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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 seed, 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 thus-far-neglected reservoir of phyllosphere microbiota. Therefore, rain microbiota were compared with phyllosphere microbiota of tomato plants either treated with concentrated rain microbiota, filter-sterilized rain, or sterile water. Based on 16S ribosomal RNA amplicon sequencing, 104 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 tomato plants exposed to rain outdoors than on tomato plants 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.

Keywords: bacteriology, genomics, metagenomics, microbiome, plant pathology, plants, rhizosphere and phyllosphere

Microbial communities associated with plants, often referred to as plant-associated microbiota and as constituents of the plant microbiome, influence a remarkable number of processes of plant biology and affect plant health and crop yield (Badri et al. 2013; Berg and Koskella 2018; Durán et al. 2018; Goh et al. 2013; Hacquard et al. 2015; Lu et al. 2018; Ritpitakphong et al. 2016; Torres-Cortés et al. 2018). The phyllosphere, considered here as the plant compartment that extends from the outside to the inside of the leaf (Morris 2002; Vacher et al. 2016), harbors a high diversity of microorganisms, with bacteria being the most abundant domain (Lindow and Brandl 2003; Vorholt 2012). Phyllosphere microbiota are exposed to fluctuating environmental stresses, including changes in UV exposure, temperature, water availability, osmotic stress, and humidity (Hirano and Upper 2000; Jacobs and Sundin 2001; Lindow and Brandl 2003; Vacher et al. 2016). The core bacterial phyla that can withstand these environmental stressors include *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* (Bulgarelli et al. 2013; Vorholt 2012). However, at lower taxonomic ranks, the phyllosphere microbiome greatly varies with changing biotic and abiotic factors (Copeland et al. 2015; Lindemann and Upper 1985; Rastogi et al. 2012; Wagner et al. 2016).

Culture-independent 16S ribosomal RNA (rRNA) amplicon analyses have expanded our knowledge of the composition of the phyllosphere microbiome of several plant species (Copeland et al. 2015; Grady et al. 2019; Kembel et al. 2014; Knief et al. 2012; Williams et al. 2013), including tomato (*Solanum lycopersicum*) (Ottesen et al. 2013, 2016). The bacterial genera *Pseudomonas*, *Erwinia*, *Sphingomonas, Janthinobacterium, Curtobacterium, Agrobacterium, Stenotrophomonas, Aurantimonas, Thermomonas, Buchnera, Enterococcus*, *Rubrobacter*, *Methylobacterium*, *Deinococcus*, and *Acidovorax* have

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all been observed to be associated with the phyllosphere of tomato grown in the field (Ottesen et al. 2013, 2016; Toju et al. 2019).

Microbes constantly cycle across interconnected habitats maintaining healthy ecosystems (van Bruggen et al. 2019). Importantly for this study, it has been shown that plants represent an important source of microbes that are released as aerosols into the atmosphere (Bowers et al. 2011; Constantinidou et al. 1990; Lighthart and Shaffer 1995; Lindemann et al. 1982; Šantl-Temkiv et al. 2013; Vaïtilingom et al. 2012). The atmosphere then serves as a vehicle for microbial dispersal not only locally but also globally (Bovallius et al. 1978; Brown and Hovmøller 2002; Schmale and Ross 2015) and airborne microbial communities are deposited back to earth surfaces as precipitation. Thus, the atmosphere represents a crucial route for the dissemination of beneficial and pathogenic species (Monteil et al. 2014, 2016; Polymenakou 2012). There is even some evidence that airborne microbes contribute to the formation of precipitation itself (Amato et al. 2015, 2017; Christner et al. 2008; Failor et al. 2017; Morris et al. 2014). *Proteobacteria*, followed by *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*, have been identified as the most common phyla in both the atmosphere (Cáliz et al. 2018; Hiraoka et al. 2017; Peter et al. 2014) and precipitation (Aho et al. 2020; Cáliz et al. 2018), notable largely the same as the core phyla of the phyllosphere microbiome mentioned earlier.

Microbial community assembly is largely influenced by deterministic (selection) and stochastic (dispersal) processes that determine the complex structure and function of microbiomes (Graham and Stegen 2017; Powell et al. 2015; Zhou and Ning 2017). This assembly process has been extensively studied in roots, which recruit microbial communities from the surrounding soil (Fitzpatrick et al. 2018; Pérez-Jaramillo et al. 2019). However, sources that drive the phyllosphere microbiome assembly are still under debate. It has been suggested that soil is the major reservoir of the phyllosphere microbiome (Grady et al. 2019; Wagner et al. 2016; Zarraonaindia et al. 2015). In fact, it has been observed that the leaf microbiome reflects soil bacterial diversity at an early stage of growth but significantly differs as plants grow and mature (Copeland et al. 2015). In contrast, Maignien et al. (2014) and Ottesen et al. (2016) found evidence that dry deposition of airborne microbes constitutes an important source of phyllosphere microbiota while Abdelfattah et al. (2021) found that seed microbiota also represent a reservoir of phyllosphere microbiota.

A few studies have also explored wet deposition of airborne microbes in precipitation as a potential reservoir of phyllosphere microbiota. For example, (Morris et al. 2008) concluded that the plant pathogen *Pseudomonas syringae* is disseminated through the water cycle because of its ubiquitous presence in compartments of the water cycle and plants. Using whole-genome sequencing, Monteil et al. (2016) confirmed these conclusions by finding that *P. syringae* bacteria isolated from diseased cantaloupe plants and *P. syringae* isolates from rain, snow, and irrigation water were members of the same population. Recently, rain was also found to affect the overall composition of plant phyllosphere microbiota but it was not determined whether rainborne bacteria were the source of the observed shifts (Allard et al. 2020). Moreover, it is well known that fungal spores are released from plants, travel long distances through the atmosphere, and can be deposited back on plants with rain (Woo et al. 2018).

Here, we hypothesized that rainborne bacteria might contribute to the assembly of phyllosphere microbiota beyond pathogenic bacteria and fungi. In support of this hypothesis, we found that the phyllosphere of tomato plants exposed to rain contained a higher bacterial population size than the phyllosphere of tomato plants not exposed to rain. Putative rainborne tomato phyllosphere colonizers were then identified in a series of controlled laboratory experiments and by comparing the composition of tomato phyllosphere microbiota of plants naturally exposed to rain with those not exposed to rain.

MATERIALS AND METHODS

Experimental design. Three groups of experiments were performed. First, the population size of tomato phyllosphere microbiota was compared between tomato plants grown either without exposure to rain or exposed to rain to determine whether rain-exposed plants carried a higher bacterial population size, possibly because of colonization by rainborne bacteria. Second, laboratory-grown tomato plants were inoculated with either concentrated rain microbiota, filter-sterilized rain, or sterile water. The taxonomic composition of the rainfall events used in these experiments was compared with that of the phyllosphere microbiota of tomato plants at two different time points after treatment to determine whether any of the taxa found in rain were able to effectively colonize the laboratory-grown tomato plants. Finally, the taxonomic composition of phyllosphere microbiota or tomato plants grown either in a commercial greenhouse protected from rain or outdoors naturally exposed to rain were compared with each other to determine whether the taxa that were found to effectively colonize laboratory-grown tomato plants were also present at higher relative abundance in tomato plants naturally exposed to rain outdoors than in tomato plants grown indoors protected from rain.

Determination of the population size of tomato phyllosphere microbiota of plants either exposed to rain or not exposed to rain. Tomato seed of the cultivar Rio Grande (Ferry Morse, Norton, MA, U.S.A.) were germinated in autoclaved (60 min/fast cycle) soil consisting of a 1:1 mix of promix BX (Premier Horticulture, Quebec, Canada) and metromix (Sungro, Sebe Beach, Canada). Plants were grown in the laboratory at approximately 22°C on shelves under 14 h of light and 10 h of darkness in 24-cell trays and fertilized after 2 weeks with liquid Miracle-Gro All Purpose Plant Food (The Scotts Company, Marysville, OH, U.S.A.) following product directions. After 3 weeks, plants were transplanted into 1-gallon (4.546 liters) black plastic pots (3 plants/pot) and either kept in the laboratory or transported to a research farm and placed on gravel near a maintained lawn (Kentland Farm, Blacksburg, VA, U.S.A.). For growth on the flat roof of the three-story Latham Hall research building at Virginia Tech (Blacksburg, VA, U.S.A.), plants were transplanted into large green home-gardening-style planters with a cinderblock at the bottom for weight to avoid tipping over under high winds.

After 4 weeks of growth under the three different conditions, the population size of phyllosphere microbiota was determined. Leaf disks were aseptically collected with a number 1 cork borer (0.52 mm^2) and placed in a 1.2-ml tube containing 200 µl of sterile 10 mM $MgSO_4$ solution and three 2-mm glass beads. Tubes were placed in a Mini Beadbeater (Biospec Products, Inc., Bartlesville, OK, U.S.A.) and shaken for 2 min to release bacterial cells. Serial dilutions were plated on R2A plates supplemented with cycloheximide (200 mg/liter) to inhibit fungal growth. Plates were incubated at room temperature and CFU were counted 4 days later.

Rain collection for treatment of laboratory-grown tomato plants. Rain was collected as previously described (Failor et al. 2017). In short, autoclavable bags were wrapped in aluminum foil and autoclaved for 40 min/fast cycle. Plastic trash cans (52 cm wide and 1 m tall) were arranged away from structures on the roof of the Latham Hall research building. Surfaces of containers were sprayed with 75% ethanol to prevent contamination. Sterile bags were placed in the cans and the lid placed back on top until the beginning of a rainfall event, at which point they were removed. The lids of three cans were then removed but one can was kept closed during the precipitation event as a negative control. After the end of precipitation events, 1 liter of sterile water was poured into the negative-control

can, simulating the precipitation event. After rainfall events ended, bags containing rain water were closed by making a knot and removed and placed at 4°C until processing.

For DNA extraction, 3 liters of rainwater was vacuum filtrated (reusable filter holders from Thermo Scientific Nalgene) through a 0.2-µm-pore filter membrane (Supor 200 PES membrane Disc Filter; PALL). Filters were removed using sterile tweezers, placed into a 15-ml Eppendorf tube, and stored at −80°C until processing. DNA extraction from the 0.22-µm filter membranes was performed using the Power Water DNA isolation kit (Qiagen) according to the manufacturer's protocol, with the following minor modifications. After adding solution PW1, tubes were incubated in a water bath at 65°C for 20 min. DNA was eluted using 30 µl of nuclease-free sterile water. Samples were incubated for 20 min prior to the elution spin. The elution step was performed twice. DNA concentration and quality were assessed by UV spectrophotometry (NanoDrop 1000; Thermo Scientific) and visualized on a 1 % agarose gel.

Treatment of laboratory-grown tomato plants with concentrated rain microbiota, filter-sterilized rain, or sterile water for determination of the taxonomic composition of phyllosphere microbiota. Tomato plants were grown in the laboratory for 4 weeks as described above for determination of the population size of phyllosphere microbiota. Two liters of rainwater were vacuum filtered as described under rain collection. To concentrate the bacterial microbiota present in rain 100-fold, the filter membranes were incubated for 10 min at room temperature in 20 ml of sterile water while stirring using a magnetic stirrer at 100 rounds/min. The bacterial suspension thus obtained was then used as inoculum (referred to as concentrated rain microbiota; hereafter, 100×-rain). The rain that passed through the filter was used as bacterial-free inoculum (referred to as filtered rain or filter-sterilized rain). Autoclaved double-distilled water was used as the sterile water treatment. Groups of four plants placed together into 33-by-41-cm plastic bags were sprayed until run off (approximately 10 ml). The bags were left open for 2 h to let the plants dry before the day-0 time point DNA extraction from two of the four tomato plants. Bags were then closed for 2 days to create a high-humidity environment favorable to plant colonization, after which they were kept open for 5 days until the day-7 time point DNA extraction from the remaining two tomato plants.

For DNA extraction, all leaves (approximately 5 g) were removed using gloves (sprayed with 70% ethanol before handling samples) and collected in a Ziploc plastic bag. Sterile distilled water was added (300 ml) and samples were sonicated for 10 min using a 1510 BRANSON sonicator (Brandsonic, Mexico) (Ottesen et al. 2013). The leaf wash was vacuum filtered onto the same kind of 0.22-µm-pore filter used for extraction of DNA from rain, and DNA extraction from membranes was performed as described for rain above.

Growth of tomato plants inside a commercial greenhouse not exposed to rain and outdoors naturally exposed to rain and leaf collection. Tomato plants (undisclosed varieties) were grown in a 8-m-high glass-covered commercial greenhouse in Virginia. Plant density was 10,000 plants/acre. Plants were grown from certified seed either hydroponically in hydroponic substratum made of washed compressed coconut husks (RIOCOCO/Ceyhinz Link) or in native soil under certified organic conditions. For both growing regimens, plants were kept in a vertical disposition without any exposure to either precipitation or irrigation water. Water and nutrients were recirculated and UV sterilized before use. Plants grown under the hydroponic regimen were located 1 m from the ground whereas plants grown under the organic regimen were grown in soil on the ground. Plants were 5 to 6 months old at the time of leaf collection. Collection of leaves was carried out during fall 2015 and spring 2016. Leaves (10 g/sample) were collected at least 1.5 m from the ground. Gloves sprayed with 70% ethanol were used and leaves were kept on ice in Ziploc plastic bags while being transported to the laboratory for DNA extraction.

Tomato plants were grown outdoors on the roof of the Latham Hall research building during July 2016 and naturally exposed to rain as described for the population size determination experiment above. DNA extraction was performed as described above for laboratory-grown tomato plants.

Library preparation and sequencing for all lab-grown, greenhouse-grown, and outdoor-grown tomato plants. For 16S rRNA amplicon sequencing, we used the barcoded primers a799wF (5′AMCVGGATTAGATACCCBG3′) and new1193R (5′ACGTCATCCCCACCTTCC3′). A 28-cycle PCR was performed using the HotStarTaq Plus Master Mix Kit (Qiagen) under the following conditions: 94°C for 3 min; followed by 28 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min; after which a final elongation step at 72°C for 5 min was performed. After amplification, PCR products obtained from the various samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, Beverly, MA, U.S.A.). All steps from PCR to paired-end (2 × 300 bp) amplicon sequencing on the Illumina MiSeq platform were performed at Molecular Research LP (MR DNA, Shallowater, TX, U.S.A.).

For metagenomic sequencing using the Illumina platform, total DNA was sequenced using 150-bp paired-end reads on the HiSeq 4000 Illumina platform, Duke University Sequencing and Genomic Technologies Shared Resource, Durham, NC, U.S.A. For metagenomic sequencing using the Nanopore platform, DNA libraries were prepared following the 1D native barcoding genomic DNA protocols (SQK-LSK109 and EXP-NBD104) provided by Oxford Nanopore Technologies (ONT).

Bioinformatic analysis. Raw 16S rRNA paired-end sequences were processed by Molecular Research LP (MR DNA) as follows: (i) reads were joined together after q25 trimming of the ends and reoriented in the 5′–3′ direction, (ii) barcodes and primer sequences were removed, and (iii) sequences shorter than 200 bp, sequences with ambiguous base calls, and sequences with homopolymer runs exceeding 6 bp were removed. Operational taxonomic units (OTUs) were assigned using the open-source Quantitative Insights into Microbial Ecology (QIIME) version 1.9.1 bioinformatic pipeline (Caporaso et al. 2010), using the open-reference protocol at 97% sequence identity, with UCLUST as the clustering tool and SILVA release 128 (Quast et al. 2013) as the database. OTU clustering was used instead of amplicon sequencing variant (ASV) clustering because ASV clustering was not available when sequencing data were first obtained and to make data comparable with earlier research on tomato phyllosphere microbiota. All OTUs annotated as mitochondria, chloroplasts, cyanobacteria, and unassigned, and OTUs with fewer than five reads, were removed from the dataset.

The QIIME-generated output file in the Biological Observation Matrix format was used for downstream data analysis and visualization in R version 3.3 using the Vegan (Oksanen et al. 2020), Phyloseq 1.26.1, and ggplot2 3.3.2 packages (McMurdie and Holmes 2013; Wickham et al. 2009).

Samples were rarefied to the lowest sample depth to compute diversity analysis. The rain core microbiome analysis was performed with the microbiome R package (Lahti and Shetty 2019) using a detection threshold of 0.1% and prevalence threshold of 100%. α Diversity was assessed using observed OTUs, Shannon, and Simpson indices. Differences in α diversity were determined by pairwise Wilcoxon rank sum test with the Holm correction method. β Diversity was analyzed based on unweighted UniFrac distance, weighted UniFrac distance, and Bray-Curtis dissimilarity. Differences in β diversity were determined using permutational multivariate analysis of variance (PERMANOVA) as implemented in adonis2 (from vegan 2.5-6 using

models with 999 permutations, adonis2[dist.matrix = approximately treatment × time point + date experiment]). Dissimilarity matrices were visualized using the principal coordinates analysis (PCoA) ordination method as implemented in the Phyloseq package. DESeq2 (Love et al. 2014) was used to identify OTUs that were differentially abundant across treatment groups and time points. OTUs were filtered using a false discovery rate cutoff of 0.01.

For metagenomic data analysis, raw 150-bp paired-end reads from Illumina were processed to remove short and low-quality reads using Trimmomatic version 0.38 (Bolger et al. 2014). Reads with an average per-base quality <30 and read length <150 bp were filtered out. Filtered reads were then classified taxonomically using Centrifuge version 1.0.4 (Kim et al. 2016) and Sourmash version 2.0.0 (Brown and Irber 2016), only retaining species that were identified by both classifiers.

RESULTS

Bacterial population size on tomato plants exposed to rain is larger than that of tomato plants not exposed to rain. Our investigation into the role of rain in shaping the phyllosphere microbiome started by comparing the bacterial population size on tomato plants grown indoors under controlled conditions with that of plants grown outside exposed to environmental disturbances, including rainfall. We observed that tomato plants grown over 4 weeks outdoors in plastic pots at the Virginia Tech Kentland research farm harbored bacterial populations of significantly larger size compared with plants grown under laboratory conditions for the same period (Fig. 1A). Interestingly, even plants grown on the roof of a campus research building, which minimized microbial dispersal from soil, and plants compared with the farm environment had bacterial populations that were significantly larger compared with those of plants grown indoors (Fig. 1B). These results suggest that airborne and rainborne bacteria through dry or wet deposition had colonized the tomato plants grown outside.

To test the effect of rain on the bacterial population size in the tomato phyllosphere under controlled conditions, we collected rain and used it as an inoculum to treat 4-week-old tomato plants that had been grown under laboratory conditions. Seven days after plants were treated with rain, they carried a significantly larger bacterial population count compared with plants that had been treated with autoclaved rain (Fig. 1C). This result suggested that at least some rainborne bacteria are able to colonize plant leaves efficiently and, thus, may affect bacterial population composition in the phyllosphere.

Rainborne microbiota in Blacksburg, VA, are highly variable. As a first step toward identifying which bacterial taxa present in rainfall may efficiently colonize the tomato phyllosphere, we characterized the bacterial diversity associated with nine rainfall events during 2015 and 2016. Rainfall was collected on the same roof of the research building previously used to grow tomato, DNA was extracted, and the 16S rRNA gene was amplified and sequenced (Table 1). In total, 1,186,365 short reads were obtained. After 97% OTU clustering and removal of all nonbacterial and unassigned reads, a total of 892,142 reads remained. All samples were rarefied to 6,419 reads/sample and 5,958 OTUs overall were identified. Rarefaction curves (Supplementary Fig. S1) show that this is an underestimate of the total number of OTUs because not all samples were sequenced to saturation. The number of OTUs per rarefied sample ranged from 541 (June 2019) to 1,782 (April 2016).

Taxonomic diversity analysis revealed *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Acidobacteria*, and *Firmicutes* to be the dominant

TABLE 1
Metadata for analyzed rain samples

Rain collection ID	Month	Year	Number of 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

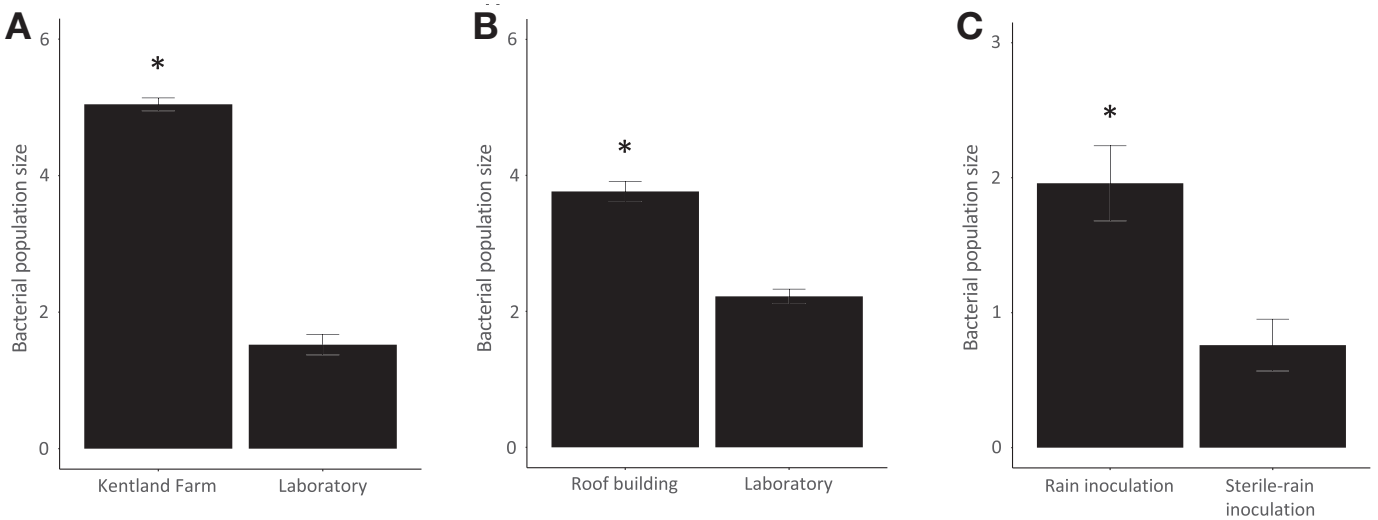

Fig. 1. Bacterial population size in the phyllosphere of tomato plants **A,** grown exposed to rain in plastic pots at the Virginia Tech Kentland Farm compared with plants grown inside the laboratory; **B,** grown on the roof of the Latham Hall research building exposed to rain compared with plants grown under laboratory conditions; and **C,** grown inside the laboratory 7 days after being treated with either rain or autoclaved rain; *t* test, $P < 0.001$.

taxa at the phylum level. At the class level, *Alphaproteobacteria* followed by *Gammaproteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Bacilli* were most abundant. However, there were considerable differences among samples. For example, *Chlamydiae* represented the most abundant taxon in the rain sample collected in August 2015 at 36% relative abundance, represented only 0.4% in the sample collected in July 2016, and were <0.1% in all other samples. *Deltaproteobacteria* were only present in August 2015, May 2016, and July 2016 at 12.79, 1.42, and 17.03% relative abundance, respectively. *Actinobacteria* were most abundant in rain samples collected in April 2016 (25.56%) and December 2016 (16.29%) whereas they represented only 2.18% of the October 2016 sample. *Gammaproteobacteria* represented 80% of relative abundance in June 2019 but averaged only 28.41% in the other samples (Fig. 2A).

Even more pronounced differences in relative abundance between samples were observed at the genus level (Fig. 2B). However, 17 OTUs were identified across all nine samples when setting a detection threshold of 0.1%. These OTUs belong to the following genera (listed in order of decreasing relative abundance): *Acidiphilium*, *Bryocella*, *Beijerinckiaceae* 1174-901-12, *Methylobacterium, Massilia, Burkholderiaceae* DQ787673.1.1527, *Pantoea*, *Pseudomonas*, and *Sphingomonas* (Supplementary Fig. S2).

Concentrated rain microbiota, filter-sterilized rain, and sterile water all affect the phyllosphere of lab-grown tomato plants. Next, we decided to determine whether inoculation with rain microbiota would not only increase the size of the tomato phyllosphere microbiota as seen in Figure 1 but also change its composition because of colonization of tomato leaves by rainborne bacteria. In six independent experiments, 100-fold concentrated rain microbiota (100×-rain) derived from six of the nine collected rainfall events described above (April 2015, August 2015, March 2016, April 2016, May 2016, and July 2016) were used to inoculate tomato plants (one set of four tomato plants each time). In parallel, a separate set of four tomato plants was treated each time with filter-sterilized rain obtained from the concentration step above. For each of the 2016 experiments, a set of four additional tomato plants was treated with sterile water. 16S rRNA amplicons were prepared and sequenced from DNA extracted from leaf washes of tomato plants 2 h after treatments (day 0) and 7 days later (day 7). For the March 2016 and May 2016 experiments, enough rain and enough plants were available so that each treatment was done in duplicate. For the April 2016 experiment, two day-7 100×-rain samples were taken. For the other three experiments, only one sample per treatment and time point was processed.

In total, 3,291,016 reads were obtained from 45 phyllosphere samples (Supplementary Table S1). After 97% OTU clustering and removing all nonbacterial and unassigned reads, a total of 3,118, 320 reads remained in the data set. Rarefaction curves show that most of the samples were deeply sequenced (Supplementary Fig. S1).

After subsampling to 6,670 reads per sample, we identified a total of 9,923 OTUs and measured the α diversity based on the total number of observed species and by Shannon and Simpson diversity indices. The α diversity values for rain compared with treated plants at day 0 and day 7 are shown in Figure 3. Although α diversity of rain microbiota was highly variable, the number of observed OTUs in rain was significantly higher than day-7 samples for all three treatments (*P* values of 0.020, 0.050, and 0.028, respectively, as determined by the pairwise Wilcoxon rank sum test with the Holm correction method) (Supplementary Table S2). Also, the number of observed OTUs significantly decreased in plants treated with 100×-rain from day 0 to day 7 (*P* value 0.027). Comparing Shannon's index, we observed a depletion in richness from day 0 to day 7 for plants treated with 100×-rain (*P* value 0.049) as well as for plants treated with filtered-rain (*P* value 0.023). No significant differences in α diversity were observed between day 0 and day 7 for plants treated with sterile-water.

PCoA derived from weighted and unweighted UniFrac distance metrics and from Bray-Curtis dissimilarity (Fig. 4) revealed that most rain samples clustered together while phyllosphere samples did not. Whereas PCoA derived from weighted UniFrac distances only separated phyllosphere samples along the second coordinate, PCoA derived from unweighted UniFrac distances and Bray-Curtis dissimilarity separated phyllosphere samples along both axes. Therefore, bacterial communities on lab-grown tomato plants were even more dissimilar from each other than the dissimilarity between rain communities. A PERMANOVA for Bray-Curtis dissimilarity revealed that date of experiment, day of sampling (day 0 versus day 7), and treatment (100×-rain, filter-sterilized rain, and sterile water) were all significantly associated with bacterial community composition. (Supplementary Table S3). In regard to the date of experiment, it can be seen (Fig. 4B and C) how samples clearly cluster by date of experiment along the x-axis. Because this clustering is independent of treatment and day of sampling, the starting tomato phyllosphere population appears to have been different between experiment dates. Second, day-7 samples differed from day-0 samples on the y-axis independently of which treatment was applied, revealing that inoculation and incubation under high humidity of plants by itself shifted the composition of the tomato phyllosphere population. Third, although treatment was significant, there was interaction between treatment and date of experiment. Therefore, the effect of 100×-rain was not significantly different compared with the effect of filter-sterilized rain or sterile water. In other words, β diversity analysis was unable to reveal whether rainborne bacteria present in the 100×-rain treatments affected the tomato phyllosphere community, possibly because of the differences in the composition of the microbial rain and phyllosphere communities between experiments.

After finding that β diversity analysis was inconclusive, we decided to compare the actual taxonomic diversity between samples. We observed an enrichment in *Proteobacteria* in the tomato phyllosphere on day 7 compared with day 0 regardless of treatment. In contrast, relative abundance of *Actinobacteria* and *Firmicutes* was dramatically reduced 7 days posttreatment in all plants treated with 100×-rain but not after treatment with filter-sterilized rain or sterile water (Fig. 5). At the class level, *Gammaproteobacteria* were significantly enriched in all tomato phyllosphere day-7 samples. *Actinobacteria* and *Bacilli* were reduced on day 7 in plants treated with 100×-rain compared with filter-sterilized rain or sterile water. Unexpectedly, no consistent increase of any taxon at either phylum, class, or genus level was observed from day 0 to day 7 for plants treated with 100×-rain alone.

In all, 104 rainborne OTUs increased significantly in relative abundance in the tomato phyllosphere exclusively after treatment with concentrated rain microbiota. Because we did not find any taxon between genus and phylum level that exclusively increased from day 0 to day 7 on tomato plants treated with 100×-rain, we wanted to determine whether we could find any individual rainborne OTUs that did so. To do this, we used DESeq2 (Love et al. 2014) because it is relatively robust to small and unequal sample sizes, as in the present study.

First, we directly compared day-0 and day-7 phyllosphere microbiota treated with 100×-rain and found that 104 OTUs (out of a total of 7,994 OTUs) significantly increased (Supplementary Table S4). These OTUs belonged to the genera *Massilia* (27 OTUs), *Pantoea* (18 OTUs), *Duganella* (13 OTUs), *Pseudomonas* (11 OTUs), *Enterobacter* (5 OTUs), *Flavobacterium* (3 OTUs), *Janthinobacterium* (2 OTUs), and *Curtobacterium* (1 OTUs). In addition, 16 and 5 unknown OTUs from the families *Burkholderiaceae* and *Enterobacteriaceae*, respectively, significantly increased in relative abundance as well

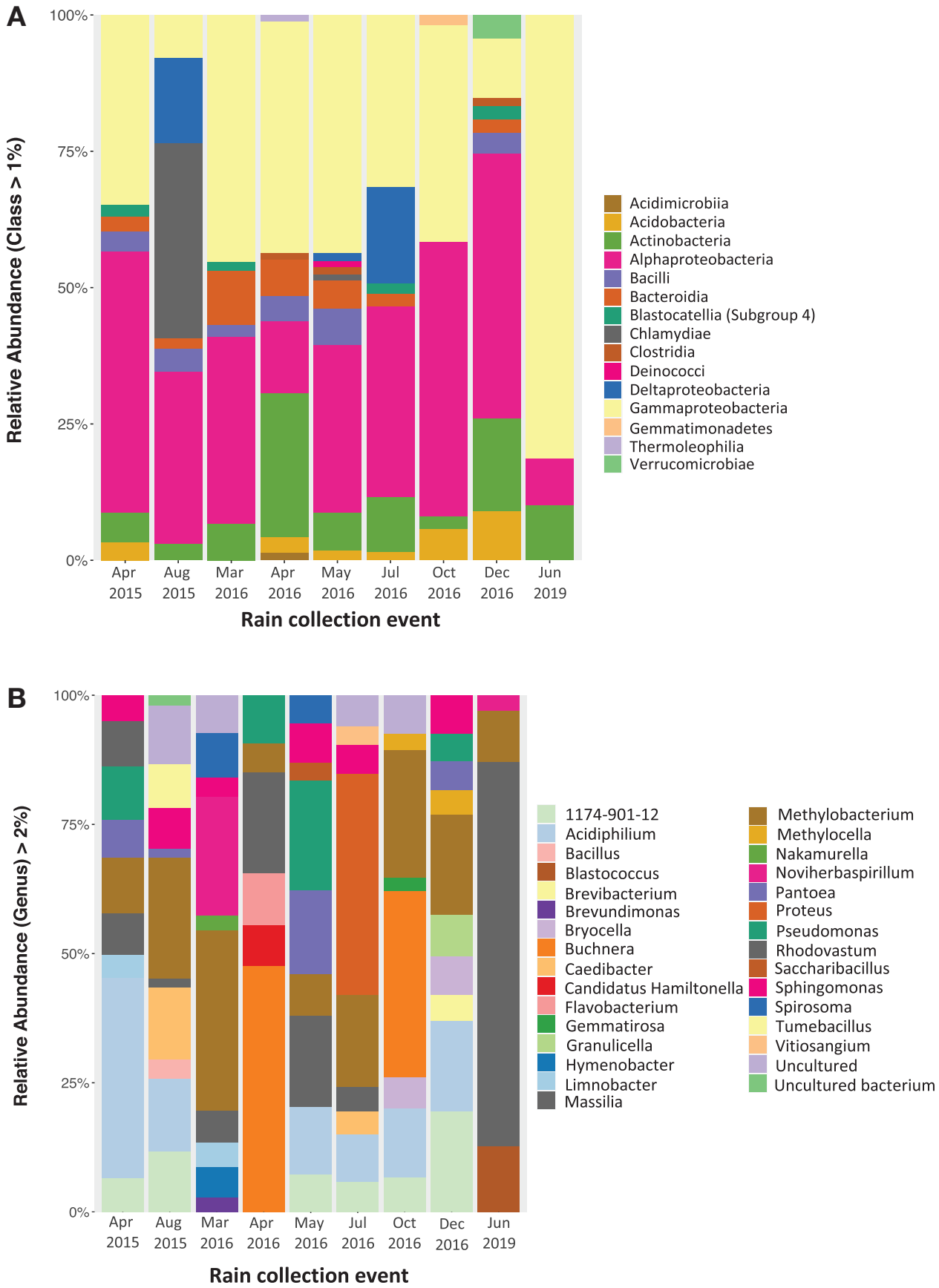

Fig. 2. Relative abundance (RA) of bacterial taxa in rainfall collected in Blacksburg, VA, on nine different days in 2015, 2016, and 2019. Samples were rarefied to 6,419 sequences. **A,** RA at the class level (only classes with RA > 1% are shown) and **B,** RA at the genus level (only generated with RA > 2% are shown). For August 2015, results are based on two technical replicates.

(Fig. 6A). Importantly, not a single OTU significantly differed in abundance between day 0 and day 7 on tomato plants treated with either filtered-rain or double-distilled water. This suggests that the OTUs which increased in relative abundance from day 0 to day 7 on tomato after treatment with 100×-rain originated from rain and were not members of the bacterial community present in the tomato phyllosphere prior to inoculation.

We then complemented the previous comparison with a slightly different analysis comparing the bacterial composition of rain microbiota with phyllosphere microbiota 7 days after treatment with 100×-rain. We observed that 35 rainborne OTUs out of a total of 5,958 OTUs had a significantly higher relative abundance on tomato at day 7 compared with their relative abundance in rain (Fig. 6B). These OTUs (in order of decreasing differential abundance) mostly belonged to the same genera as the genera identified in the day-0 to day-7 comparison: *Massilia, Pseudomonas*, *Pandoraea*, *Streptomyces*, and *Pantoea*. The genus *Pantoea* was the genus that most consistently increased in relative abundance. In fact, *Pantoea* was detected in all rain collections (Fig. 7A) and reached a high relative abundance (between 4 and 44%) in the tomato phyllosphere after 7 days each time its abundance in rain was >1% (observed four of six times). Genus-level abundance in rain and phyllosphere samples was also shown for *Flavobacterium, Janthinobacterium*, *Pseudomonas, Methylobacterium*, and *Massilia*, which all successfully colonized the tomato phyllosphere each time they were detected in rain (Fig. 7B to E).

In contrast, 61 OTUs found in rain samples significantly decreased in relative abundance by day 7, suggesting that these rainborne taxa were definitely not able to colonize tomato leaves. These OTUs belonged to the following genera (list of the first 10 bacterial genera in order of decreasing differential abundance): *Acidiphilium*, *Beijerinckiaceae*-1174-901-12, *Bryocella, Actinomycetospora, Methylobacterium, Methylocella*, *Granulicella, Belnapia, Modestobacter*, and *Blastococcus.* The genus *Acidiphilium* best exemplifies this group of taxa. It was detected at high relative abundance in most rain samples, it was observed on plants treated with 100×-rain on day 0, but it was never found on plants treated with 100×-rain on day 7 (Fig. 7G). This clearly shows that *Acidiphilium* is a common rainborne bacterial genus that does not include any members able to colonize the tomato phyllosphere.

Several other pairwise comparisons were made to gain additional insights into differences in composition between microbiota at the OTU level. For example, we determined which OTUs were present in significantly higher abundance in rain compared with tomato treated with sterile water on day 0 to identify OTUs that are

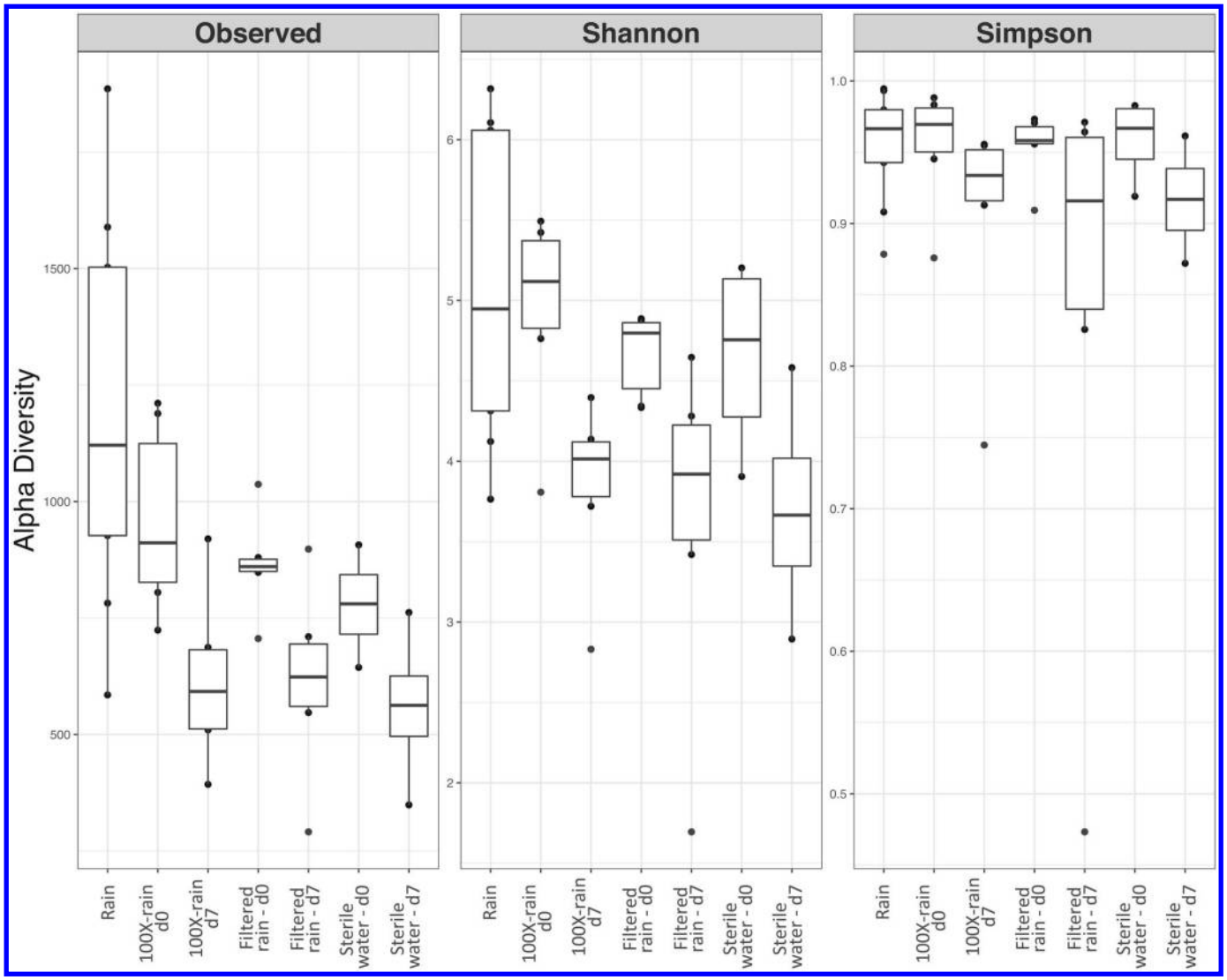

Fig. 3. α Diversity measurements for rain compared with treated plants at day 0 versus day 7. Three measures of α diversity (observed operation taxonomic units, Shannon diversity index, and Simpson diversity index) were used. For rain, only samples used for plant inoculations were included.

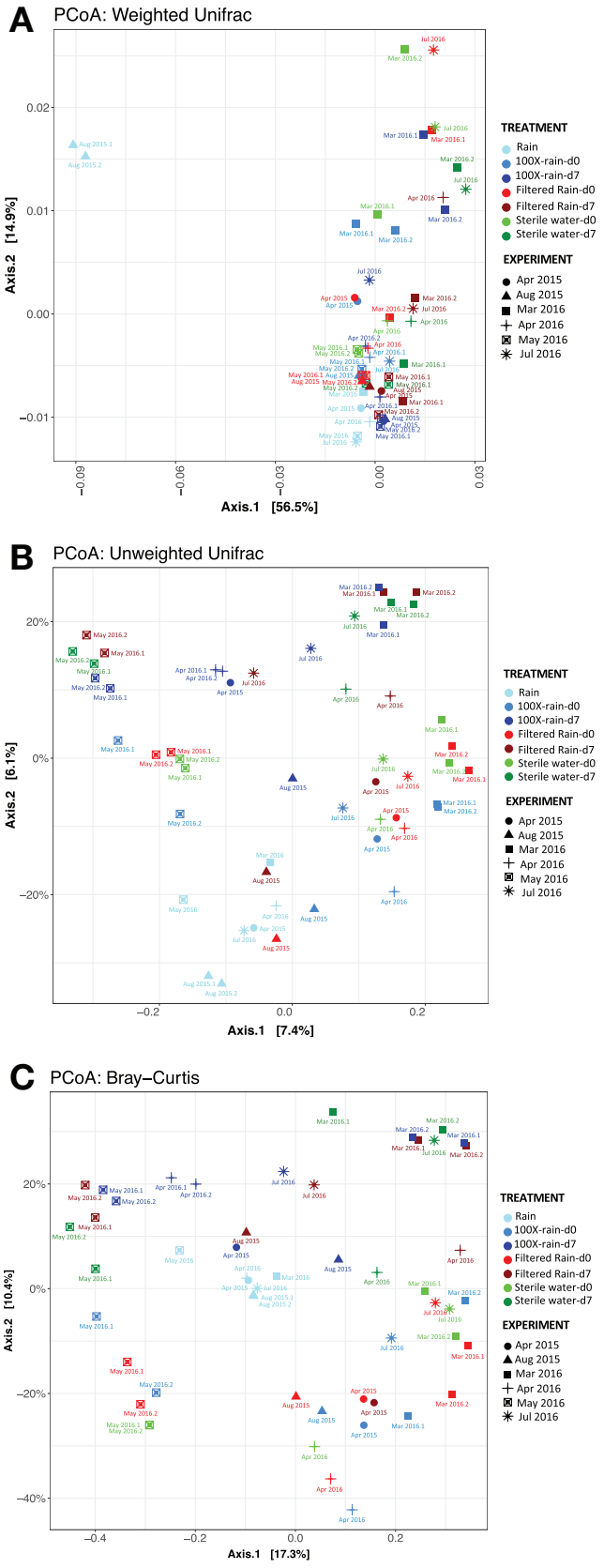

Fig. 4. Principal coordinates analysis (PCoA) derived from **A,** weighted UniFrac distances; **B,** unweighted UniFrac distances; and **C,** the dissimilarity matrix of Bray-Curtis. Only rain samples used for plant inoculation were included. For experiments for which replicates were available, the replicate samples are labeled as "month year.1" and "month year.2".

commonly present in rain but present in low abundance (or not at all) on laboratory-grown tomato plants (Supplementary Fig. 3A). In all, 174 such OTUs (out of a total of 5,958 OTUs present in rain) were identified. Most of these OTUs belonged to the following genera (list of the first 10 bacterial genera in order of decreasing differential abundance in rain): *Caedibacter*, *Tumebacillus*, *Acidiphilium*, *Methylobacterium, Rhodovastum, Belnapia, Sphingomonas, Methylocella*, *Bacillus*, and *Pantoea.*

On the other hand, 109 OTUs had significantly higher relative abundance in laboratory-grown tomato (tomato at day 0 after being treated with sterile water) than in rain (Supplementary Fig. S3A). Thus, these taxa are common inhabitants of tomato grown under our laboratory conditions in the absence of rain. They mostly belonged to the following genera (list of the first 10 bacterial genera in order of decreasing differential abundance): *Hyphomicrobium*, *Rhodanobacter*, *Chryseobacterium*, *Burkholderia*, *Paenibacillus*, *Nocardioides, Pandoraea*, *Bacillus*, *Novosphingobium*, and *Streptomyces*.

One genus that did not fall into any of the above categories was the genus *Bacillus*: *Bacillus* OTUs were observed at high relative abundance in rain samples as well as in the tomato phyllosphere at day 0 independent of treatment and on day 7 after sterile-rain and double-distilled water treatments but not after 100×-rain treatments (Fig. 7H). This suggests that *Bacillus* OTUs were present in rain as well as on lab-grown tomato but were outcompeted by other rainborne bacteria added with the 100×-rain treatments.

To more precisely identify the OTUs that represented the most efficient tomato colonizers, we used metagenome shotgun sequencing to resequence the microbiota associated with rain samples and with plants treated with 100×-rain on day 0 and day 7. The metagenome shotgun sequencing approach generated 260,035,170 short reads. After quality control, 7,543,305 reads remained, of which 98.26% were identified as bacterial reads. The results, listing the bacterial species present in rain that numerically increased in abundance from day 0 to day 7 and ranked from high to low based on their relative abundance on day 7, are summarized in Table 2. Based on this analysis, rainborne species *Pantoea vagans* and *P. agglomerans* were the most effective tomato phyllosphere colonizers, followed by *Pseudomonas citronellolis*, *Novosphingobium resinovorum*, an unnamed *Buttiauxella* sp., *Erwinia gerundensis*, *P. fluorescens*, *Cedecea neteri*, and an unnamed *Massilia* sp. Additional *Pantoea*, *Massilia*, *Pseudomonas*, *Janthinobacterium*, and *Enterobacter* spp. ranked highly as well. Thus, the metagenomic analysis mostly confirmed and refined our 16S rRNA results of which rainborne taxa are the most effective colonizers of the tomato phyllosphere.

There are consistent differences between phyllosphere microbiota of tomato plants never exposed to rain and naturally exposed to rain outdoors. After identifying bacterial taxa present in rain that efficiently colonized tomato leaves under laboratory conditions, we tested the hypothesis that these taxa would be abundant in tomato plants grown outdoors in pots containing autoclaved soil on the roof of the same research building where we had collected rain samples earlier (seven samples) but be missing, or at least be underrepresented, in phyllosphere microbiota of greenhouse-grown tomato that had never been exposed to rain. This second set of plants included tomato plants grown in a hydroponic system (29 samples) and tomato plants grown in soil (18 samples), both in a commercial greenhouse (Supplementary Table S5). In total, we obtained 4,166,519 reads. After 97% OTU clustering and removing all nonbacterial and unassigned reads, a total of 3,080,204 reads remained. Samples were rarefied to 2,546 reads per sample and 10,525 OTUs overall were identified. Taxonomic diversity analysis revealed *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* to be the dominant bacterial taxa (Fig. 8A), the same phyla identified on the lab-grown tomato. α

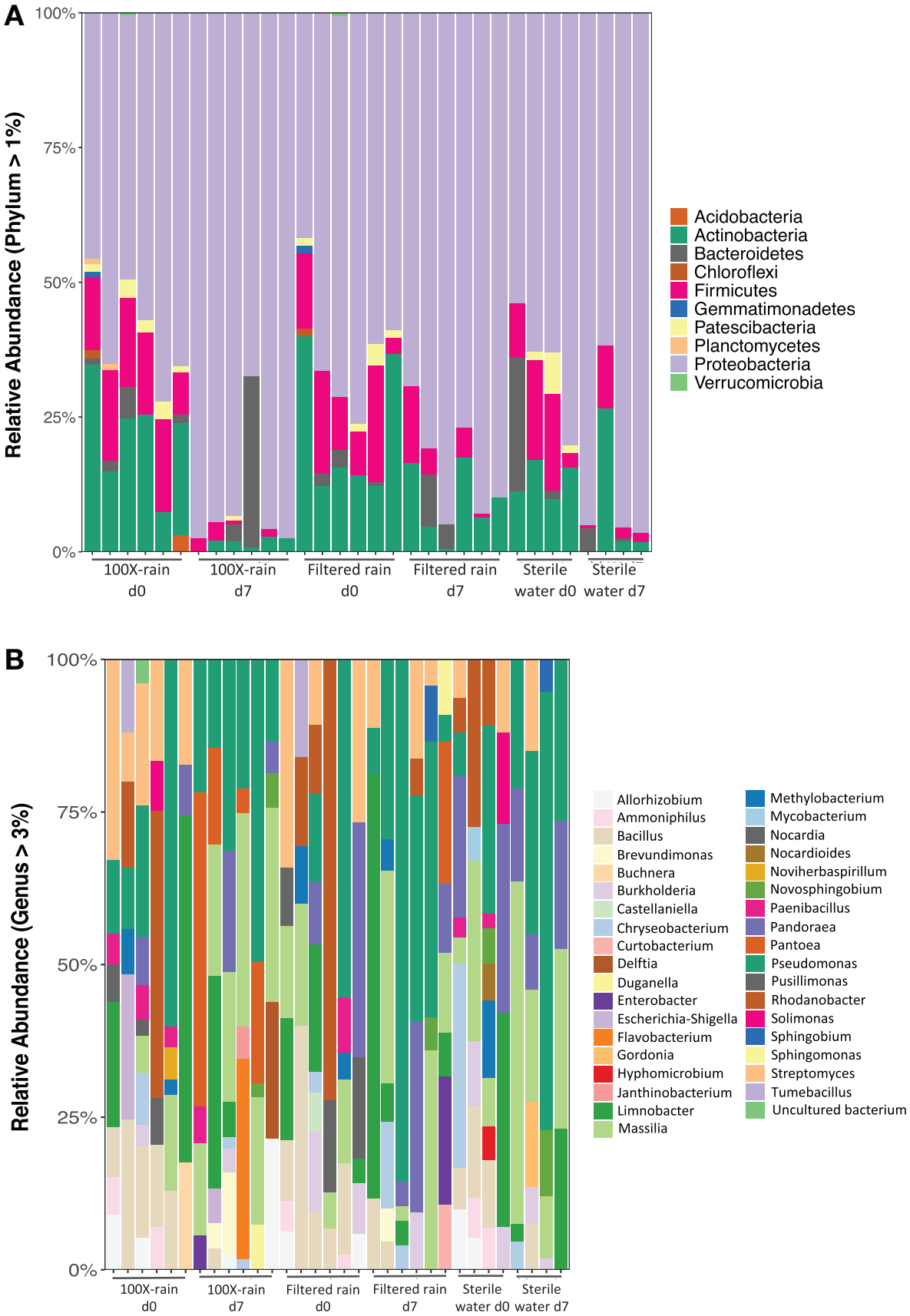

Fig. 5. Relative abundance (RA) of bacterial taxa of plants treated with either concentrated rain microbiota (100×-rain), filtered rain, or sterile water at day 0 and day 7. Experiments are listed by dates from left to right (April 2015, August 2015, March 2016, April 2016, May 2016, and July 2016). **A,** RA at the phylum level (abundance > 1%) and **B,** RA at the genus level (abundance > 3%). For experiments for which replicates were available, RA is based on all replicates.

Diversity analysis supported by the total number of observed species and by the Shannon and Simpson diversity indices (Fig. 8B), together with a pairwise comparisons using the Wilcoxon rank sum test, showed significant differences in the number of OTUs between the hydroponic and the soil system (Supplementary Table S6).

To identify any OTUs present at significantly higher relative abundance in tomato plants grown outside exposed to rain compared with the tomato plants grown in the greenhouse hydroponically in the absence of rain, DESeq2 was used again. In all, 40 OTUs from the genera *Pandoraea*, *Curtobacterium*, *Massilia*, *Gemmatirosa*, *Kineococcus*, *Methylobacterium*, *Sphingomonas*, *Buchnera*, *Alloiococcus*, *Pseudomonas*, and *Streptococcus* were found (Fig. 9A). Similarly, 58 OTUs from the genera *Pantoea*, *Pelomonas*, *Kineococcus*, *Methylobacterium*, *Massilia*, *Aureimonas*, *Buchnera*, *Alloiococcus*, *Pseudomonas*, *Streptococcus*, and *Sphingomonas* were of significantly higher relative abundance on the tomato plants grown outside exposed to rain compared with the tomato plants growing in soil never exposed to rain in the greenhouse (Fig. 9B). Note that, as we hypothesized, OTUs in the genera *Massilia*, *Curtobacterium*, *Pseudomonas*, and *Pantoea* were among the same genera as the OTUs identified to significantly increase in relative abundance in the phyllosphere of tomato plants treated with concentrated rain microbiota 7 days postinoculation. However, unexpectedly, the actual OTUs of these genera identified in this comparison were not the same as those identified in the controlled laboratory experiments (see Discussion section for possible explanations).

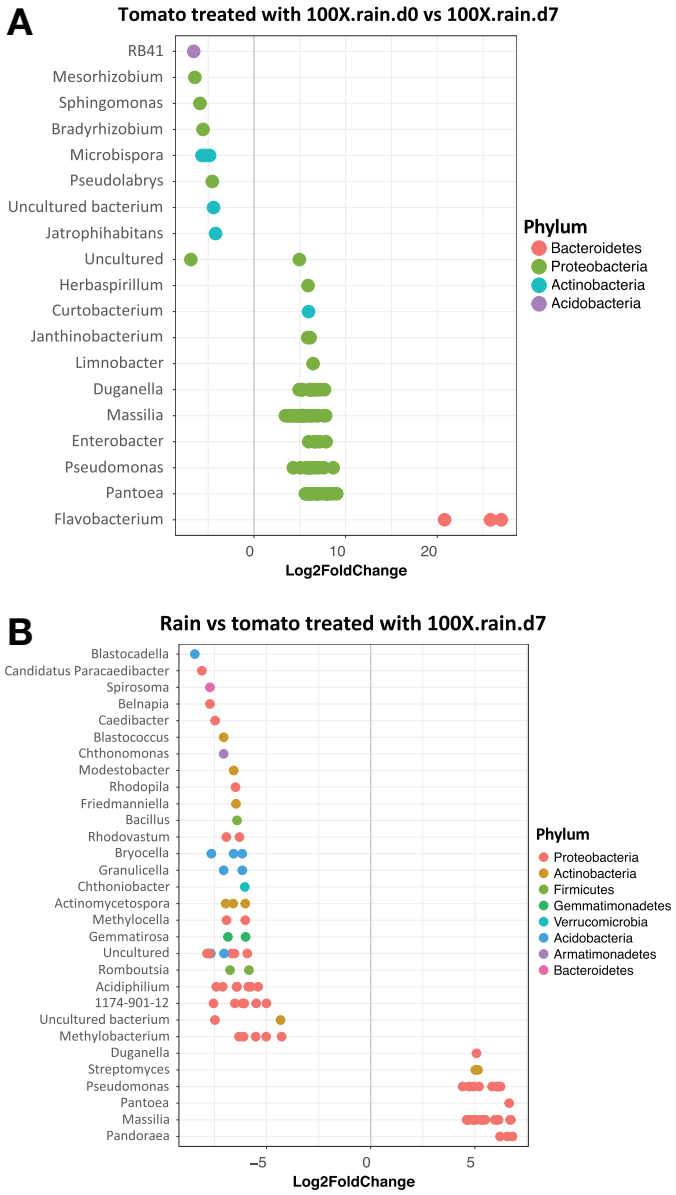

Fig. 6. Differential abundance analysis at the level of operational taxonomic unit (OTU) using DESeq2 (Love et al. 2014). The fold change is shown on the x-axis and genera are listed on the y-axis. Each colored dot represents a separate OTU. **A,** Comparison of phyllosphere microbiota of plants treated with concentrated rain microbiota (100×-rain) between day 0 and day 7 and **B,** comparison between rain microbiota and phyllosphere microbiota 7 days after treatment with the respective 100×-rain sample.

DISCUSSION

Although our general understanding of the plant microbiome has increased dramatically over the last few years, the basic question of where the bacteria that colonize and inhabit the phyllosphere originate from has remained unanswered. The main approach in trying to answer this question has been to make comparisons of the composition of the phyllosphere microbiome with the microbiomes of putative reservoirs (Grady et al. 2019; Maignien et al. 2014; Ottesen et al. 2016; Wagner et al. 2016; Zarraonaindia et al. 2015). To complement this approach, here, we used controlled laboratory experiments.

We decided to focus on rain because previous studies provided evidence that at least the bacterial leaf pathogen *P. syringae* may be disseminated by precipitation and efficiently colonize the plant phyllosphere (Monteil et al. 2016; Morris et al. 2008). Moreover, at least temporary shifts in the composition of phyllosphere communities after rain events were observed (Allard et al. 2020). In a first step, we found that tomato plants naturally exposed to rain outdoors, or simply sprayed with rainfall when grown in the lab, carried significantly higher bacterial populations sizes compared with lab-grown plants that had not been treated with rainfall or that were treated with sterilized rainfall. Although plants grown outside may have also been colonized by airborne bacteria through dry deposition or by soilborne bacteria after being splashed during rainfall events, the observation that treating lab-grown plants with rain caused the bacterial population size to increase more than spraying lab-grown plants with sterilized rain was a strong indication that the observed increase in population size was due to the colonization and growth of rainborne bacteria.

To dig deeper into the possibility that rain harbors bacteria that can effectively colonize and grow on tomato leaves, we decided to characterize the taxonomic composition of rainfall events and then determine whether any of the identified members of the rain microbiota would increase in relative abundance on tomato plants treated with concentrated rain microbiota (100×-rain) but not on tomato plants treated with filter-sterilized rain or sterile water. We decided on using concentrated rain microbiota instead of rain because we had previously found that rain contained as few as 4×10^3 CFU liter^{-1} (Failor et al. 2017) and the maximum volume that we can spray on a tomato plant before water runs off the leaves is only 10 ml. Therefore, as few as 40 bacteria may get inoculated on an entire plant using rain as inoculum in a lab experiment, which we deemed not to be enough to lead to a bacterial population size that could be analyzed using 16S rRNA amplicon sequencing. Thus, we used 100×-rain concentrate, acknowledging that we artificially aided rainborne bacteria in our experiment.

The most important observation from the characterization of the rainfall microbiota was that each collected rainfall event harbored a very different bacterial community. This is in line with other recent results on rainfall collected in the United States, Europe, and Asia (Aho et al. 2020; Cáliz et al. 2018; Woo and Yamamoto 2020) that

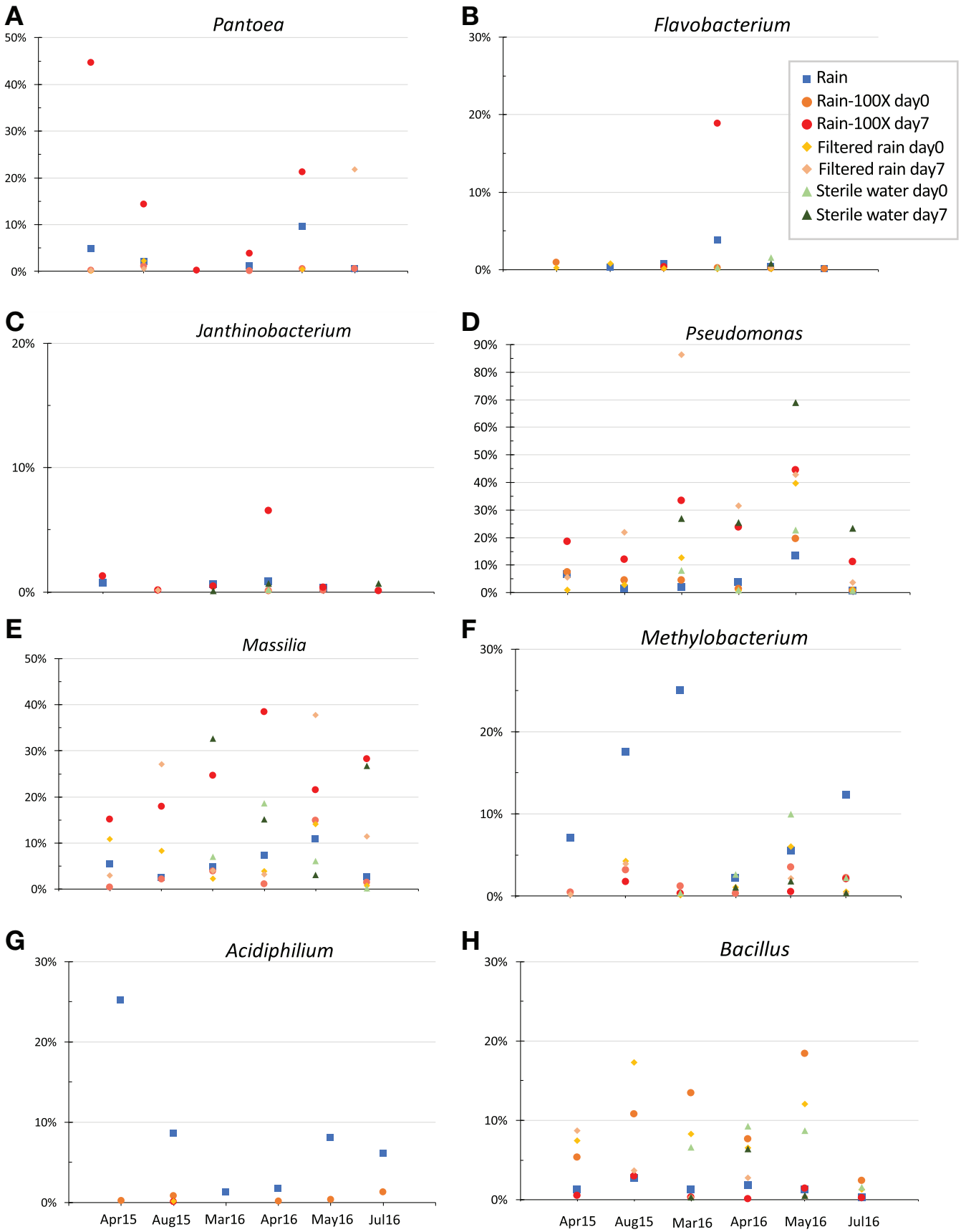

Fig. 7. Relative abundance of a representative selection of rainborne genera that either failed or succeeded in colonizing the tomato phyllosphere. Dates of experiments are listed on the x-axis of panels G and H. Relative abundance is shown on the y-axis for rain, tomato plants on day 0 and day 7 after being treated with concentrated rain microbiota (100×-rain), filtered rain, or sterile water. **A,** *Pantoea*; **B,** *Flavobacterium*; **C,** *Janthinobacterium*; **D,** *Pseudomonas*; **E,** *Massilia*; **F,** *Methylobacterium*; **G,** *Acidiphilium*; and **H,** *Bacillus*.

showed that the taxonomic composition of microbiota in rain changes with origin of air masses and season. For our experiment, this meant that we could not expect to find the same taxa to colonize and grow on tomato in each inoculation experiment.

It was also important to use appropriate controls when inoculating lab-grown tomato plants with 100×-rain. Importantly, tomato plants were not grown in sterile conditions. Therefore, they already carried microbiota at the time of inoculation and simply spraying these plants with water and incubating them at high humidity (as we did to favor plant colonization) could be expected to lead to changes in relative abundance of the preexisting microbiota. Moreover, rain contains nutrients (Salve et al. 2008) and may contain bacteriophages that are not removed by filtering and that will also affect preexisting microbiota. Therefore, we used both filter-sterilized rain (including

TABLE 2
Species with highest relative abundance on tomato plants on day 7 after treatment with concentrated rain microbiota (100×-rain) based on metagenomic sequencing

Species	Pos[a]	Average abundance[b]			Highest[c]
		In rain	On day 0	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

[a] Number of positive day-7 samples out of five sample.
[b] Only considering experiments for which the species was present on day 7.
[c] Highest abundance on day 7.

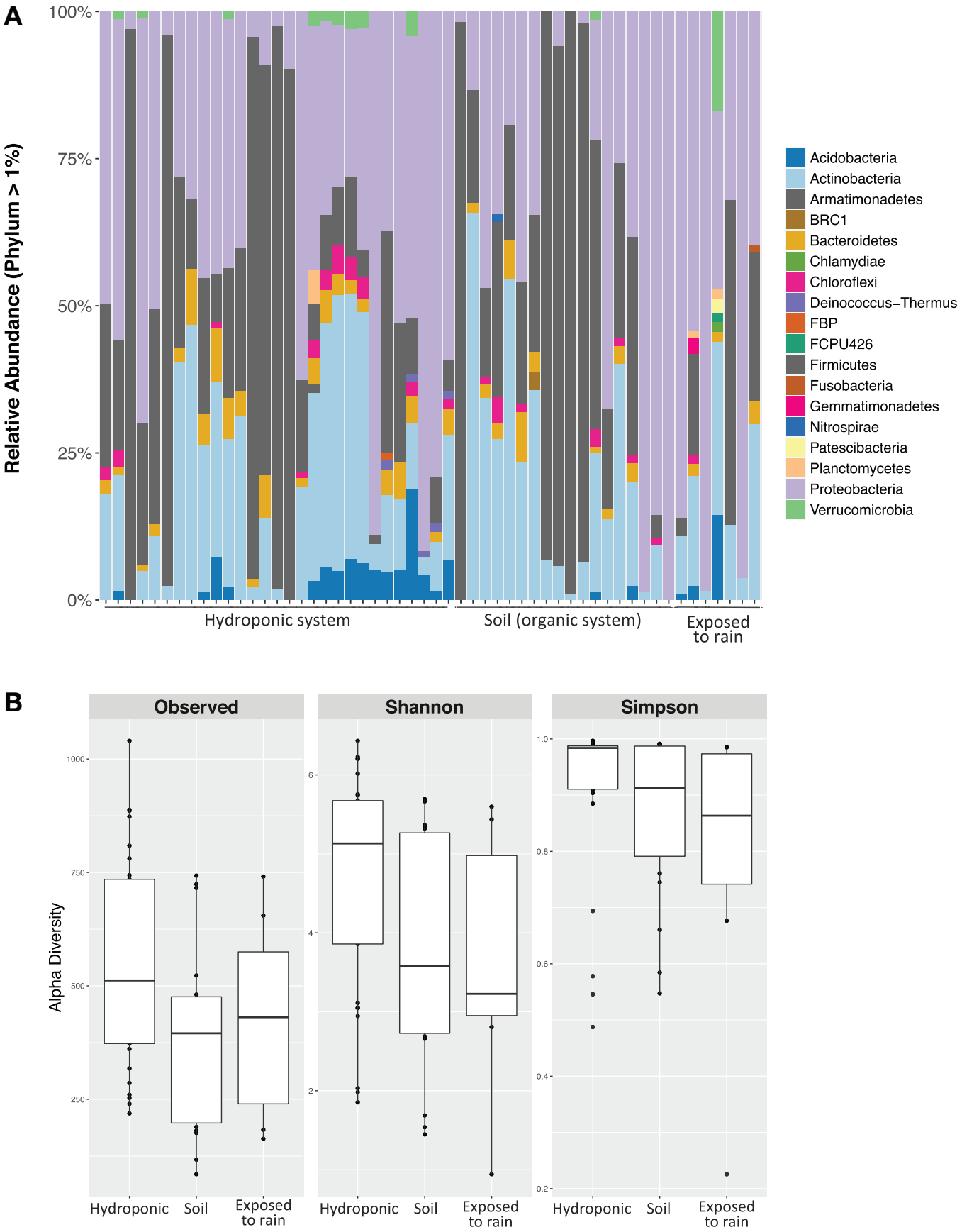

Fig. 8. Taxonomic composition and α diversity of phyllosphere microbiota of tomato plants grown hydroponically or in soil (both in a commercial greenhouse) and of tomato plants grown outside (on the roof of the Latham Hall research building). **A,** Relative abundance (RA) at the phylum level (only phyla with RA > 1% are shown) and **B,** α diversity (observed OTUs, Shannon diversity index, and Simpson diversity index).

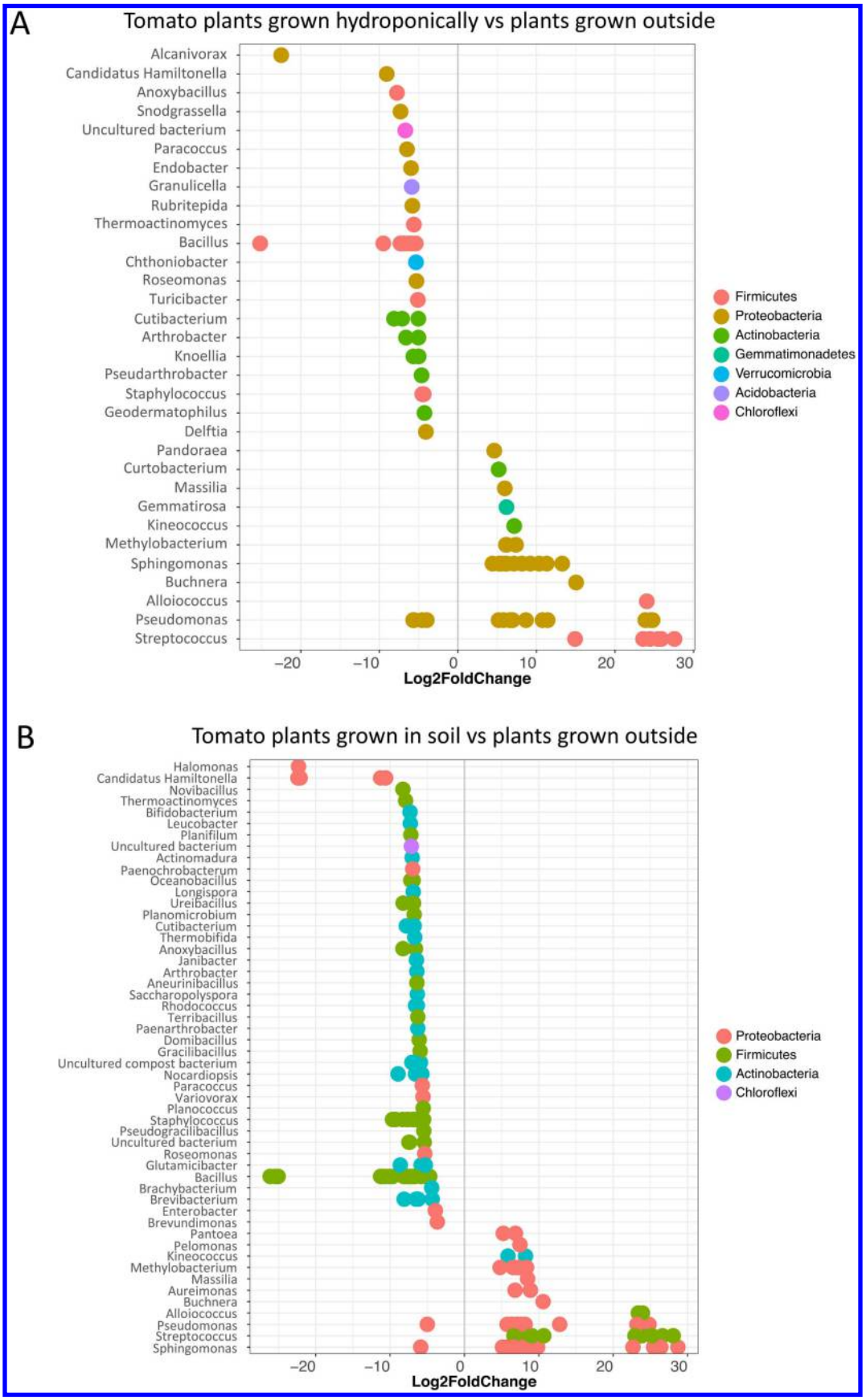

Fig. 9. Differential abundance analysis at the level of operational taxonomic unit (OTU) using DESeq2 (Love et al. 2014). The fold change is shown on the x-axis and genera are listed on the y-axis. Each colored dot represents a separate OTU. **A,** Plants grown hydroponically in a greenhouse compared with plants grown on the roof of the Latham Hall research building and **B,** plants grown in soil in a greenhouse compared with plants grown on the roof of the Latham Hall research building.

nutrients and possibly bacteriophages but no bacteria) and autoclaved double-distilled water (expected to contain neither nutrients nor bacteriophages nor bacteria) as controls.

Comparison of rain microbiota with phyllosphere microbiota on day 0 and day 7 for the three different treatments yielded some results confirming our hypothesis that rain contains effective colonizers of tomato leaves, whereas other results were ambiguous. First, the observed drop in α diversity for phyllosphere microbiota from day 0 to day 7 after treatment with 100×-rain is in agreement with the expected increase in relative abundance of some effective tomato leaf colonizers accompanied by a decrease in relative abundance of many rainborne bacteria that are not adapted to tomato leaves and that, thus, are not effective tomato colonizers. The observed decrease in α diversity from day 0 to day 7 for the sterile rain treatment may have been due to growth of some preexisting tomato phyllosphere members that thrived under the high humidity conditions applied for 2 days after inoculation and the nutrients added with the rain water, which helped them outcompete other members of the preexisting microbiota. It is also possible that bacteriophages present in the filter-sterilized rain reduced the relative abundance of some phyllosphere members to below the detection limit because it has been shown that bacteriophages strongly influence community composition of tomato phyllosphere microbiota (Morella et al. 2018).

In regard to β diversity, we had expected the composition of the day 0 microbiota to be similar to each other because tomato plants of the same cultivar were grown in autoclaved soil in relatively stable laboratory conditions. Therefore, it was surprising to find phyllosphere samples to differ more from each other (even on day 0 after sterile water treatments) compared with the differences among rain samples. This was the case for weighted UniFrac distances, unweighted UniFrac distances, and the Bray-Curtis dissimilarity static. A possible explanation is that the composition of the phyllosphere microbiota of our lab-grown tomato was determined by stochastic processes because of the low concentration of plant-associated bacteria present in the indoor air and the autoclaved soil that was used for growing.

Another unexpected result was that β diversity significantly changed from day 0 to day 7 for all three treatments. Although the high humidity maintained for 2 days after inoculation was expected to have some effect on the phyllosphere community, we still expected 100×-rain to have a stronger effect on β diversity than sterile water. Similarly unexpected, the taxonomic composition at the phylum, class, and genus level on day 0 and day 7 did not reveal any consistent change unique to the treatment with 100×-rain compared with filter-sterilized rain or sterile water. Taken together, these results suggested that, if they existed, tomato leaf colonizers present in rain were individual species and changes in abundance of these individual species were not evident from the overall comparison of β diversity or taxonomic composition at higher taxonomic ranks.

Therefore, we decided to look at changes at the OTU level. To do this, we used DESeq2 (Love et al. 2014), a tool originally developed to identify significant changes in gene expression in RNA-sequencing experiments, which have challenges similar to OTU tables, and which has been shown to be effective for smaller OTU datasets like ours (Weiss et al. 2017). We made several comparisons. Most importantly, not a single OTU significantly increased from day 0 to day 7 after treatment with filter-sterilized rain or sterile water but 104 OTUs increased significantly after treatment with 100×-rain. Because none of these OTUs significantly increased from day 0 to day 7 after treatment with filter-sterilized rain or sterile water, they were most likely rainborne.

The 104 OTUs belong to 10 genera, with one of them being the genus *Pantoea*. The DeSeq2 analysis identified 18 OTUs of this genus that significantly increased in relative abundance from day 0 to day 7 after being treated with 100×-rain. Importantly, *Pantoea* OTUs were also present in all rain samples. Moreover, the species *Pantoea agglomerans* and *P. vagans* were identified as the most abundant species in several of the tomato phyllosphere day-7 samples after being treated with 100×-rain based on metagenomic sequencing. Also, two *Pantoea* OTUs were present in significantly higher relative abundance in tomato grown outside compared with tomato grown organically inside a commercial greenhouse. *P. agglomerans* and *P. vagans* were also both identified in a rain metagenome as well as in a metagenome of a tomato plant exposed to rain but not in a tomato plant not exposed to rain when using ONT MinION sequencing (a short description of this single experiment and the obtained results are provided in Supplementary Table S7). Moreover, the genus *Pantoea* is well known to include plant-pathogenic bacteria and beneficial plant-associated bacteria (Coutinho and Venter 2009; Mechan Llontop et al. 2020; Walterson and Stavrinides 2015). We previously identified 192 *Pantoea* isolates in a culture-dependent study of precipitation samples (Failor et al. 2017), and *Pantoea* spp. were recently identified in rainfall both before and after falling through a forest canopy, with higher relative abundance in the throughfall samples (Ladin et al. 2021). Therefore, based on the results obtained here and data in previous literature, members of the genus *Pantoea* are likely phyllosphere inhabitants that originate from rainfall.

Another rainborne genus that includes species that appear to successfully colonize tomato plants is *Massilia*. Members of this genus were found in all rain samples (those analyzed by 16S rRNA amplicon sequencing and the one sequenced with ONT's Minion). Twenty-seven OTUs of this genus significantly increased between day-0 phyllosphere samples and day-7 samples for 100×-rain treated plants. Two *Massilia* spp. were also found among the species with the highest relative abundance in the metagenomic sequences of the 100×-treated tomato samples on day 7. One *Massilia* OTU each was more abundant in tomato grown outdoors compared with hydroponically or organically grown tomato in the commercial greenhouse, respectively. Four *Massilia* spp. were found in the rain sample and tomato sample grown outdoors but not in the tomato plant not exposed to rain when using ONT MinION sequencing (Supplementary Table S7). As with *Pantoea*, *Massilia* spp. were previously cultured by us out of precipitation (Failor et al. 2017) and were recently found in rain and rain that had fallen through a forest canopy (Ladin et al. 2021). Finally, OTUs and named species belonging to the genus *Massilia* have been found in plants, soil, and even extreme environments (Bodenhausen et al. 2013; Holochová et al. 2020; Ofek et al. 2012; Purahong et al. 2018; Rastogi et al. 2012; Singh et al. 2019). Therefore, members of the genus *Massilia* may cycle through multiple environments and some of them may be transported by rain to leaf surfaces, where they colonize the phyllosphere.

Other rainborne genera likely to colonize the tomato phyllosphere based on our data include *Janthinobacterium* which, like the genus *Massilia*, is a member of the *Burkholderiaceae* family. Four *Janthinobacterium* spp. were also found in rain and in the rain-exposed tomato plant but not in the tomato plant protected from rain in the ONT MinION experiment (Supplementary Table S7). However, *Janthinobacterium* was not found at significantly higher relative abundance in tomato plants grown outside compared with greenhouse-grown tomato. Its inconsistent presence in rain may explain this result.

Finally, we found evidence for OTUs and named species of the genus *Pseudomonas* to colonize tomato plants. Unexpectedly, though, we did not find a single member of the *Pseudomonas syringae* species complex (*P. syringae* sensu lato), which includes many plant-pathogenic and commensal lineages (Monteil et al. 2016; Vinatzer et al. 2016). We do not have any good explanation for why we found *P. syringae* neither at relatively high abundance in

the analyzed rain samples nor in our phyllosphere samples treated with 100×-rain, although we had previously cultured *P. syringae* from rain (Failor et al. 2017) and from plants in our geographic area (Clarke et al. 2010). We conclude that, although *P. syringae* pathogens are present in rain and disseminated by precipitation, they may not be a major component of precipitation microbiota, at least not in the geographic area where the experiments here described were performed.

Importantly, though, although our data and the literature suggest that members of some common phyllosphere genera are rainborne, the fact that we found different enrichment of OTUs from these genera across experimental conditions (lab versus greenhouse) precluded the identification of likely rainborne tomato phyllosphere colonizers at the species level. One possible explanation for this is that each rain event harbored such different taxa and this increased variation coupled with low replication meant that OTUs with higher relative abundance on tomato grown outside were simply missed in the lab experiments. Conversely, the small number of analyzed tomato plants grown outside and exposed to rain made it difficult to find significant differences compared with the tomato grown inside and not exposed to rain. Finally, laboratory conditions may not have allowed some of the OTUs found outside to effectively grow on tomato plants inside the laboratory. On the other hand, artificial light and almost constant temperature and humidity may have favored tomato colonization of related but different species compared with the most effective colonizers of tomato plants grown outside, where plants are exposed to natural sunlight, including UV radiation and dramatic temperature and humidity changes. These differences in environmental conditions may explain why members of the genera *Sphingomonas* and *Methylobacterium* were consistently found in rain and in significantly higher abundance on tomato plants grown outside than in greenhouse-grown tomato but OTUs of these genera did not significantly increase in our controlled laboratory experiments after 100×-rain treatments. Finally, the use of concentrated rain microbiota instead of rain may have led to increased competition between rainborne bacteria and suppressed the colonization efficiency and growth of some while favoring the growth of others.

In summary, by comparing the effect of concentrated rain microbiota with the effect of filter-sterilized rain and sterile water on community composition of tomato phyllosphere microbiota in controlled experiments, it was possible to pinpoint the changes in community composition that were due to the rainborne bacteria present in rain from and not due to spraying leaves with rainwater and incubating plants at high humidity. However, on the flip side, this experimental design did not reconstitute a natural outdoor environment in which plants are naturally exposed to rain. Therefore, to follow up on the results reported here and to gain further insight into the relative importance of seed, soil, the atmosphere, and precipitation as reservoirs of phyllosphere microbiota, we propose to expand the kind of controlled experiments we performed here by growing tomato outdoors from either sterilized or nonsterilized seed, either not limiting exposure to precipitation or limiting exposure to precipitation (for example, through the use of mobile rain-out shelters), and growing plants in native versus sterile soil. Moreover, strain-level metagenomics (Olm et al. 2021) of all reservoir microbiota and phyllosphere microbiota could provide the necessary strain-level resolution to identify which strains from which reservoirs are the most important colonizers of the phyllosphere.

Data deposition. Sequences and metadata were deposited at NCBI under BioProject DPRJNA719680. All read processing steps, bioinformatic workflows, and scripts used in this research are available on GitHub (https://github.com/marcoeml/VinatzerLab-Mechan-rain-phyllosphere-microbiota-2020).

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