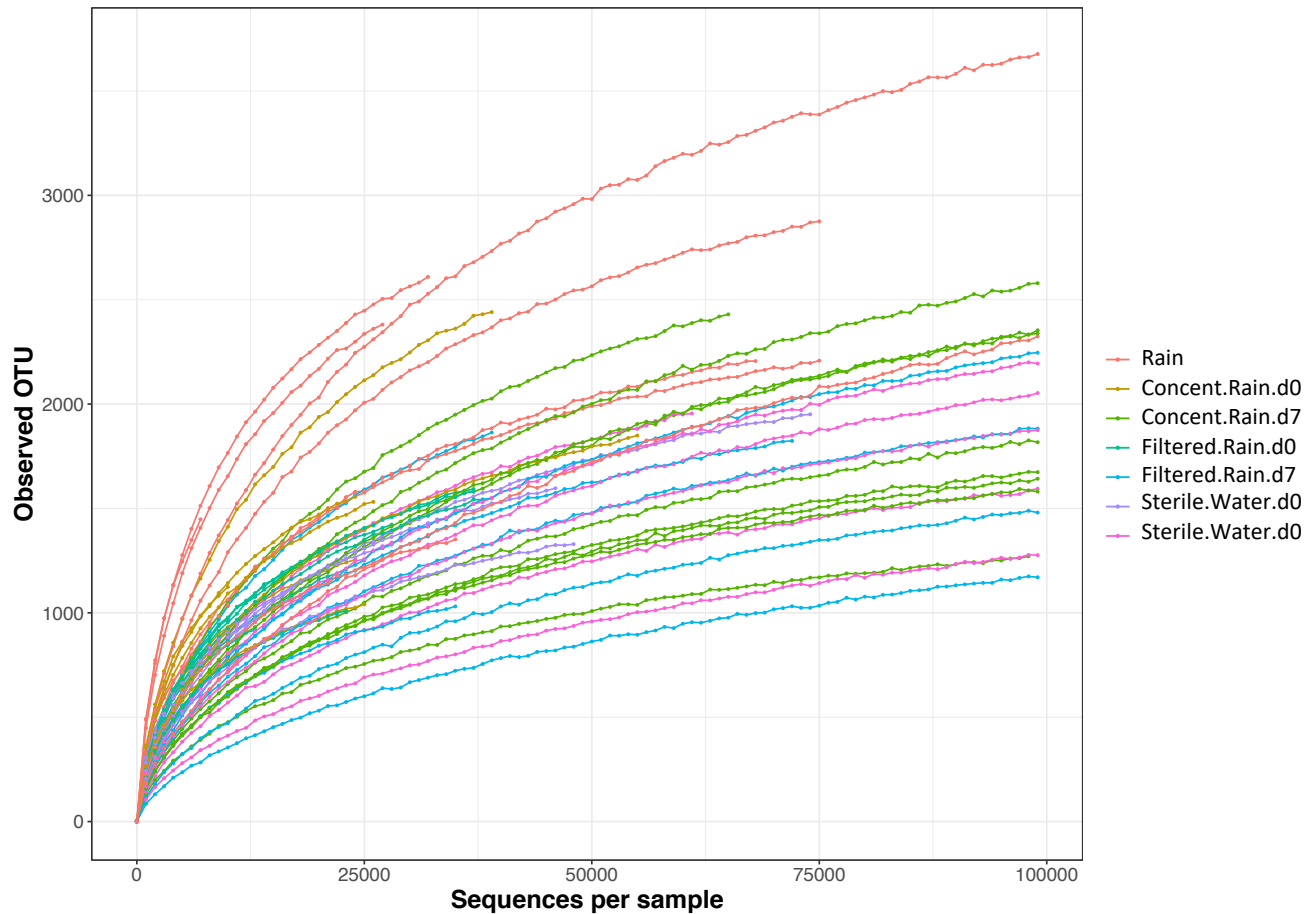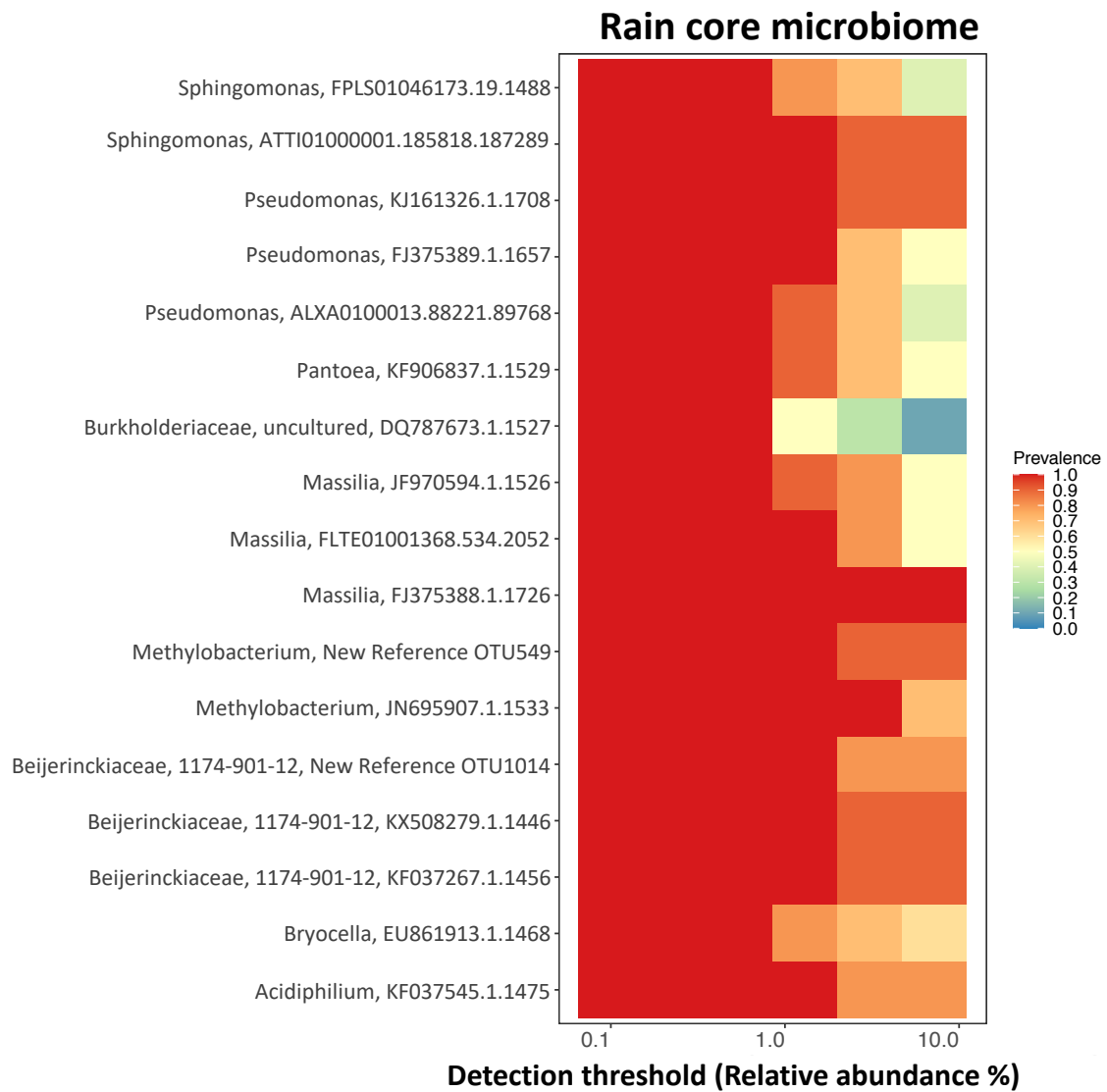
Supplementary Figure 2. The core rain microbiome. Rain-associated OTUs at a detection threshold of 0.1% and 100% as a prevalence threshold.

Supplementary Figure 3. Differential abundance analysis at the OTU level usingDESeq2 (Love et al., 2014). A) Comparison of rain microbiota with the tomato phyllosphere treated with sterile water at day-0 during the same experiment, B) Comparison of rain microbiota with the tomato phyllosphere microbiota on the day there were treated with 100X-rain (day-0).