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ORIGINAL PAPER

The aeroponic rhizosphere microbiome: community dynamics in early succession suggest strong selectional forces

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Abstract In the last decade there has been increased interest in the manipulation of rhizosphere microbial communities in soilless systems (hydroponics) through the addition of plant growth promoting microbes (PGPMs) to increase plant nutrition, lower plant stress response, and control pathogens. This method of crop management requires documenting patterns in communities living in plant roots throughout the growing season to inform decisions on timing of application and composition of the supplemental PGPM consortium. As a contribution to this effort, we measured changes in the bacterial community through early succession (first 26 days) in plant root biofilms growing in an indoor commercial aeroponic system where roots were sprayed with a mist of nutrient-amended water. By 12 days following seed germination, a root-associated community had established that was distinct from the source communities found circulating in the system. Successional patterns in the community over the following 2 weeks (12–26 days) included changes in abundance of bacterial groups that have been documented in published literature as able to utilize plant root exudates, release plant hormones, or augment nutrient availability. Six bacterial families/genera (Hydrogenophilaceae, *Rhizobium*, Legionellaceae, *Methylophilus*, *Massilia*, or *Herbaspirillum*) were the most abundant

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in each root sample, comprising 8–37% of the microbiome. Given the absence of soil-associated microbial communities in hydroponic systems, they provide an ideal design for isolating plant–microbial interactions and identifying key components possibly contributing to plant health.

Keywords Community structure · Illumina sequencing · Hydroponics · Proteobacteria

Introduction

A number of negative impacts are associated with conventional, soil-based agriculture, including: large land and water requirements, soil nutrient depletion, high pesticide and fertilizer use, and transfer of harmful chemicals to the environment via runoff (Almuktar et al. 2018; Alshrouf 2017; Barbosa et al. 2015). In modern times in the 1860s (Hofmeister 1865), solution hydroponics offers several inherent advantages over traditional agriculture. For example, since dissolved nutrients in water itself are used to meet the nutritional requirements of crops (Alshrouf 2017), soil nutrient depletion does not occur. These systems reduce water use, fertilizer inputs, and pesticide application (Lee and Lee 2015). The systems, especially vertical applications, are also space-efficient, increasing yield per unit area by as much as 18 times over that of a traditional field operation (Barbosa et al. 2015; Graamans et al. 2018), and enable perpetual harvests (Barbosa et al. 2015). These systems are being used or tested in specialty applications such as isolated human outposts like McMurdo Station Antarctica (Campiotti et al. 2000) and the International Space Station (Ferl et al. 2002; Wheeler 2017). Moreover, they are becoming a viable alternative to traditional agriculture in locations where water is scarce, space is limited, growing seasons are short, or fertile soils are absent, such as in Israel, Japan, and the Netherlands (Sheridan et al. 2017; Alshrouf 2017).

Despite advancements in hydroponic technology and increased adoption of this method for plant production, knowledge of the microbiology of the hydroponic rhizosphere remains understudied, referred to here as the rhizosphere microbiome (Bartelme et al. 2018). The rhizosphere comprises the narrow region surrounding root tissue that is directly influenced by root secretions. Compounds in root secretions select for microbial species with specialized functions thought to support plant growth. Plant growth-promoting microbes (PGPM) in the rhizosphere include both prokaryotes and fungi and serve as agents of biocontrol, bioremediation, and biofertilization (Hardoim et al. 2015). Rhizosphere biofertilizers act primarily through nitrogen fixation and by increasing the availability of essential nutrients, such as potassium, iron, and phosphorus, through alteration of soil minerals (Philippot et al. 2013; Calvaruso et al. 2006; Bhat et al. 2015; Pii et al. 2015). PGPMs also reduce abiotic stress through production of a bacterial deaminase in the rhizosphere, increasing drought and flooding tolerance through the regulation of plant auxin and ethylene production, as well as ameliorating plant stress in response to changes in salinity, which can be of particular concern in hydroponic systems using closed loop water circulation (Glick 2014). Rhizosphere microbes can also suppress the growth of plant pathogens through antibiotic production or competitive exclusion (Lee and Lee 2015; Compant et al. 2005). In return for the benefits PGPMs supply, bacteria and fungi acquire polysaccharides, amino acids and growth factors, which are provided by plant root exudates secreted into the rhizosphere (Philippot et al. 2013; Pii et al. 2015). Interest in commercial mixtures of PGPMs has increased recently, although for decades farmers have added fungal spores to soils before planting to increase the chances of development of mycorrhizal associations to promote stress resistance and nutrient acquisition (Rouphael et al. 2015).

Development of a PGPM-rich community is considered integral to crop production, with differentiation between bulk soil and rhizosphere microbiome composition occurring in as early as 3 weeks in an annual grass species (Shi et al. 2015). Work in soil crops has documented selection pressure by plant root exudates, which creates a less diverse and distinct microbiome on root material in comparison to bulk soils (Tkacz et al. 2015). Soils also create long-term "memory," which has been used to explain consistent inter-annual patterns in rhizosphere communities of crops grown in the same soil matrix through multiple seasons (Lapsansky et al. 2016; Shi et al. 2015). Hydroponic systems do not possess this memory. Therefore, microbial rhizosphere communities begin

development on a relatively denuded surface, allowing the opportunity for divergence in community structure from one plant to another. When sand was used in place of soil, opportunistic bacterial species established in the rhizosphere of the plants within 4 weeks following germination, suggesting that rhizosphere community composition in soilless crops selects for *r*-selected, fast growing species found in source microbial communities (Tkacz et al. 2015). In contrast, hydroponic rhizosphere communities measured 10 weeks after germination did not share similar patterns with the microbial communities found in the system's recirculating water (Sheridan et al. 2017).

Identifying, and eventually managing microorganisms associated with the plants in hydroponic systems, can lead to more efficient growing methods. In hydroponics, it is common for farmers to add commercial mixtures of PGPM (both bacteria and fungi), but studies testing the effectiveness of these applications have found variable results depending on the plant species, growing conditions, and method of application. The only published work that comprehensively categorized the hydroponic microbiome using next-generation sequencing found the addition of a commercial PGPM mixture influenced the community composition in the water system, but produced variable results within the rhizosphere community measured at harvest (Sheridan et al. 2017). Virtually unexplored, however, is whether the microbiome structure within the rooting zone of hydroponic plants follows a predictable successional sequence through time that results in selection for plant-health-promoting bacterial groups regardless of the source populations. To begin to address these knowledge gaps, we collaborated with a local plant producer to monitor the microbial rhizosphere in a recirculating aeroponic system growing lettuce (*Lactuca sativa*) with the following objectives: (a) to determine if source communities share key characteristics with root-associated communities to evaluate the strength of selection by root exudates, (b) to document early succession trajectories, and (c) to compare microbial rhizosphere composition and successional changes over time to patterns published for soil crops. Here we describe a sampling effort to characterize root biofilms of nine lettuce plants that transition from hydroponic trays to an indoor aeroponic unit using Illumina high-throughput sequencing, documenting patterns in community development in early succession.

Materials and methods

Hydroponic system description

Aeroponic farming is a form of hydroponics where plant roots are suspended in a chamber filled only with air, and water is applied as a mist to the roots. Although the aeroponic system used in this research is a proprietary product of Indoor Farms of America (IFOA), it utilizes design elements common to many hydroponic systems. Plant growth was performed in vertical panels ($\sim$ 1.2 $\times$ 2.4 m) with high-density growth site placement. The system was a recirculating, closed system (fresh water was not introduced to the system each delivery cycle) using Las Vegas Valley Water District municipal water source supplemented with AmHydro (Arcata, CA) GroMagnon fertilizer. This nutrient solution was recirculated for 2 weeks with only pH monitoring and adjustment. Application of the solution to the plant roots was routed first through IFOA's "Rock Box" to reestablish mineral levels, followed by a chilled (20.5–26 °C), aerated sump tank and pump system that led to a 50-gallon covered reservoir made of opaque HDPE, then finally distributed through PVC pipes to hanging planters. The spray heads delivered the nutrient solution from the reservoir directly to the plant roots every 15 min. Seed germination was performed in custom germination plugs made of coco coir, which were then transferred into the IFOA aeroponic system. Seed surfaces and germination plugs were not sterilized prior to germination. Vegetative and fruiting stages of growth were supported by horticulture-grade triple-band LED light bars (DQM spectra, Solidlite Corporation, HsinChu, Taiwan) placed in front of the vertical growth panels. Ambient air temperature was maintained at 25–27 °C. A digital monitoring system controlled climate, water, ventilation, and lighting specifics.

Sample collection and aqueous chemistry

Plant roots and recirculating water samples were collected over a 2 week period (21 September, 28 September, and 4 October 2016), which coincided with IFOA's water replacement schedule. Water in the

aeroponic system was replaced with Las Vegas Valley Water District municipal water prior to sample collection on 21 September. The first sampling included plants incubated under grow lights in trays supplied with nutrient-rich water; the plants were 12 days old at this time (TP1). At the second sampling, the plants were 19 days old and had been in the aeroponic units for 1 week (TP2). The third sampling occurred 2 weeks after moving into the aeroponic units; the plants were 26 days old (TP3). Just prior to the second sampling event, the aeroponic units moved locations to a new business space. In the process, there was a power interruption that persisted for approximately 12 h. Sampling occurred after the power interruption and addition of pH Down solution (General Hydroponics, Santa Rosa, CA).

Water temperature, conductivity, total dissolved solids (TDS), salinity, pH, oxidation reduction potential (ORP), and dissolved oxygen (DO) concentrations were measured with a YSI 6920 sonde (YSI Inc., Yellow Springs, OH) deployed into the recirculation reservoir prior to sample collection. Filtrate from 0.22 μm Sterivex polyethersulfone filters (EMD Millipore, Darmstadt, Germany) was collected for dissolved aqueous chemistry analysis (dissolved ions, dissolved organic carbon (DOC), and dissolved nutrients (nitrate, nitrite, ammonium, and orthophosphate). Samples collected for dissolved ions and DOC analysis were stored at 4 °C and samples collected for dissolved nutrient analysis were stored at dissolved nutrient analysis were stored − 80 °C. Dissolved ion and organic carbon analyses were conducted by ACZ Laboratories (Steamboat Springs, CO) according to EPA Methods 200.7 for cations, SM4500Cl-E for chloride, D516-02/-07—Turbidimetric for sulfate, and SM5310B for dissolved organic carbon. Dissolved nutrient concentrations were determined using a Hach DR 5000 UV–Vis spectrophotometer, including nitrate (cadmium reduction, Method 8171), ammonium (USEPA accepted standard method 10023), orthophosphate (USEPA accepted standard method 8048), total phosphorus, (Standard Method 8190), and biochemical oxygen demand (USEPA accepted standard method 8000). To distinguish between changes in water chemistry concentrations due to hydrologic modifications versus biotic or abiotic uptake and release, changes in ionic concentrations within the reservoir water were evaluated through calculation of the molar ratio of each dissolved constituent to the molar ratio of chloride measured on the same day, with the assumption that chloride acted as an inert tracer.

Plant root samples from nine leafy green lettuce seedlings (*Lactuca sativa*) were collected with flame-sterilized dissection scissors and forceps at each sampling event. No differentiation was made between prokaryotes closely adhering to the root surface (rhizoplane) versus prokaryotes growing inside plant root tissue (phylosphere); both were contained in a single sample. Roots from the same nine plants were sampled at each time point: the first collection was from seedling roots in three separate seed germination trays (three seedlings from each of three trays) and the second and third collections were from the roots of the same plants growing next to each other in vertical growth panels. The proximity of the plants to each other in the aeroponic unit did not allow for roots to contact each other. Samples from possible contributing sources of prokaryotes to the rooting community were also collected at each time point, including planktonic biomass from water in the recirculation tank (1 L per sample), which was concentrated onto 0.22 μm Sterivex polyethersulfone filters (EMD Millipore). Another possible source of prokaryotes to the roots was rock chips and sediment from the Rock Box, which was collected into 50 mL conical centrifuge tubes from each sampling event. Additionally, an uninoculated artificial medium plug used for physical support of the plant (labeled "Growth Plug" in figures) and powdered hydroponic fertilizer (AmHydro Gro-Magnon 10-6-17 Fertilizer, Bags A and B [AmHydro, Arcata, CA], labeled "Fertilizer" in figures) were collected for prokaryotic community analysis as possible sources to the rhizosphere. All samples for prokaryotic community analysis were stored on dry ice during transportation to the lab and at − 80 °C until DNA extraction.

In addition to documenting possible sources of microorganisms to the rhizosphere community, control samples were generated to document any unintended contamination of sequencing samples during field and laboratory processing. These samples included "trip blanks" where Milli-Q water was carried into the field on each of the three sampling dates, and the container was opened during sampling before being sealed again and returned to the lab. A laboratory "extraction blank" was processed through the full extraction protocol to detect contamination from kit reagents or the laboratory environment.

Finally, a "sterile water" control, molecular-grade water was submitted for sequencing to detect contamination from outside of the Desert Research Institute laboratory.

DNA extraction, library preparation, and 16S rRNA gene sequencing

Total genomic DNA was isolated from plant root samples (10–40 mg), and from 0.22 µm Sterivex filters with the MoBio PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer's instruction, with the addition of a freeze–thaw step (30 min at − 80 °C followed by 10 min at 65 °C) prior to bead beating. Total genomic DNA was isolated from Rock Box bulk material (18–33 g) with the MoBio PowerMax DNA Isolation Kit (MoBio) according to manufacturer's instructions, with the addition of a freeze–thaw step (30 min at − 80 °C followed by 10 min at 65 °C) prior to bead beating and an additional centrifugation (2500×*g* for 4 min) after incubation of Solution C3 to pellet out debris. Library preparation and Illumina DNA sequencing were performed at MR DNA (Shallowater, TX). Library preparation was carried out via PCR using modified primer sequences targeting the V4 hypervariable region of the 16S rRNA gene found in Prokaryotes (F515 [5′-GTGYCAGCMGCCGCGGTAA-3′] and 806R [5′-GGACTACHVGGGTWTCTAAT-3′]) (Hou et al. 2013) and were sequenced in one Illumina MiSeq instrument run using the 2x250 MiSeq Reagent Kit v2. The raw 16S rRNA gene sequences were deposited in the European Nucleotide Archive under project accession number PRJEB31440.

Prokaryotic community analysis

Sequencing reads were processed with QIIME 1.9.1 (Caporaso et al. 2010b). Paired-end reads were merged according to the fastq-join method (Aronesty 2011) using default parameters. Merged reads were demultiplexed and quality-filtered (reads containing ambiguous ['N' characters] and low-quality base calls [Phred score < 30] were removed) with the split_libraries_fastq.py command. Chimeric sequences were identified with the usearch61 algorithm (Edgar 2010) and removed. Operational taxonomic units (OTUs) were generated from the 5,321,019 high-quality nonchimeric sequences, based on 97% sequence similarity, and taxonomy assignments made with a subsampled open-reference OTU-picking strategy using usearch61 and uclust (Edgar 2010) against the SILVA_128 database (Pruesse et al. 2007; Quast et al. 2013; Yilmaz et al. 2014). OTUs with plant chloroplast and mitochondrial taxonomic assignments were removed. Remaining OTUs supported by less than 0.005% of all sequences were removed, resulting in a final dataset comprised of 2,667,800 sequences. A phylogenetic neighbor-joining tree (Price et al. 2010), based on PyNAST-aligned OTU sequences (Caporaso et al. 2010a), was generated and used for alpha and beta diversity metrics. Lastly, the OTU table was rarefied to a depth of 10,000 sequences per sample to account for differences in sequencing depth. Alpha diversity metrics [Faith's Phylogenetic Diversity index, Shannon's index, and Simpson's Index of Diversity (1-D)] and pairwise Bray–Curtis and UniFrac (Lozupone et al. 2011) distances between samples were calculated from 100 rarefied OTU tables.

Statistical analyses

Principal coordinate analysis (PCoA) and analysis of similarity tests (ANOSIM) of weighted and unweighted UniFrac distances and Bray–Curtis distances were conducted in R (R Core Team 2013) using the vegan package (Oksanen et al. 2015). Clustering of prokaryotic communities was evaluated by constructing a dendrogram based on unweighted pair group method with arithmetic mean-clustering (UPGMA) of weighted and unweighted UniFrac distances. Node support values were calculated from 100 rarefied OTU tables of 10,000 sequences per sample. Similarity percentage (SIMPER) analysis, based on Bray–Curtis dissimilarity, was performed to identify OTUs responsible for differences between groups of samples. Diversity indices were compared through time in root samples using repeated measures ANOVA executed in Sigma Plot (14.0). Data were transformed prior to analysis if they did not meet the assumption of normality (Shapiro–Wilk). T-tests for identifying differences in alpha diversity between source communities and plant roots were completed using the Data Analysis package in Microsoft Excel.

Results

Aqueous chemistry

Reservoir water temperatures ranged from 20.5 to 25.9 °C; however, during the 12 h power outage at the second sampling point (1 week in the aeroponic system), water temperature temporarily reached 30.7 °C (Table 1). Conductivity ranged from 2456 to 3222 μS cm^{-1}, values above normal municipal water conductivity due to the addition of nutrient solution and pH adjustment. pH was maintained between 5.4 and 6.4; however, our second sampling date coincided with an addition of pH Down solution (phosphoric acid), creating a transient pH reading of 4.85. Dissolved O_2 remained near saturation at all time points. Most dissolved constituents increased in concentration over the 2 week period (Table 1) and inorganic nutrients and cations were well above those typical of natural, urban, and agricultural aquatic ecosystems (Mulholland et al. 2008). Nitrate, which did not follow any trend, ranged from 60 to 120 mg L^{-1}. Ammonium, however, increased from an initial value of 5.6 to 40.6 mg L^{-1} after 2 weeks. Total P was high throughout the sampling period, measured at 109.2 mg L^{-1} at 1 week in the recirculating reservoir, and then 273 mg L^{-1} at 2 weeks, with 41–57% of the P pool present as orthophosphate. Potassium was relatively consistent, ranging from 179 to 211 mg L^{-1} (Table 1). Aluminum, antimony, arsenic, beryllium, bismuth, chromium, gallium, lead, nickel, scandium, silver, thallium, tin, titanium, and vanadium were undetectable in all samples, and therefore are not reported in Table 1. Molar ratios (ion: Cl) varied by less than 30% within the 2 week time period for barium, calcium, cobalt, magnesium, manganese, nitrate, potassium, silica, silicon, sodium, strontium, sulfate, and sulfur (Table 1), while molar ratios of ammonium, boron, copper, DOC, iron, lithium, manganese, molybdenum, orthophosphate, total P, and zinc increased over the 2 week period (> 50% increase in ratios from first to last sampling) (Table 1).

Prokaryotic microbiome analysis

In total, 41 samples were submitted for 16S rRNA gene amplicon sequencing, which resulted in 63,342 ± 23,425 quality-screened sequences per sample and 1601 OTUs (Appendix S1). Prokaryotic microbial communities in plant root samples were found to be entirely bacterial, except for eight Thaumarchaeal OTUs found only in a single sample (the Growth Plug, comprising 20.6% of sequences recovered in the sample); hence, the remaining text refers to microbial communities in this work as bacterial. Microbial eukaryote community composition and diversity were not considered as part of this work. The majority of OTUs were classified as Proteobacteria (Fig. 1). Alphaproteobacteria was the most abundant class within the Proteobacteria for root samples, comprising on average 41% of the total (range 0.10–80%). Betaproteobacteria and Gammaproteobacteria followed in abundance in the root system, comprising an average of 38% (range 18–76%) and 16% (range 2–33%) of the profiles of each sample, respectively. In contrast, samples collected as possible sources of bacteria to the roots, such as the Rock Box, exhibited microbial profiles that were distinct from plant root samples. Acidobacteria, Nitrospirae, and Planctomycetes (total from all three groups was 38–39% of sequences) dominated the community profile of Rock Box samples, whereas these groups were rare in root samples (0–1% of root sample sequences) (Fig. 1). Another possible source community to the roots was the reservoir water, which clustered distinctly from root samples in the UPGMA clustering dendrogram. The reservoir samples taken at the first two sampling dates were similar to the Rock Box sample, but 2 weeks after initial filling of the reservoirs (TP3), the reservoir community was dominated by Gammaproteobacteria (59%) (Fig. 1). The reservoir sample at TP3 clustered with the two samples from the fertilizer added to the reservoir, which were dominated by Firmicutes (26% in A, 10% in B) and Gammaproteobacteria (51% in A, 50% in B) (Fertilizers A and B, Fig. 1). A third potential source of bacteria was from the uninoculated plugs used for seed germination and root support during the experiment. The community living on this material was the most distant of any source from the root samples, with similarities to the Rock Box samples (Growth Plug, Fig. 1). Trip Blanks, Sterile Water, and Extraction Control samples flanked the root samples in the dendrogram as separate clusters (Fig. 1).

To further evaluate differences between the microbial communities developing through early succession on the plant roots and the source communities

Table 1 Physical parameters and aqueous chemistry of reservoir samples collected at three time points

	Sampling event			
	12 day old plants in trays (9/21/16)	1 week in aeroponic unit (9/28/16)	2 weeks in aeroponic unit (10/4/16)	% Change in molar ratio (ion:Cl)
Physical measurements				
Temperature (°C)	25.91	30.66	20.52	
Conductivity (μS/cm)	2456	3222	2803	
TDS (g/L)	1.569	1.890	1.993	
Salinity	1.24	1.50	1.60	
pH	6.37	4.85	5.40	
ORP (mV)	283.9	213.9	233.0	
DO (% saturation)	110.1	98.4	97.7	
DO (mg/L)	8.89	7.30	8.71	
COD (mg/L)	28	67	57	
Dissolved ions (mg/L)				
Chloride	91.1	109	125	–
Sulfate	356	409	379	6
Barium	0.125	0.093	0.115	− 8
Boron	0.18	0.3	0.37	106
Cadmium	< 0.005	0.011	0.012	–
Calcium	179	156	173	− 3
Cobalt	0.01	–	0.01	0
Copper	0.14	0.79	1.21	764
Iron	0.26	1.47	2.55	881
Lithium	0.041	0.053	0.063	54
Magnesium	47.9	57.7	60.1	25
Manganese	0.492	0.416	0.79	61
Molybdenum	–	0.02	0.04	–
Phosphorus	17.4	56.1	53.5	124
Potassium	179	211	201	12
Silica	7.9	10.2	10.2	− 6
Silicon	3.7	4.7	4.7	27
Sodium	90.5	112	118	30
Strontium	1.68	1.71	1.99	18
Sulfur	107	120	113	6
Zinc	0.15	0.85	1.2	700
Dissolved nutrients (mg/L)				
DOC	5.5	19.1	16.5	119
NO_3^- as N	120	60	110	8
NH_4^+ as N	5.6	33.1	40.6	625
Total P as PO_4^{3-}	109.2	294	273	82
Orthophosphate as PO_4^{3-}	44.4	168	156	251

TDS total dissolved solids, *ORP* oxidation/reduction potential, *DO* dissolved oxygen, *COD* chemical oxygen demand, *DOC* dissolved organic carbon. No measurement or calculation indicated with –

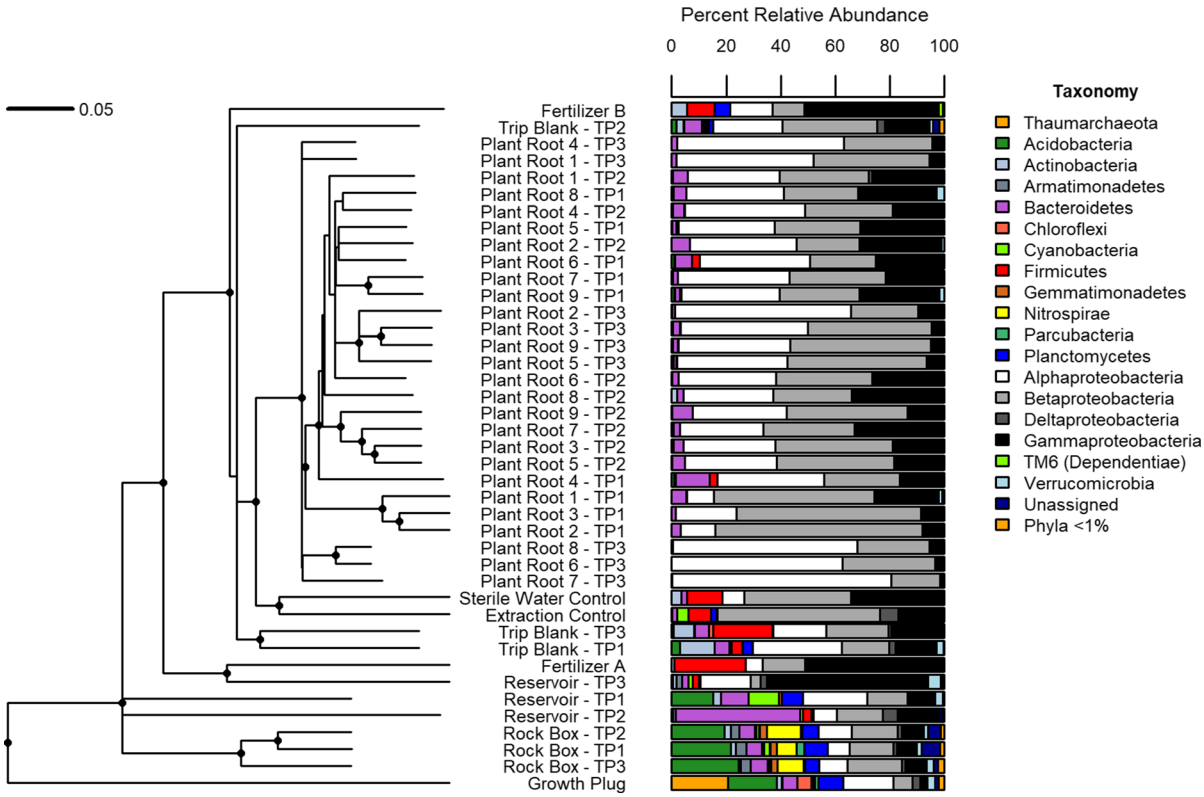

Fig. 1 Left: unweighted pair group method with arithmetic mean (UPGMA) clustering dendrogram based on pairwise abundance-weighted UniFrac distances. Bootstrap values $\geq$ 80% based on 100 rarefactions are shown as black circles at the nodes. Scale bar represents 5% dissimilarity. Right: phylum-level taxonomic bar chart of prokaryotic communities. Samples are listed according to their order in the UPGMA clustering dendrogram. The Proteobacteria phylum is presented at the class level. Groups with $<$ 1% relative abundance in all samples are included collectively as 'Phyla $<$ 1%'

supplying bacteria to the roots, principal coordinate analysis (PCoA) of pairwise unweighted and weighted UniFrac distances was performed. The first two component axes explained 35.6% and 46.8%, unweighted and weighted, respectively, of the variation between the sample communities (Fig. 2). Unique community groupings for each source sample (Rock Box, Reservoir Water, Growth Plug) were found, and they did not fall within the same PCoA space as the root samples communities (Fig. 2). Similar results were found with control samples, which were distinct from the rhizosphere samples and the source communities. One exception was in the weighted UniFrac analysis, where the sterile water sample that was used to detect contamination by the sequencing laboratory grouped with the first samples taken when plants were growing in germination trays. However, there was little similarity in community composition between control samples and root samples using the unweighted UniFrac PCoA and the clustering analysis (Figs. 1, 2).

Differences between sampling time points in microbial rhizosphere communities were evaluated separately from source and control sample communities using weighted UniFrac PCoA analysis. Component axes 1 and 2 combined explained 57.2% of the variation measured between the rhizosphere communities over time (Fig. 3). Root samples from each time point clustered in different portions of the coordinate space, and intra-sample variability was lowest in plant samples taken 1 week after transfer to the aeroponic system (TP2) compared to the other two time points (Fig. 3).

Variability in diversity among the nine plants followed through the time series was evaluated using two alpha diversity indices, Faith's Diversity and Shannon Index. Average diversity did not change when plants were transitioned from incubation trays to

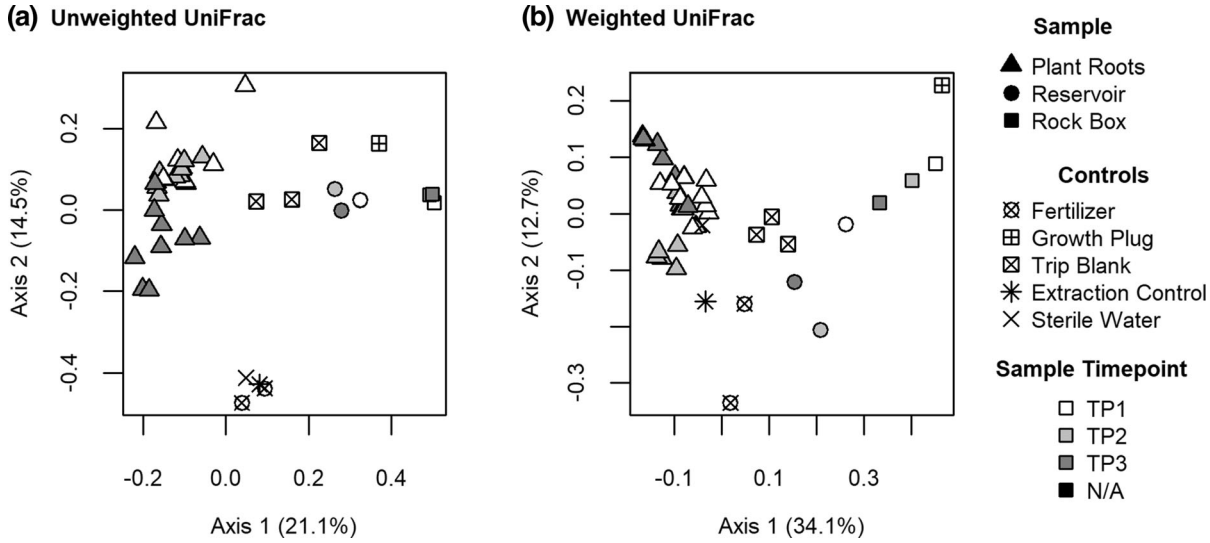

Fig. 2 Weighted and unweighted UniFrac PCoA analysis demonstrating similarities between plant root, source community, and control samples

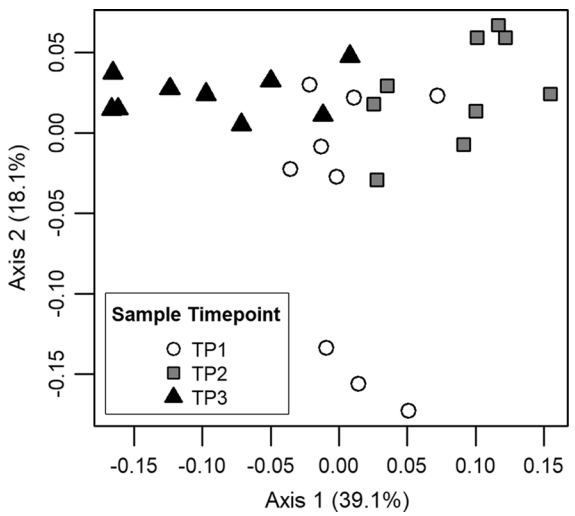

Fig. 3 Weighted Unifrac PCoA analysis including only root samples collected 12 days after seed germination (TP1), at 19 days following 1 week of growth in the aeroponic unit (TP2), and after 2 weeks growing in the aeroponic unit (TP3)

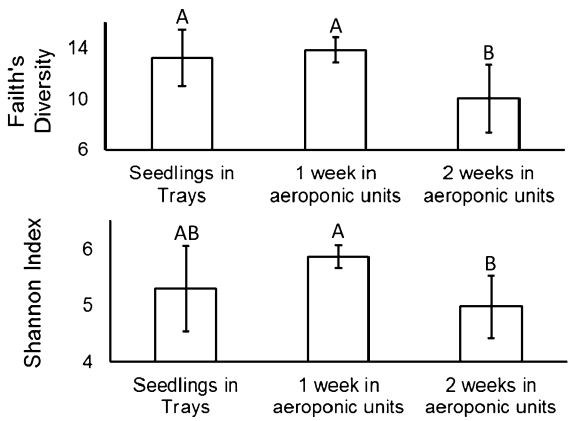

Fig. 4 Measures of alpha diversity in root samples collected over the 2 week period, including Faith's Diversity and Shannon Index. Bars with variable letter designations are statistically different from each other using a one-way ANOVA, $p < 0.05$. Error bars represent standard deviations

the aeroponic unit (TP1 to TP2), but there was less variability in alpha diversity indices within the nine root samples after 1 week in the aeroponic unit (TP2), evidenced by the comparison of error bars indicating standard deviations (Fig. 4). Plants growing in the aeroponic system saw a decrease in average Faith's and Shannon diversity when comparing 19-day-old plants incubated for 1 week in the aeroponic unit versus 26-day-old plants in the aeroponic unit (TP2 to TP3), but along with this decrease in alpha diversity was an increase in the variability between the nine samples (Fig. 4, $ANOVA_{Faith's}$, $df = 2$, $F = 8.546$, $p = 0.003$, transformed by squaring, Bonferroni post hoc test; $ANOVA_{Shannon}$, $df = 2$, $F = 5.121$, $p = 0.019$, Bonferroni post hoc test). In contrast, Rock Box alpha diversity was higher than that found in rhizosphere samples (t $stat_{Faith's} = 6.83$; $p < 0.001$; t $stat_{Shannon} = 2.81$, $p = 0.0089$), and the values in Rock Box samples maintained a narrow range over the 2 week period, with Faith's Diversity indices ranging

from 22.4 to 23.3, and Shannon indices ranging from 6.37 to 6.51. Reservoir samples also had higher Faith's Diversity indices than rhizosphere samples (t $stat_{Faith's}$ = 5.10; $p < 0.001$), while values for the Shannon Index of diversity were not statistically significantly different between reservoir and rhizosphere samples (t $stat_{Shannon}$ = 1.10; $p = 0.276$). Faith's Diversity indices in reservoir samples ranged from 19.1 to 20.9, and Shannon indices ranged from 5.3 to 6.6.

ANOSIM also revealed statistically significant differences between source communities (Rock Box and reservoir) and rhizosphere communities, as well as controls and samples (Table 2). The SIMPER routine identified the top five OTUs contributing to differences between source communities and root samples, as well as changes in root communities through time, with 20–27% of the variability in these communities explained solely by variation in these five OTUs (Table 3). When comparing source communities to root samples, there were higher abundances of Blastocatellaceae Subgroup 4 and *Nitrospira* OTUs in the Rock Box compared to root samples, while the water reservoir had higher abundances of *Flectobacillus* and *Acinetobacter* OTUs when compared to root samples. Both the Rock Box and reservoir had much lower abundances of *Methylophilus*, *Rhizobium*, and Rhizobiaceae OTUs as compared to the root samples (Table 3).

Changes in the microbial communities over time indicate a dynamic structure in the roots of these nine plants. When focusing on the five OTUs contributing the greatest to differences in microbial diversity, seedling root communities sampled at the first time point had higher abundances of Hydrogenophilaceae and Legionellaceae OTUs when compared to the same plant roots growing in the aeroponic system (Table 3, Fig. 5). Seedlings growing in the tray at the first time point also had elevated abundances of Rhizobiaceae (13–20% higher) and *Rhizobium* OTU_740 (3.5–5% higher) OTUs compared to the root communities found when transferred to the aeroponic system (Table 3, Fig. 5). Seedling root communities sampled at the first time point had *lower* abundances of *Methylophilus*, *Rhizobium* OTU_768, and *Herbaspirilum* OTUs as compared to these same plants while growing in the aeroponic system (Table 3, Fig. 5). Finally, comparison of plant roots growing in the aeroponic system sampled 1 week apart had increased abundances of *Rhizobium*, *Herbaspirilum*, and *Massilia* OTUs after 2 weeks in the aeroponic system, and concurrently a decline in *Methylophilus* and *Cellvibrio* OTUs (Table 3, Fig. 5).

Discussion

Recirculation of water in hydroponic systems, while increasing water use efficiency tremendously during food production, has also been cited as a system weakness, due to the potential for system-wide plant infection by pathogens once they escape into the hydrologic flow (Lee and Lee 2015). However, our work demonstrated a strong selection pressure early in plant seedling development for a community of plant rhizosphere bacteria that was distinct from groups found in the circulating water, addressing the first goal

Table 2 Analysis of similarity (ANOSIM) analysis of abundance-weighted UniFrac, abundance-unweighted UniFrac, and Bray–Curtis distances

	Weighted UniFrac	Unweighted UniFrac	Bray–Curtis
Samples versus controls[a]	R = 0.4342, p = 0.004	R = 0.3942, p = 0.003	R = 0.5681, p = 0.001
Growth plug versus reservoir[b]	R = 0.6296, p = 0.1	R = 0.7778, p = 0.1	R = 0.6667, p = 0.1
Rock Box versus root	R = 0.9158, p = 0.001	R = 0.9416, p = 0.001	R = 1.0, p = 0.001
Reservoir versus root	R = 0.9958, p = 0.001	R = 0.7998, p = 0.002	R = 0.9933, p = 0.001
Root samples by date	R = 0.5572, p = 0.001	R = 0.4629, p = 0.001	R = 0.5112, p = 0.001

R- and p values shown were calculated from 999 unconstrained permutations of pairwise UniFrac or Bray–Curtis distances between samples from an OTU table rarefied to 10,000 sequences per sample. R-values closer to zero indicate no difference between groups of samples and R-values close to 1 indicate that samples within the same grouping are most similar to one another

[a]Control samples include: Fertilizer, Growth Plug, Trip Blanks, Extraction Control, and Sterile Water Control

[b]Statistics calculated from nine unconstrained permutations due to limited sample number

Table 3 SIMPER analysis showing the top five OTUs responsible for the Bray–Curtis dissimilarity between groups of samples

OTU ID	Phylum	Genus[1]	Mean abund. Rock Box (%)	Mean abund. root (%)	Contribution to dissimilarity (%)	Cumulative contribution (%)
Rock Box versus root						
OTU_25	Acidobacteria	f_Blastocellaceae Subgroup 4	11.94	0.04	6.17	6.17
OTU_492	Nitrospirae	*Nitrospira*	9.59	0.02	4.96	11.13
OTU_1145	Betaproteobacteria	*Methylophilus*	0.10	7.93	4.06	15.19
OTU_740	Alphaproteobacteria	*Rhizobium*	0.10	6.94	3.55	18.74
OTU_725	Alphaproteobacteria	f_Rhizobiaceae	0.18	6.21	3.12	21.86

OTU ID	Phylum	Genus[1]	Mean abund. reservoir (%)	Mean abund. root (%)	Contribution to dissimilarity (%)	Cumulative contribution (%)
Reservoir versus root						
OTU_182	Bacteroidetes	*Flectobacillus*	10.51	0.62	5.54	5.54
OTU_1145	Betaproteobacteria	*Methylophilus*	0.14	7.93	4.18	9.72
OTU_1358	Gammaproteobacteria	*Acinetobacter*	6.89	0.09	3.70	13.42
OTU_740	Alphaproteobacteria	*Rhizobium*	0.53	6.94	3.44	16.86
OTU_725	Alphaproteobacteria	f_Rhizobiaceae	0.24	6.21	3.21	20.07

OTU ID	Phylum	Genus[1]	Mean abund. TP1 (%)	Mean abund. TP2 (%)	Contribution to dissimilarity (%)	Cumulative contribution (%)
Plant roots: TP1 versus TP2						
OTU_1136	Betaproteobacteria	f_Hydrogenophilaceae	10.51	3.78	9.13	9.13
OTU_1145	Betaproteobacteria	*Methylophilus*	5.88	11.23	6.17	15.30
OTU_725	Alphaproteobacteria	f_Rhizobiaceae	6.85	5.72	3.78	19.08
OTU_740	Alphaproteobacteria	*Rhizobium*	7.13	6.89	3.59	22.67
OTU_1344	Gammaproteobacteria	f_Legionellaceae	3.86	1.25	3.14	25.81

OTU ID	Phylum	Genus[1]	Mean abund. TP1 (%)	Mean abund. TP3 (%)	Contribution to dissimilarity (%)	Cumulative contribution (%)
Plant roots: TP1 versus TP3						
OTU_768	Alphaproteobacteria	*Rhizobium*	0.27	11.29	8.39	8.39
OTU_1136	Betaproteobacteria	f_Hydrogenophilaceae	10.51	0.75	7.84	16.23
OTU_1097	Betaproteobacteria	*Herbaspirilum*	0.58	6.01	4.12	20.35
OTU_740	Alphaproteobacteria	*Rhizobium*	7.13	6.79	3.59	23.94
OTU_725	Alphaproteobacteria	f_Rhizobiaceae	6.85	6.07	3.25	27.19

OTU ID	Phylum	Genus[1]	Mean abund. TP2 (%)	Mean abund. TP3 (%)	Contribution to dissimilarity (%)	Cumulative contribution (%)
Plant roots: TP2 versus TP3						
OTU_768	Alphaproteobacteria	*Rhizobium*	0.24	11.29	8.83	8.83
OTU_1145	Betaproteobacteria	*Methylophilus*	11.23	6.67	5.10	13.93
OTU_1097	Betaproteobacteria	*Herbaspirilum*	0.48	6.01	4.42	18.35
OTU_1286	Gammaproteobacteria	*Cellvibrio*	4.98	0.33	3.71	22.06
OTU_1105	Betaproteobacteria	*Massilia*	0.91	5.04	3.63	25.69

Mean abundances and contributions to dissimilarity were calculated from an OTU table rarefied to 10,000 sequences per sample

[1]The f_ prefix indicates family-level taxonomy

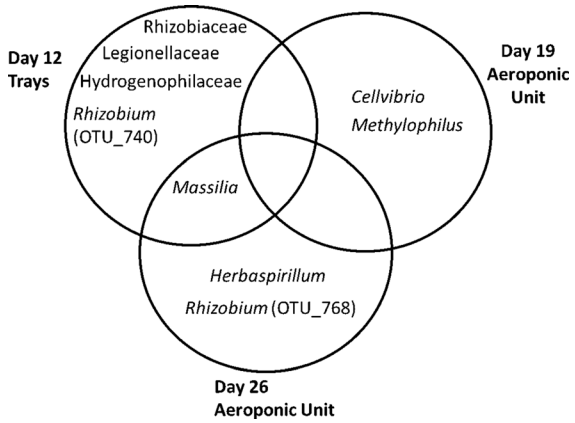

Fig. 5 Venn diagram indicating OTUs that contributed the greatest amount to differences in plant root samples over time, as determined using the SIMPER analysis (Table 3). Commonalities among samples are not shown to emphasize changes in dominant members

of this study. Although not measured nor observed in our study, it is plausible that selection for bacteria by root exudates as early as 12 days after seed germination could provide biocontrol properties through allelopathic chemical release, making bacterial biofilms more resistant to invasion by pathogens, as demonstrated elsewhere (Wu et al. 2015; Strobel and Daisy 2003). While we have no measurements of plant condition in our study, personal observation confirmed the plants grew to harvest and leaf color remained dark green (Evans and Martin 2016), suggesting that the aeroponic system maintained conditions that supported plant productivity. None of the most common bacterial plant pathogens were identified as OTUs in any of our samples (Mansfield et al. 2012), even though their presence has been a concern in the use of hydroponics for food production (Rosberg et al. 2014). However, we acknowledge that genus-level identifications from short 16S rRNA gene sequences (< 300 base pairs) should be interpreted with caution and the absence of particular genera in the sequencing libraries does not necessarily indicate complete absence of those organisms in the sampled environment. PGPBs can produce antibiotics that exclude harmful bacteria and alert the plant's immune system to the presence of pathogens and tissue damage created by insects that feed on the leaves and stalks (Compant et al. 2005). We hypothesize that the unique root biofilms characterized in our work contributed to plant success within the first weeks of development through competitive exclusion, which will be tested in future experiments.

We also found that nutrient supply in the circulating water likely met the physiological needs of the plants. None of the calculated molar ratios of dissolved ions to chloride concentrations indicated significant depletion of the nutrients measured, and in fact, ammonium, boron, copper, DOC, iron, lithium, manganese, molybdenum, orthophosphate, total P, and zinc were released to the water circulation system during the 2 weeks. Increases in these ions suggest possible solubilization of metals (Fe, B, Cu, Li, Mb, Mn, Zn) through release of chelating organic acids from roots or the production of siderophores from root-associated bacteria, which could also trigger P release from previously adsorbed or precipitated mineral forms (Radzki et al. 2013; Pii et al. 2015). All other measured ions appeared to fluctuate based on water addition or evaporation, as their molar ratios did not vary significantly through time (< 30% difference over the 2 week period).

Not only was root-associated community composition apparently changed through selection by the plants, but community diversity was lower compared to source communities, which is a common pattern in published soil literature (Tkacz et al. 2015; Shi et al. 2015). The distinct OTUs in source samples were not those often identified as root-associated, but rather bacterial groups, based on their phylogenetic identity, with the potential to perform nitrification and reduction of complex organic molecules. One exception was the 28 OTUs identified as *Acinetobacter* (Gammaproteobacteria), some of which are known to provide beneficial functions for plants in the rhizosphere (Rokhbakhsh-Zamin et al. 2011; Kang et al. 2012). *Acinetobacter* were abundant in the dry fertilizer, reservoir, and Rock Box, but were virtually missing in root samples with the exception of one OTU, being found in only 5 of the 27 plant root samples at very low abundance. This suggests that seeding the water system with PGPBs could be ineffective in establishing beneficial microorganisms in the roots of the plants, even in recirculating systems.

A second goal of this research was to monitor successional changes in the microbiome associated with plant roots growing in an aeroponic system. For the first 12 days, plants were grown horizontally in compartments sitting in a tray with shallow standing water, then moved to vertically arranged

compartments where roots are suspended in air and misted with nutrient solution. *Rhizobium* species were consistently a dominant member of the root community when compared to source communities in this study, regardless of the sampling time point. *Rhizobium* species are typically associated with plant root nodules in leguminous crops through a mutualistic relationship, where they fix nitrogen that is then utilized by the plant (Mia and Shamsuddin 2010). Given the very high nitrate and ammonium concentrations of the nutrient solution, and that nodules are not formed in lettuce plants, *Rhizobium* in these plant root samples were either indifferent to their rhizosphere habitat, or provided alternative PGPB functions that have been documented in other studies, such as plant hormone production to stimulate growth [indole-3-acetic acid (IAA) and cytokinins], siderophore production for solubilization of iron, or protection from fungal infection (García-Fraile et al. 2012; Mia and Shamsuddin 2010; Flores-Félix et al. 2013). Turnover in the OTU identity of different *Rhizobium* species throughout the experiment contributed to the unique composition of the community at 12 days and 26 days (SIMPER analysis), but whether these different *Rhizobium* groups had distinct functional roles in the root zone cannot be determined within the context of this study.

Aside from the consistent contribution by *Rhizobium* species to community composition in root samples, the SIMPER analysis documented important shifts in the distinctive groups found in the root zone as the plants matured. When plants were growing in trays for initial incubation, Hydrogenophilaceae and Legionellaceae OTUs were more abundant in plant root samples as compared to source samples, and neither family is a typical PGPB found in published literature. The implications of the slightly elevated abundance of members from the family Legionellaceae at this first sampling time point when compared to all other samples is unknown. There were Legionellaceae OTUs in all samples, and half of all species documented in the family are not classified as human pathogens, some being parasitic on amoeba (Llewellyn et al. 2017). The higher abundance of these two families does imply a very different rhizosphere environment for the plants when grown in trays as compared to plants in the aeroponic unit, suggesting that plant developmental stage or water delivery method may select for different PGPBs, depending on plant needs.

When the plants were moved from trays to the aeroponic unit, community shifts in two distinctive genera may have resulted from the stress of a new water delivery method. One community member was *Methylophilus*, which was most abundant at the second sampling following 1 week of aeroponic spray. Members of this genus have been found to solubilize inorganic P, produce siderophores, and generate the plant hormone IAA (Correa-Galeote et al. 2018). These methylotrophic bacteria degrade methanol, which is a plant hormone generated during growth, but also produced in response to physical stress, such as accidental leaf damage (Dorokhov et al. 2018). Along with this documented spike in *Methylophilus* as plants were transitioned from trays to aeroponic water delivery was a drop in abundance of *Massilia*, followed eventually by an increase again of *Massilia* at the final sampling. Twenty-eight OTUs were identified as *Massilia* in our study, found only in the plant root samples (no source samples). *Massilia* are known to produce the plant hormone IAA, as well as siderophores for iron capture to allow competitive exclusion of pathogens, including the common fungal disease, *Phytophthora infestans* (Ofek et al. 2012). We hypothesize that these community shifts in *Methylophilus* and *Massilia* genera were an indication of bacterial-mediated manipulation of plant hormones for the purpose of increasing root exudates and lowering stress response, both of which could benefit bacterial growth.

Two additional community members which contributed substantially to differences in root samples over time (SIMPER analysis) had contrasting patterns at the third sampling time point, with one declining in abundance and the other increasing. *Cellvibrio* was more abundant at the beginning of plant growth (plants in trays) as compared to when plants were in the aeroponic system. This genus is composed of aerobic, cellulolytic bacteria, with the ability to degrade pectin and mannan, among other polysaccharides (Ofek-Lalzar et al. 2014). Plants have been shown to purposely secrete pectin in the rhizosphere to stimulate non-pathogenic bacteria for biocontrol purposes (Wu et al. 2015), and similarly, the presence of mannan in rhizosphere polymers was found to enhance biofilm formation of PGPBs, providing protection against environmental stressors for the plant (Pham

et al. 2017). *Cellvibrio*'s presence could be a side effect of plant production of these compounds. In contrast to *Cellvibrio*, *Herbaspirillum* spp. comprised eight OTUs throughout the study, but did not reach high abundances until the last sampling time point. *Herbaspirillum* living in plant roots have been documented producing IAA, siderophores, and 1-aminocyclopropane 1-carboxylic deaminase (ACC deaminase) (Glick 2014). ACC deaminase is an enzyme that bacteria produce to block the ethylene production pathway in plants, as ethylene at high concentrations causes plant tissues to degrade, plant growth to decline, and an eventual loss of leaves under environmental stress (drought, heat, salinity). Therefore, bacteria producing ACC deaminase promote root production and continued exudate release even under these suboptimal environmental conditions, which is one hypothesis as to why *Herbaspirillum* dominance was triggered in our study.

Our third goal was to compare rhizosphere community composition in an aeroponic growth unit to patterns found in traditional soil agriculture, evaluating whether successional patterns during plant growth are possibly universal in early stages of rhizosphere development. Work with plant–microbe interactions in soils identified that bulk soil geochemistry can be the dominant characteristic influencing endophytic microbiome structure, with plant growth stage a secondary control (Lundberg et al. 2012; Breidenbach et al. 2016). In hydroponic systems, the absence of soil allows researchers to fully evaluate the role of rhizodeposits (root exudates) in establishing a microbiome based solely on the interdependencies of the plant–microbe relationship, teasing apart the influence of bulk soil geochemistry and plant-mediated selection forces.

Phylum-level comparisons in published studies show both similar and distinct patterns in composition of root communities between soils and our aeroponic system, including the consistent presence of Bacteroidetes and Proteobacteria in both plant rhizosphere community types, along with increasing abundance of these phyla throughout plant growth (Bulgarelli et al. 2015; Lundberg et al. 2012; Oberholster et al. 2018; Sugiyama et al. 2014). Other phyla dominating communities in soils, such as Actinobacteria, were not found in 58% of our root samples, and in samples with Actinobacteria, abundance was $< 1\%$ of the community. While the Rock Box and reservoir samples contained groups found in other soil analyses of root communities (Firmicutes, Gemmatimonadetes, Cloroflexi, Nitrospirae, Planctomycetes, and Verrucomicrobia), these groups were absent in the majority of root samples in our study, and when present, comprised $< 1\%$ of the community (an exception was Verrucomicrobia, present at 1–3% in two root samples) (Chaparro et al. 2014; Oberholster et al. 2018). The presence of these groups in the water of the aeroponic system may have benefited plants by excluding pathogens in circulation. The lack of these groups in our root samples could indicate that in soil-based studies they are transient when on the surface of roots, maintaining source populations in soils directly surrounding roots. Alternatively, the missing groups in our samples could have been less efficient at root colonization.

In contrast to these phylum-level differences between our findings and published literature, strong concurrence was found with OTUs identified as *Rhizobium* (Alphaproteobacteria) and *Massilia*, which increased in abundance through time in our samples, and in sorghum and sunflowers growing in soils (Oberholster et al. 2018). The OTUs with the highest number of sequences in each of the 27 root samples in this study came from just six families/genera, all found within Proteobacteria: Hydrogenophilaceae, *Rhizobium*, Legionellaceae, *Methylophilus*, *Massilia*, or *Herbaspirillum*. The dominant OTU in each sample ranged from 8 to 37% of the community, with obvious temporal trends (discussed above). These dominant genera are known soil community members, some with documented plant associations within rhizosphere communities. Given our assertion that aeroponic systems exclude transient microbial community members that are primarily soil-based and not root-associated, these six OTUs should be of high interest for further research.

Conclusion

This study is one of only a few published with a focus on microbial root communities of plants grown in an aeroponic system, which is a hydroponic method gaining popularity worldwide. Hydroponic systems using a recirculating water system create the need for nutrient supplementation and concern over the spread of plant disease. Farmers often manipulate rhizosphere

communities through addition of PGPMs, with the goals of pathogen biocontrol, reducing plant stress response, and increasing nutrient supply to plants. Therefore, additional work in soilless crop systems will direct these management practices so they are more effective. We documented early successional patterns in microbial root communities and determined that direct application of water to roots did not prevent a distinctive community of root-associated bacteria from establishing very early in succession (day 12). These communities were temporally dynamic, and successional patterns suggest a response by the root-associated microbiome to water delivery method and plant development. Although there were only a few similarities in phylum- and genus-level OTUs found in this work when compared to soil studies, the dominant taxa we identified (all Proteobacteria) are documented in published research as PGPBs with demonstrated capabilities for increasing plant health. Our work suggests hypotheses to be tested directly in future experiments, using a hydroponic system that avoids the confounding presence of soil particles and isolates plant–microbe interactions.

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Author's contribution JE (co-first author) aided with sampling, generated water chemistry data, analyzed portions of sequence data, and wrote and edited the original draft. JS (co-first author) designed study, lead sampling effort, coordinated lab analyses, analyzed sequence data, wrote portions of the original draft and edited the original draft. HL contributed to laboratory sample processing and to writing portions of the original draft. HH contributed to writing portions of the original draft. DM conceived and designed the study, aided with sampling, and edited the original draft.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on request.

Compliance with ethical standards

Conflict of interest Authors JWE, JDS, HL, HLH, DPM declare they have no conflict of interest.

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