2017

Spatial Variation of Bacterial Communities on the Leaves of a Southern Magnolia Tree

Emily Q. Nguyen
University of Mississippi. Sally McDonnell Barksdale Honors College

SPATIAL VARIATION OF BACTERIAL COMMUNITIES ON THE LEAVES OF A SOUTHERN MAGNOLIA TREE

By

Emily Nguyen

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford May 2016

Approved by

_____

Advisor: Dr. Colin Jackson

_____

Reader: Dr. Ryan Garrick

_____

Reader: Dr. Stephen Brewer

Abstract:

The leaf surface, or phylloplane, is a dynamic environment for its microbial inhabitants, which can be subjected to many environmental factors. Existing phylloplane studies have focused on differences in bacterial community structure between trees of the same species, in different geographical locations, or between trees of different species. Few studies have examined the spatial distribution of bacterial communities on the leaves of a single tree. In this study, leaf samples from different areas of the canopy were obtained from a single *Magnolia grandiflora* tree. Samples were taken from the high (3.5-4.0 m above the ground), middle (1.5 m), inner middle (1.5 m but close to the trunk), and low (0.5 m) portions of the tree canopy, following cardinal directions (north, south, east, west). Following DNA extraction procedures, dual index barcoding was used to sequence the V4 region of the 16S ribosomal RNA gene. Phylloplane communities were dominated by Alphaproteobacteria, Actinobacteria, and Bacteroidetes, which are common inhabitants of plants. Patterns in alpha and beta diversity suggested that the height from which the sample was taken had a strong influence on the shaping of communities, while cardinal direction was not a significant predictor of diversity. Interestingly, the inner leaves of the canopy had higher species abundance than the leaves on the outside of the canopy, which could suggest that the surrounding canopy protects the inner leaves from ultraviolent light, desiccation, or other factors that may limit bacterial growth.

TABLE OF CONTENTS

Acknowledgements

First and foremost, I would like to thank Dr. Colin Jackson for being my thesis advisor! I truly appreciate his patience and guidance throughout this experience. The knowledge that I acquired from lab work and data analysis is something I will carry with me forever, and a door I would have never been able to open without him. I also want to thank my readers Dr. Stephen Brewer and Dr. Ryan Garrick for their advice on what I could have done better. I highly valued their input, and I could not have asked for a better thesis committee! I would also like to thank Mr. Brad Campbell for organizing biology thesis workshops for the Honors College throughout the 2016 fall semester. Mr. Campbell showed great interest in all of our honors projects despite not being in the field himself, but his guidance aided in the structuring and grammar component of our theses. Next, I would like to thank Bram Stone and Eric Weingarten for their help in the lab, especially during the DNA extraction process. Both of them gave me great advice and would stop anything they were working on to answer a question I had. Finally, I would like to thank the Sally Barksdale Honors College for giving me this opportunity!

Introduction:

Microorganisms are ubiquitous on plants, where many exist as pathogens or symbionts, and others are grazers feeding on other microbes. Studies of the phyllosphere as a habitat for microorganisms began in the 1950s and have been an important area of microbiological research since the 1970s (Morris, 2001). Since then, thousands of bacteria, mycelial fungi, yeasts, and protozoa have been detected in the phyllosphere (Chernov *et al*., 2013). While there are different types of microorganisms found on plants, bacteria are by far the most abundant inhabitants of the phyllosphere, the aerial portion of the plant (Turner *et al*., 2013). While phyllosphere is the more common term, many studies on microorganisms associated with plants really focus on the phylloplane, the surface of the leaf, as a habitat for microorganisms (Levetin & Dorsey, 2006). Given that plants cover a significant portion of the global land area and that each plant has several leaves, until we consider the millions of bacteria that each leaf harbors, we cannot fully appreciate the enormity and importance of leaf-microbe interactions (Hirano & Upper, 2000). In terms of total area, it is estimated that the terrestrial leaf surface area colonized by microbes is about 6.4×10^8 km^2 (Morris & Kinkel, 2002).

To understand the ecology and evolution of microorganisms, it is critical for microbiologists to study microorganisms in different environments. The phyllosphere as a habitat for microorganisms is of particular interest as it is much more dynamic than the rhizosphere (the microbial community in soil around the roots) because of variation in

temperature, wind, insects, moisture, and radiation (Turner *et al.*, 2013). The microorganisms in the phyllosphere or phylloplane are important, and the plant microbiome is a key determinant of plant health and productivity (Berendsen *et al.*, 2012) that can influence plant fitness through mechanisms such as plant hormone production and protection against pathogens (Innerebner *et al.*, 2011; Ritpitakphong *et al.*, 2016). Evidence has shown that microbes can alter plant phenotypic plasticity in response to changing environmental conditions by alleviating stress and increasing plant fitness (Goh *et al.*, 2013). During periods of rapid change in the environment, it is possible that microorganisms can aid the holobiont (the host and its microbial symbionts) in surviving (Zilber-Rosenberg & Rosenberg, 2008). A testament to the importance of plant-microbe interactions is mycorrhizal fungi; molecular evidence suggests that their association with green algae was fundamental to the evolution of land plants 700 million years ago (Turner *et al.*, 2013). While mycorrhizal symbioses take place in the rhizosphere, it is not unreasonable to expect that microbial inhabitants of the phyllosphere could be as important to plant fitness. More broadly, Zilber-Rosenberg (2008) developed the hologenome theory of evolution based on four generalizations: 1) All animals and plants form symbiotic relationships with microorganisms. 2) Symbiotic microorganisms are transmitted between generations. 3) The association between host and symbionts affects the fitness of the holobionts. 4) Variation in the hologenome can be brought about by changes in either host or microbiota genomes.

In recent years, the phyllosphere of plants has received substantial attention, however we still have a limited understanding of the diversity and biogeography of phyllosphere bacterial communities, especially at fine scales (Redford *et al.*, 2010;

Turner *et al.*, 2013). The problem lies in the fact that a large number of bacterial species are not easily cultivated; therefore, many bacterial taxa associated with plants have not been captured in culture-based surveys (Yang *et al*., 2001; Yashiro *et al*., 2011). Recent studies, however, such as one on *Arabidopsis thaliana* (Bulgarelli *et al*., 2012), have yielded a better understanding of the diversity and spatial distribution of bacterial communities. The importance of phyllosphere ecology was recognized as early as 1961, when J. Ruinen published a paper entitled "The Phyllosphere - an Ecologically Neglected Milieu" (Ruinen, 1961). Until then, interest in the phylloplane tended to focus on distribution and population dynamics of single microbial species and their relation to foliar diseases (Hirano & Upper, 2000; Stone & Jackson, 2016). Studies on spatial patterns of microbial activity in the phyllosphere have also tended to focus on patterns of disease; thus, autoregressive-moving average (ARMA) models are commonly used (Clayton & Hudelson, 1995). However, Stone & Jackson (2016) state that these models are best when applied unidirectionally, while distance methods are probably more appropriate to compare plants not distributed regularly. Based on research on plant and animal biogeography, it is expected that communities within close proximity will be more similar to each other than communities that are geographically distant (Lomolino *et al.*, 2006), a concept that was generally supported in studies of phyllosphere bacterial communities of magnolia trees on scales of meters to hundreds of meters apart (Stone & Jackson, 2016).

In a recent study, Laforest-Lapointe *et al*. (2016), investigated intra- and inter-individual variation of phyllosphere communities among host tree species. They demonstrated that 65% of the intra-individual variation in bacterial community structure

was attributed to inter-individual and inter-specific differences, while canopy location was not significant. In comparison, host tree species explained 47% of inter-individual and inter-specific variation followed by individual identity (32%) and canopy location (6%) (Laforest-Lapointe *et al.*, 2016). However no other studies have examined variation in the composition of bacterial phyllosphere community on leaves of the same tree. In this study, I will investigate intra-individual variation in the bacterial phyllosphere within a single tree in order to see how a smaller geographic distance may influence community structure, with a particular focus on the diversity and spatial distribution of bacterial populations.

Methods:

In September 2016, leaves were sampled from a *M. grandiflora* tree in a garden in Oxford, Mississippi. Leaf samples were obtained from the high (3.5-4.0 m above the ground), middle (1.5 m), inner middle (1.5 m but close to the trunk), and low (0.5 m) portions of the tree canopy, and following cardinal directions (north, south, east, west) whenever possible given the orientation of the tree. Collected leaves were immediately placed in sterile plastic bags and frozen within 30 minutes for subsequent DNA extraction. The samples were labeled based upon their location on the tree in a two-character system. The first character reflects the height of where the sample was taken. For example, "H" is an abbreviation for "high". The second character represents the cardinal direction of the sample. While four samples from each height of the tree were desirable, accessibility to one part of the tree was restricted so that the HS (high, south) sample could not be collected. An additional sample, HE, was later removed from the analysis because it yielded a low number of sequences (<2,000). Thus, the final study focused on 14 samples, representing four samples (each direction) from the low, inner, and middle parts of the canopy, and two samples (north, west) from the highest part of the canopy.

The phyllosphere community was recovered from each sample by brushing the leaf on both sides for 1 minute with a sterile toothbrush in 4 mL Tris-EDTA (TE) pH 8.0 buffer. The resulting suspension was centrifuged (10,000x*g*) for 2 minutes and the

resulting pellet was frozen (-20° C) until DNA extraction. DNA was extracted using a PowerSoil extraction kit (MoBio, Carlsbad, CA) following the manufacturer's standard procedures. Agarose gel electrophoresis was used to confirm the presence of DNA.

A dual index barcoding strategy was used to target the V4 region of the 16S ribosomal RNA (rRNA) gene (Kozich *et al*., 2013). Primers (1 µL each) and AccuPrime Pfx Supermix (Invitrogen, Grand Island, NY) (17 µL) were mixed with 1 µL DNA. DNA was then amplified for 30 cycles at 95 °C for 20 s, 55 °C for 15 s, and 72 °C for 2 min after an initial denaturation step at 95 °C for 2 min and with a final elongation step of 72 °C for 10 min. The amplification products were standardized with SequalPrep Normalization Plates (Life Technologies, Grand Island, NY) and pooled before sequencing with Illumina MiSeq platform at the Molecular and Genomics Core Facility at the University of Mississippi Medical Center (UMMC).

Raw data files (FASTQ) were processed using Mothur software following procedures recommended by Schloss, *et al.* (2011) and Kozich, *et al.* (2013). The sequences were aligned with the Silva V4 reference database, and non-informative or erroneous data, chimeras, and mitochondrial or chloroplast DNA were removed. The Greengenes database was used to establish sequences into operational taxonomic units (OTUs) with 97% sequence similarity. Diversity analysis was conducted after classifying the OTUs and by subsampling (1000 iterations) the number of reads to the lowest remaining sample (2,030). Alpha diversity was determined as the number of OTUs, coverage, inverse Simpson index, S_{Chao} index, Shannon index, and S_{ACE} index. The abundance-based theta index was used for analysis of molecular variance (AMOVA) which was used to test for differences in phyllosphere community composition based

on height and cardinal direction. In addition, analysis of similarities (ANOSIM) was used to compare species abundance based on the same criteria.

Results:

After removal of sequences identified as Archaea, Eukarya, chloroplast, or mitochondria, the total number of sequences across all leaf samples was 128,625 consisting of 3,090 unique sequences. The number of sequences obtained from each leaf was highly variable and unrelated to its location on the tree (Figure 1). The medium height northern direction sample (MN) had the fewest sequences at 2,030, while the western sample at this height (MW) had the greatest number of sequences at 18,531. In terms of community composition, Proteobacteria was the dominant phylum (Figure 2) in the dataset and accounted for 82,140 of the sequences (63.9%), with class Alphaproteobacteria accounting for 53,145 of those sequences (64.7%). Acidobacteria accounted for 7,326 of the bacterial sequences (5.7%), Actinobacteria accounted for 10,598 of the sequences (8.2%), Bacteroidetes accounted for 11,780 of the sequences (9.2%), Cyanobacteria accounted for 3,899 of the sequences (3.0%), Firmicutes accounted for 1,647 of the sequences (1.3%), Planctomycetes accounted for 3,615 of the sequences (2.8%), and Verrucomicrobia accounted for 1,679 of the sequences (1.3%). Other phyla found at <1% were Armatimonadetes with 945 sequences (0.7%), Chlamydiae with 24 sequences (0.01%), Chlorobi with 6 sequences (0.004%), Chloroflexi with 413 sequences (0.3%), Deinococcus-Thermus with 261 sequences (0.2%), Elusimicrobia with 16 sequences (0.01%), Gemmatimonadetes with 416 sequences (0.3%), Nitrospirae with 54 sequences (0.04%), and Tenericutes with 35 sequences (0.03%).

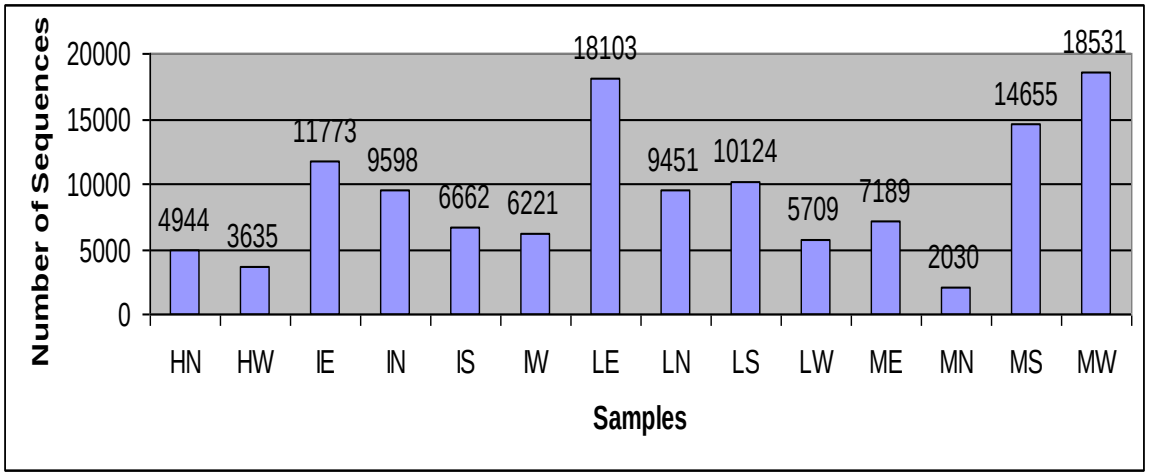

Figure 1: Numbers of 16S rRNA sequence reads obtained from next generation sequencing of phyllosphere bacterial communities on an individual magnolia (*M. grandiflora*) leaves sampled from a single tree. Leaves were collected from high (H) in the canopy, internal in the canopy (I), low in the canopy (L), and of medium (M) height in the canopy, at each of the cardinal directions (second letter designating N, E, S, W). Samples corresponding to HE and HS were not analyzed, and therefore were not shown.

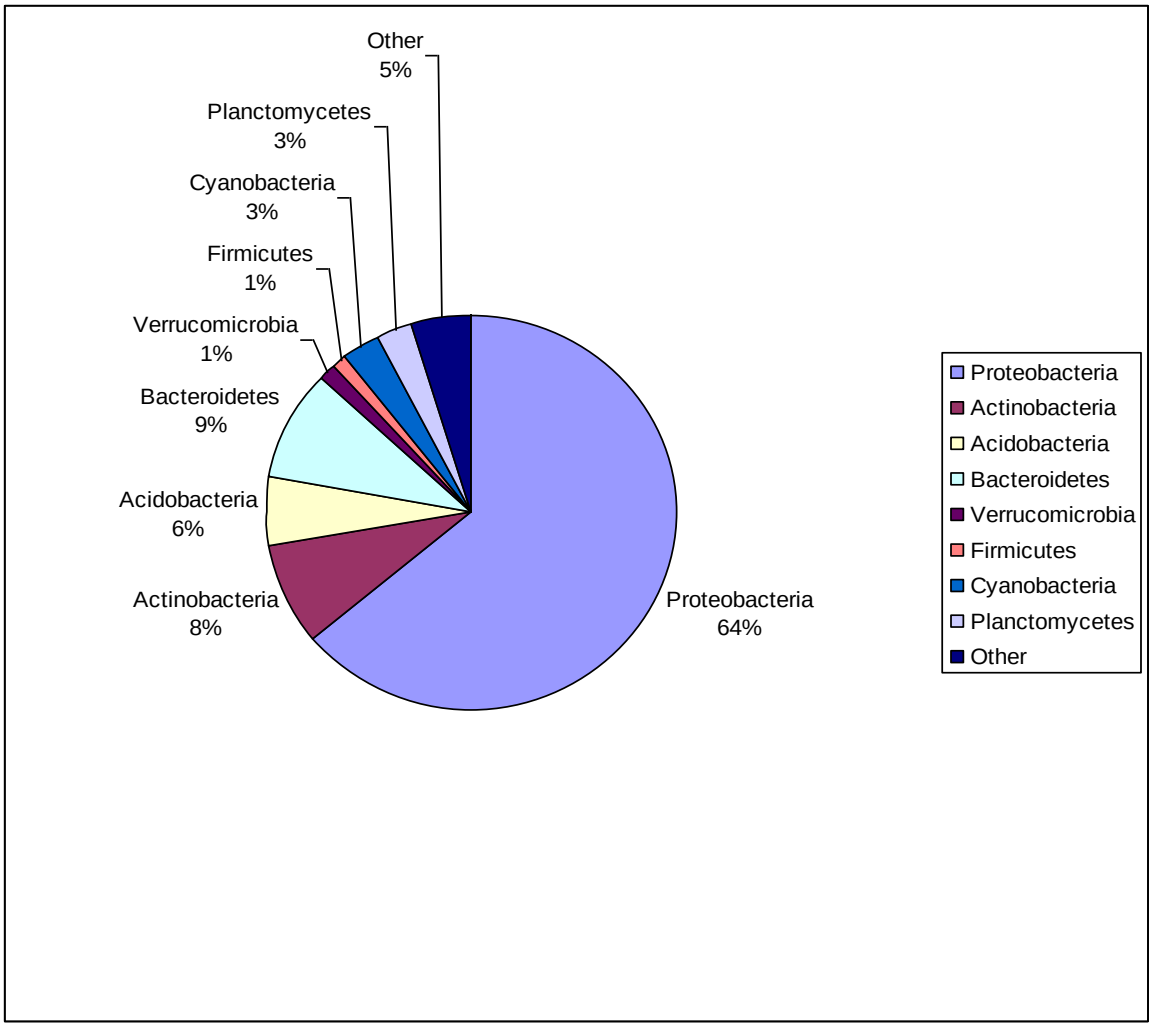

Figure 2: Percentages of major bacterial phyla occupying a single *M. grandiflora* tree. Any phyla accounting for >1% of total sequences (128,625) are shown. Proteobacteria accounted for most of the sequences (64%), while Bacteroidetes (9%), Actinobacteria (8%), and Acidobacteria (6%) also represented a significant amount of the sequences. Phyla that accounted for <1% were grouped into the "other" category.

At a finer taxonomic level, 17 OTUs had >1000 reads and accounted for 66,870 sequences or 52.0% of the total (Table 1). A member of the Alphaproteobacteria, order Rhizobiales, contained the most reads out of all the OTUs and accounted for 12,800 sequences out of the total 128,625 sequences (10.0%). Other members of Alphaproteobacteria in the 17 most prominent OTUs were identified as being members of the genus *Methylobacterium* with 5,529 sequences (4.3%), order Rhizobiales with 2,457 sequences (1.9%), genus *Sphingomonas* (99) with 7,387 sequences (5.7%), genus *Sphingomonas* (100) with 3,722 sequences (2.9%), and genus *Wittichii* with 3,233 sequences (2.5%). Betaproteobacteria, genus *Massilia*, contained 5,844 sequences (4.5%); Deltaproteobacteria, genus *Cystobacter* contained 2,557 sequences (2.0%), and family *Cystobacterinea* contained 2,655 sequences (2.1%). A member of the Gammaproteobacteria, species identified as *E. coli*, contained 5,259 sequences (4.1%), but of all the major OTUs, this was the only one identified without much certainty (68% match; Table 1). Acidobacteria, family Acidobacteriaceae, contained 3,423 sequences (2.6%), and genus *Terriglobus* contained 1,059 sequences (0.8%). Actinobacteria, family Microbacteriaceae, contained 3,575 sequences (2.8%) and genus *Actinomycetospora* had 1,732 sequences (1.3%). Bacteroidetes, genus *Hymenobacter*, contained 2,850 sequences (2.2%). Cyanobacteria contained 2,788 sequences (2.2%).

Table 1: Ranking of the 17 most abundant OTUs with the number in parenthesis representing the confidence interval of whether the phylum, class, order, family, genus or species was properly identified. Each of these OTUs was comprised of >1,000 sequence reads.

Ranking of Most Abundant OTUs	Number of OTUs obtained	Taxonomy

1	12,800	p__Proteobacteria(100);
		c__Alphaproteobacteria(100);
		o__Rhizobiales(100);
		o__Rhizobiales_unclassified(100)

2	7,387	p__Proteobacteria(100);
		c__Alphaproteobacteria(100);
		o__Sphingomonadales(100);
		f__Sphingomonadaceae(100);
		g__Sphingomonas(99)

3	5,844	p__Proteobacteria(100);
		c__Betaproteobacteria(100);
		o__Burkholderiales(100);
		f__Oxalobacteraceae(100);
		g__Massilia(98)

4	5,529	p__Proteobacteria(100);
		c__Alphaproteobacteria(100);
		o__Rhizobiales(100);
		f__Methylobacteriaceae(100);
		g__Methylobacterium(100);

5	5,259	p__Proteobacteria(100);
		c__Gammaproteobacteria(100);
		o__Enterobacteriales(100);
		f__Enterobacteriaceae(100);
		g__Escherichia(68);s__coli(68);

6	3,722	p__Proteobacteria(100);
		c__Alphaproteobacteria(100);
		o__Sphingomonadales(100);
		f__Sphingomonadaceae(100);
		g__Sphingomonas(100);

7	3,575	p__Actinobacteria(100);
		c__Actinobacteria(100);
		o__Actinomycetales(100);
		f__Microbacteriaceae(100);
		f__Microbacteriaceae_unclassified(100)

8	3,233	p__Proteobacteria(100);

		c__Alphaproteobacteria(100);
		o__Sphingomonadales(100);
		f__Sphingomonadaceae(100);
		g__Sphingomonas(100);
		s__wittichii(97)

9	2,850	p__Bacteroidetes(100);
		c__Cytophagia(100);
		o__Cytophagales(100);
		f__Cytophagaceae(100);
		g__Hymenobacter(100)

10	2,788	p__Cyanobacteria_unclassified(100)

11	2,655	p__Proteobacteria(100);
		c__Deltaproteobacteria(100);
		o__Myxococcales(100);
		f__Cystobacterineae(100);
		f__Cystobacterineae_unclassified(100)

12	2,557	p__Proteobacteria(100);
		c__Deltaproteobacteria(100);
		o__Myxococcales(100);
		f__Cystobacteraceae(100);
		g__Cystobacter(100);
		s__fuscus(90)

13	2,457	p__Proteobacteria(100);
		c__Alphaproteobacteria(100);
		o__Rhizobiales(100)

14	2,128	p__Acidobacteria(100);
		c__Acidobacteriia(100);
		o__Acidobacteriales(100);
		f__Acidobacteriaceae(100);
		f__Acidobacteriaceae_unclassified(100);

15	1,732	p__Actinobacteria(100);
		c__Actinobacteria(100);
		o__Actinomycetales(100);
		f__Pseudonocardiaceae(100);
		g__Actinomycetospora(100);

16	1,295	p__Acidobacteria(100);
		c__Acidobacteriia(100);
		o__Acidobacteriales(100);
		f__Acidobacteriaceae(100);
		f__Acidobacteriaceae_unclassified(100);

17	1,059	p__Acidobacteria(100);
		c__Acidobacteriia(100);
		o__Acidobacteriales(100);
		f__Acidobacteriaceae(100);
		g__Terriglobus(100);

In addition to the dominant OTUs, the number of unique sequences in each phylum was determined out of a total of 3,090 unique sequences, as well as the number of unique sequences of that phyla found per sample. Below, I included phylum listed within the 17 most abundant OTUs without taking into account the number of times each unique sequence appears (it is only counted once) to emphasize the concentration of bacterial species in the inner leaves of the tree:

- Acidobacteria accounted for 165 unique sequences with 19 in HN, 20 in HW, 58 in IE, 59 in IN, 35 in IS, 40 in IW, 30 in LE, 44 in LN, 31 in LS, 7 in LW, 55 in ME, 14 in MN, 25 in MS, and 22 in MW.
- Actinobacteria accounted for 258 unique sequences with 16 in HN, 67 in HW, 108 in IE, 84 in IN, 50 in IS, 60 in IW, 75 in LE, 99 in LN, 57 in LS, 31 in LW, 70 in ME, 40 in MN, 53 in MS, and 46 in MW.
- Alphaproteobacteria accounted for 487 unique sequences with 85 in HN, 102 in HW, 187 in IE, 133 in IN, 119 in IS, 172 in IW, 133 in LE, 146 in LN, 103 in LS, 58 in LW, 117 in ME, 78 in MN, 151 in MS, and 132 in MW.
- Bacteroidetes accounted for 388 unique sequences with 32 in HN, 38 in HW, 87 in IE, 87 in IN, 59 in IS, 122 in IW, 57 in LE, 86 in LN, 65 in LS, 16 in LW, 50 in ME, 30 in MN, 92 in MS, and 125 in MW.
- Betaproteobacteria accounted for 79 unique sequences with 10 in HN, 15 in HW, 27 in IE, 23 in IN, 20 in IS, 20 in IW, 19 in LE, 19 in LN, 10 in LW, 22 in ME, 13 in MN, 14 in MS, and 13 in MW.

- Cyanobacteria accounted for 72 unique sequences with 10 in HN, 16 in HW, 21 in IE, 18 in IN, 13 in IS, 17 in IW, 12 in LE, 18 in LN, 11 in LS, 5 in LW, 16 in ME, 9 in MN, 15 in MS, and 14 in MW.
- Deltaproteobacteria accounted for 159 unique sequences with 20 in HN, 20 in HW, 49 in IE, 40 in IN, 28 in IS, 44 in IW, 29 in LE, 30 in LN, 22 in LS, 15 in LW, 20 in ME, 9 in MN, 39 in MS, and 47 in MW.
- Gammaproteobacteria accounted for 89 unique sequences with 14 in HN, 23 in HW, 29 in IE, 20 in IN, 16 in IS, 25 in IW, 16 in LE, 24 in LN, 21 in LS, 12 in LW, 23 in ME, 11 in MN, 13 in MS, and 18 in MW.

Patterns in Alpha and Beta Diversity

Alpha diversity and beta diversity were determined after subsampling each sample to 2,030 sequences (the number of sequences in the lowest remaining sample). Coverage (how well the community was sampled) was generally good with >0.9 being ideal (Table 2). At a basic level, the number of OTUs observed in each sample is a simple assessment of alpha diversity, and the most OTUs (475) were found in the western focused inner canopy sample (IW) and the least (161) in the western lower canopy (LW) (Figure 3).

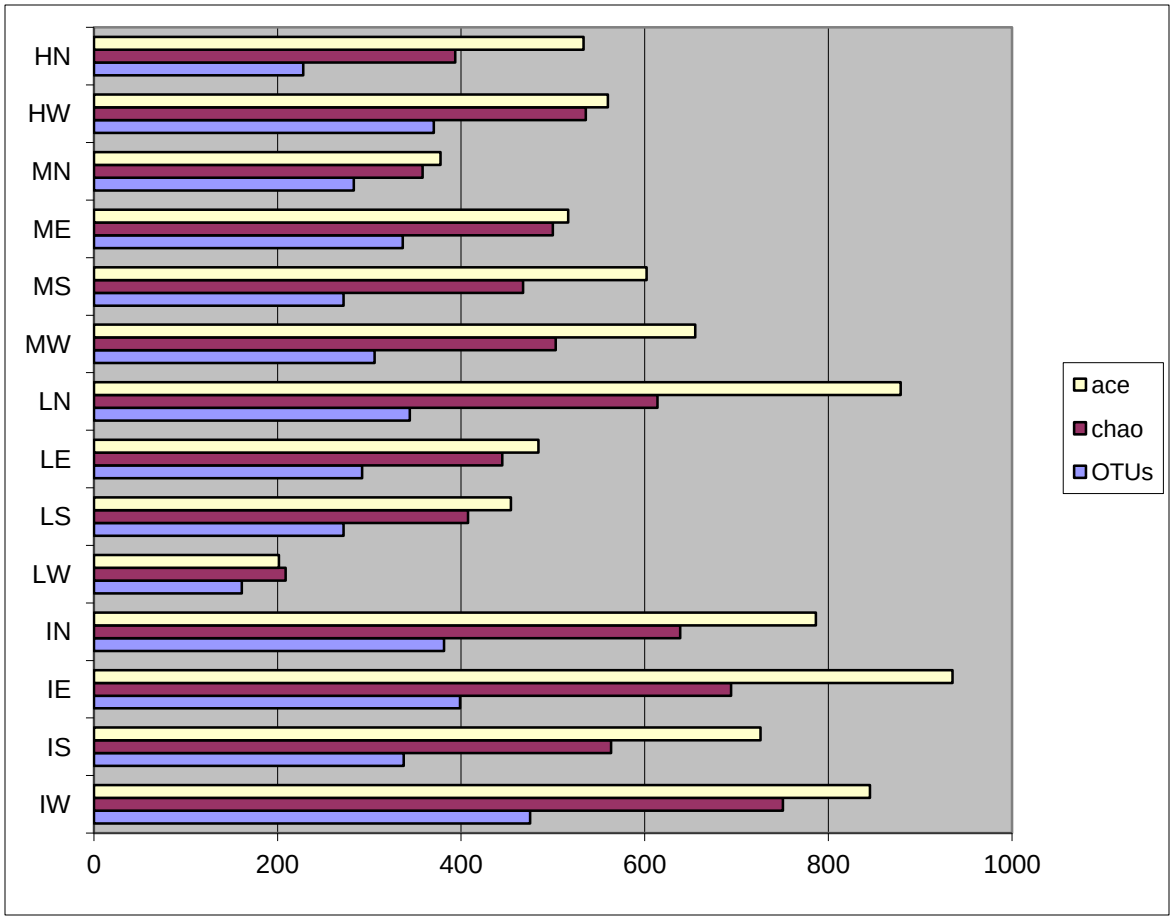

Figure 3: Comparison of S_{obs}, S_{chao}, and S_{ace} indices for *M. grandiflora* after subsampling to 2,030 sequences. The S_{chao} and S_{ace} indices estimated that the actual number of OTUs was greater than what S_{obs} revealed. S_{chao} and S_{ace} are nonparametric indexes that estimate species richness by adding a correlation factor to the number of observed species, where rare OTUs may be found more frequently. S_{chao} accounts for singletons and doubletons, while S_{ACE} incorporates data from species with <10 reads (Chao, 1984).

Species diversity and richness varied across different locations on the tree but did show noticeable patterns (Table 2). Sample IW had the highest observed OTUs, species richness, and diversity. LW showed the least observed OTUs, species richness, and diversity out of all samples. The ranking of samples was IW > HW > IE > MW > MN > MS > IN > HN > ME > LN > LE > LS > IS > LW for inverse Simpson, and IW > IE > IN > LN > IS > HW > MW > ME > MS > LE > LS > HN > MN > LW for S_{chao}. The height of where the sample was taken seemed to indicate more differentiation between bacterial communities than cardinal direction, as it generally did not affect the inverse Simpson or S_{chao} index.

The samples with the highest inverse Simpson were IW (70.8), HW (46.4), and IE (39.1), while the samples with the highest S_{chao} index were IW (750.4), IE (693.9), and IN (638.5). Inverse Simpson indexes were generally higher in samples categorized by "W" as the top four samples with the highest index included IW, HW, and MW. This is the only incidence where cardinal direction seemed to affect bacterial community structure. LW had the lowest inverse Simpson index and S_{chao} index but this may be because of the height of where the sample was taken. Samples in the inner, high, and middle parts of the tree tended to have higher diversity and richness than samples in the low parts of the tree. Sample IS was an exception to this as it had the second lowest inverse Simpson index. Including sample LN, the inner samples had the highest S_{chao} indexes. MW, ME, and MS tended to have higher S_{chao} indexes than the samples from the lower part of the tree, with the exception of MN, which had a S_{chao} index of 358.1 (second lowest S_{chao}). Overall, patterns in alpha diversity suggested that: 1) the height of where the sample was taken seemed to shape bacterial communities more than cardinal direction, 2) the inner leaves

of the tree had the highest species richness and diversity, and 3) samples on the lower leaves of the tree tended to have lower diversity and richness.

Table 2: Alpha diversity indices S_{obs}, coverage, inverse Simpson, Shannon, S_{chao}, and S_{ace} were used to estimate species diversity and richness. The coverage determines sampling effort with 1.0 being perfect and ~0.9 acceptable. S_{obs} (number of observed OTUs) was used by S_{chao} and S_{ace} calculators to obtain a closer estimate to species richness (also see Figure 3). Inverse Simpson and Shannon diversity indexes simultaneously took into account the different species in the dataset and how evenly they were distributed to generate a "score" of diversity with the higher scores indicating higher diversity.

Group	OTUs	Coverage	I. Simpson	Shannon	S_{chao}	S_{ace}
IW	475	0.89	70.8	5.18	750	845
IE	399	0.90	39.1	4.69	694	935
IN	381	0.91	30.3	4.55	638	786
HW	370	0.93	46.4	4.84	536	560
LN	344	0.91	26.6	4.35	614	879
IS	337	0.92	22.8	4.30	563	726
ME	336	0.93	27.2	4.39	500	517
MW	306	0.93	37.9	4.48	503	655
LE	292	0.94	24.3	4.16	445	484
MN	283	0.95	35.7	4.42	358	378
MS	272	0.94	33.6	4.27	468	602
LS	272	0.94	24.2	4.12	408	454
HN	228	0.95	29.3	4.05	394	534
LW	161	0.98	17.0	3.79	209	202

Examining patterns of beta diversity, it was highly suggestive that canopy height or position influenced community composition when analyzed by AMOVA ($p=0.08$) and significant when analyzed by ANOSIM ($p=0.016$). In contrast, cardinal direction did not influence community composition, an outcome validated by both AMOVA ($p=0.778$) and ANOSIM ($p=0.868$).

Discussion:

Microbial inhabitants of the phyllosphere have the potential to influence plant biogeography and ecosystem function by improving a plant's performance in response to its environment (Fürnkranz et al., 2008; Friesen et al., 2011; Meyer & Leveau, 2012). However, most studies of phyllosphere communities have assumed within-plant variation in the bacterial community to be negligible, an exception being a study focused within the canopy of a single *Ginkgo biloba* tree (Leff *et al*., 2015). Typically for tree phyllosphere studies, samples are taken from leaves at the bottom of the canopy or at mid-canopy height near the trunk (Laforest-Lapointe *et al*., 2016), likely because of easy access by researchers. Samples can also be taken from specific canopy locations (e.g. Kembel *et al*. 2014; Kembel & Mueller, 2014) or from multiple leaves around the canopy at the same height (e.g. Redford & Fierer, 2009; Redford *et al*., 2010; Jackson & Denney, 2011). The aim of this study was to compare phyllosphere bacterial communities within a single *M. grandiflora* tree by taking samples from different heights, while also gathering samples from each cardinal direction (North, West, East, and South) at each height.

Certain bacterial groups re-establish themselves on plants from year to year, such that it has been argued that plants harbor local reservoirs of bacteria (Feil *et al*., 2005). Analysis of phyllosphere bacterial communities on pine trees found that species variability was lower within a plant species than between different plant species, even over large geographical distances (Redford *et al*., 2010). Similar results have been found

for *A. thaliana* but geographical site is also important factor (Knief *et al*., 2010). Variability in dominant bacterial communities found in the phyllosphere, soil (Lauber *et al*., 2009), and air (Bowers *et al*., 2009) suggests that phyllosphere bacteria are not simply transient inhabitants but are closely associated with host tree species (Redford *et al*., 2010).

Many of the bacterial phyla identified in this study have also been found in other *M. grandiflora* studies. These include the Proteobacteria (dominant class: Alphaproteobacteria), Bacteroidetes, and Actinobacteria (Jackson & Denney, 2011; Stone & Jackson, 2016). Alphaproteobacteria are common inhabitants of plants and include extracellular or intracellular plant mutualists (Delmotte *et al*., 2009). Bacteroidetes and Actinobacteria are also commonly represented in the phyllosphere of many plants including *A. thaliana*, soybean, clover, and rice (Vorholt, 2012). Acidobacteria, Armatimonadetes, Chloroflexi, Cyanobacteria, Firmicutes, Planctomycetes, and Verrucomicrobia were represented at smaller quantities but have also been previously identified in the *M. grandiflora* phylloplane during various seasons (Jackson & Denney, 2011; Stone & Jackson, 2016).

The most prominent OTU identified in this study was classified as a member of order Rhizobiales (Alphaproteobacteria), a group of nitrogen fixing bacteria commonly found in soil that must colonize a host plant for survival (Prell & Poole, 2006). This suggests that leaf-associated communities can contain more typically soil bacteria. Less abundant OTUs are most likely transient species introduced by the atmosphere, precipitation, and contact with animal and plant dispersal vectors (Kembel *et al*., 2014). Interestingly, a member of Enterobacteriaceae was the fifth most prominent OTU. This

was identified as being *E. coli* but with only a 68% certainty, the lowest percentage of any major OTU. While the presence of *E. coli* could suggest contamination during sample processing, the low percentage match more likely reflects another enteric bacterium of birds and mammals, and the likelihood of fecal contamination of the phyllosphere by such species.

Of the 17 most prominent OTUs, there were many unique sequences among the inner leaves of the canopy, especially in the innermost eastern part of the canopy (IE). For example, Acidobacteria accounted for 165 unique sequences with the most abundant sequences (58) being in IE. The pattern of IE containing the most sequences continues with Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Cyanobacteria, Deltaproteobacteria, and Gammaproteobacteria. Alpha diversity results indicated that the innermost western sample (IW) had the overall most OTUs and highest inverse Simpson, Shannon, and S_{chao} indexes. The only major phyla from the 17 most prominent OTUs that did not have the most reads in the inner leaf samples was Bacteroidetes, which was concentrated in the middle leaves of the tree. The lower western (LW) part of the tree also consistently showed the least diversity as shown with Actinobacteria, Alphaproteobacteria, Bacteroidetes, Betaproteobacteria, and Cyanobacteria. ANOSIM and AMOVA did not detect cardinal direction as significant drivers of community composition but detected the sample height (H-I-M-L) as significant. While AMOVA was suggestive of population differentiation depending on height, ANOSIM was shown to be more significant in terms of sample height influencing species abundance. Results from alpha and beta diversity analyses suggested overall patterns in species diversity and richness (high in inner leaves, low in lower leaves), however there were many exceptions

to the general pattern. The phylloplane is a highly dynamic environment, unlike the rhizosphere which is surrounded by soil in which microorganisms can survive in a dormant state for many years or even decades. The microorganisms of the phyllosphere must adjust to fluctuations in the season and the day/night cycle and developmental, morphological, and anatomical dynamics of the plant from the bud, to the senescing leaf, to the flower, and to the fruit (Bringel & Couée, 2015). Variation in community composition within the canopy is therefore likely, and more than the sample height is necessary to explain why differences occur in bacterial community structure.

A multitude of factors could influence phyllosphere bacterial communities on leaves including wind (Laforest-Lapointe *et al*., 2016), ultraviolet (UV) light (Laforest-Lapointe *et al*., 2016; Bringel & Couée, 2015), radiation (Kadiver & Stapleton, 2003; Bringel & Couée, 2015), pollution (Vorholt, 2012), temperature (Bringel & Couée, 2015), desiccation (Beattie, 2011; Bringel & Couée, 2015), leaf age, limitation in nutrients such as carbon and nitrogen (Bringel & Couée, 2015), and the presence of other microorganisms (Vorholt, 2012). In addition, host genetic factors (Bodenhausen *et al*., 2014; Horton *et al*., 2014) and taxonomic identity (Redford *et al*., 2010; Kembel *et al*., 2014) seem to be important drivers of phyllosphere bacterial community structure. Numerous studies of host-microbe interactions have found that microbial biodiversity is a trait that forms as part of the extended phenotype of the host organism (Benson *et al*., 2010). Plant genetics determines leaf texture and types of metabolites released to the surface of the leaf, which may promote or inhibit bacterial growth (Rastogi *et al*., 2013).

Because of the many chemical and physical factors that influence the growth of bacteria, there must be selection for bacterial phenotypes that can overcome these

limitations, such as phenotypes that can modify the microhabitat in order to increase nutrient availability (Lindow & Brandl, 2003). Physical factors that may influence what types of bacteria colonize a specific leaf include the size, texture, and thickness of the leaf. Photosynthetic activity and water content are also important attributes of the leaf that should be considered (Lichtenthaler *et al*., 1981), although the extent that these vary between leaves of a single tree is generally unknown. Comparisons between plant species are much more common in phyllosphere studies; for example, the number of culturable bacteria from broad-leaf succulent herbaceous plants such as cucumber, lettuce, and bean is significantly higher than that of grasses or waxy broad-leaf plants such as cabbage and citrus (O'Brien & Lindow, 1989; Lindow & Andersen 1996; Kinkel *et al.* 2000).

Before bacteria can colonize a leaf surface, they must first encounter the waxy surface of the leaf (cuticle) which serves as a diffusion barrier to minimize water and solute loss, minimize temperature fluctuations, confer water repellence, and provide protection against pathogens (Beattie, 2002). *M. grandiflora* has leaves with a waxy coating that makes them resistant to damages from salt and air pollution (Sternberg *et al*., 2004). As the leaves grow older, the cuticle erodes and wettability increases, allowing for microorganisms to inhabit the leaf easier (Beattie, 2002). Phyllosphere bacteria produce polysaccharides that form cell aggregates that protect against desiccation (Lindow & Brandl, 2003; Delmotte *et al*., 2009; Rastogi *et al*., 2013), as well as potential biosurfactants that increase wettability (Bunster et al., 1989; Schreiber *et al*., 2005).

Bacterial abundance on leaves has been correlated with leaf position, as influenced by wind and local leaf humidity (Medina-Martínez *et al*., 2015). Wind exposure can reduce leaf moisture and induce stomata closure (Grace *et al*., 1975), which

could impact the diffusion of nutrients and reduce the size of microbial aggregates (Leveau & Lindow, 2001; Miller et al., 2001). The characteristics of the upper and lower phylloplane (Eglinton & Hamilton, 1967; Schreiber *et al*., 2004; Reisberg *et al*., 2013) are able to affect the interactions between microorganisms by modulating access to nutrients (Ruinen, 1961; Bulgarelli *et al*., 2013), providing more or less protection from sunlight (Atamna-Ismaeel *et al*., 2012), or presenting gateways for penetration into the plant (Hirano & Upper, 2000; Schreiber *et al*., 2004; Bringel & Couée, 2015). Because the inner leaves of the canopy are more shielded from harmful UV light, partial shade and protection provided by the outside leaves could result in higher species diversity and richness on inside leaves, as seen in this study.

Phyllosphere-associated microorganisms live in a sunlight-exposed habitat. Culture-independent analyses indicate that tolerance or intolerance to UV radiation is an important selection pressure for survival and growth of bacteria (Kadivar and Stapleton 2006; Stapleton and Simmons 2006), and most phyllosphere bacteria on the leaf are capable of withstanding high UV radiation though the formation of pigments (Sundin, 2002). Some phyllosphere bacteria are photosynthetic, so they can benefit from light and use light energy to fix carbon (Atamna-Ismaeel *et al*., 2012; Stiefel et al., 2013).

Leaf morphology and age could also be considered in the sense of factors that may allow for successful colonization of a leaf. Further work must involve colonization and growth of bacterial communities on leaves in order to understand the drivers of phyllosphere bacterial community structure (Stone & Jackson, 2016). In addition, random colonization via atmospheric air flow (Barbeán *et al*., 2015) or animals (Scheffers *et al*., 2013), competition between bacterial populations (Vorholt, 2012); or intra-individual

variation in leaf functional traits (Hunter *et al*., 2010; Reisberg *et al*., 2012) are needed to understand the dynamics driving intra-individual variability in bacterial community structure.

Bibliography:

1. Atamna-Ismaeel N., Finkel O., Glaser F., Von Mering C., Vorholt J.A., Koblížek M., Belkin S., Béjà O. (2012) Bacterial anoxygenic photosynthesis on plant leaf surfaces. Environmental Microbiology Reports. 4(2):209–216. doi: 10.1111/j.1758-2229.2011.00323.x.
2. Barberán A., Ladau J., Leff J.W., Pollard K.S., Menninger H.L., Dunn R.R., Fierer N. (2015) Continental-scale distributions of dust-associated bacteria and fungi. Proceedings of the National Academy of Sciences, USA. 112(18):5756–5761. doi: 10.1073/pnas.1420815112.
3. Beattie G.A. (2002) Leaf surface waxes and the process of leaf colonization by microorganisms. In: Phyllosphere Microbiology. Lindow S.E., Hecht-Poinar E.I., Elliott V.J., editors. St. Paul, USA: APS Press. pp. 3–26.
4. Beattie G. A. (2011) Water relations in the interaction of foliar bacterial pathogens with plants. Annual Review of Phytopathology. 49: 533–555. doi: 10.1146/annurev-phyto-073009-114436.
5. Benson A.K., Kelly S.A., Legge R., Ma F., Low S., Kim J., Zhang M., Oh P., Nehrenberg D., Hua K., Kachman S.D., Moriyama E.N., Walter J., Peterson D.A., Pomp D. (2010) Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proceedings of the National

Academy of Sciences. 107(44):18933–18938. doi: 1
0.1073/pnas.1007028107.

6. Berendsen R. L., Pieterse C. M., Bakker P. A. (2012) The rhizosphere microbiome and plant health. Trends in Plant Science. 17(8): 478–486. doi:10.1016/j.tplants.2012.04.001.
7. Bodenhausen N., Bortfeld-Miller M., Ackermann M., Vorholt J. A. (2014). A synthetic community approach reveals plant genotypes affecting the phyllosphere microbiota. PLOS Genetics. 10(4):e1004283. doi: 10.1371/journal.pgen.1004283.
8. Bowers R., Lauber C., Wiedinmyer C., Hamady M., Hallar A., Fall R., Knight R., Fierer N. (2009) Characterization of airborne microbial communities at a high elevation site and their potential to act as atmospheric ice nuclei. Applied and Environmental Microbiology. 75(15):5121–5130. doi: 10.1128/AEM.00447-09.
9. Bringel F., Couée I. (2015) Pivotal roles of phyllosphere microorganisms at the interface between plant functioning and atmospheric trace gas dynamics. Frontiers in Microbiology. 6: 486. doi: 10.3389/fmicb.2015.00486.
10. Bulgarelli D., Rott M., Schlaeppi K., Ver Loren van Themaat E., Ahmadinejad N., Assenza F., Rauf P., Huettel B., Reinhardt R., Schmelzer E., Peplies J., Gloeckner F.O., Amann R., Eickhorst T., Schulze-Lefert P. (2012) Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. Nature. 488: 91–95. doi: 10.1038/nature11336.

11. Bulgarelli D., Schlaeppi K., Spaepen S., Ver Loren van Themaat E., Schulze-Lefert P. (2013) Structure and functions of the bacterial microbiota of plants. Annual Review of Plant Biology. 64 807–838. doi: 10.1146/annurev-arplant-050312-120106

12. Bunster L., Fokkema N. J., Schippers B. (1989) Effect of surface-active *Pseudomonas* spp. on leaf wettability. Applied and Environmental Microbiology. 55(6): 1340-1345.

13. Chao, A (1984) Non-parametric estimation of the number of classes in a population. Scandinavian Journal of Statistics. 11:265-270.

14. Chernov T.I., Zhelezova A.D., Manucharova N.A., Zviagintsev D.G. (2013) Monitoring of the chitinolytic microbial complex of the phylloplane. Bulletin of the Russian Academy of Sciences. 40: 527-532. doi: 10.1134/S1062359013060034

15. Clayton M.K., Hudelson B.D. (1995) Confidence intervals for autocorrelations based on cyclic samples. Journal of the American Statistical Association. 90: 753–757. doi: 10.2307/2291088

16. Delmotte N., Knief C., Chaffron S., Innerebner G., Roschitzki B., Schlapbach R., Von Mering C., Vorholt J.A. (2009) Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proceedings of the National Academy of Sciences, USA. 106(38): 16428–16433. doi: 10.1073/pnas.0905240106.

17. Eglinton G., Hamilton R. J. (1967) Leaf epicuticular waxes. Science. 156: 1322–1335. doi: 10.1126/science.156.3780.1322.

18. Feil H., Feil W.S., Chain P., Larimer F., DiBartolo G., Copeland A., Lykidis A., Trong S., Nolan M., Goltsman E., Thiel J., Malfatti S., Loper J.E., Lapidus A., Detter J.C., Land M., Richardson P.M., Kyrpides N.C., Ivanova N., Lindow S.E. (2005). Comparison of the complete genome sequences of *Pseudomonas syringae* pv. syringae B728a and pv. tomato DC3000. Proceedings of the National Academy of Sciences, USA. 102(31): 11064–11069. doi: 10.1073/pnas.0504930102.

19. Friesen M.L., Porter S.S., Stark S.C., von Wettburg E.J., Sachs J.L., Martinez-Romero E. (2011) Microbially mediated plant functional traits. Annual Review of Ecology, Evolution, and Systematics. 42: 23–46. doi: 10.1146/annurev-ecolsys-102710-145039.

20. Fürnkranz M., Wanek W., Richter A., Abell G., Rasche F., Sessitsch A. (2008) Nitrogen fixation by phyllosphere bacteria associated with higher plants and their colonizing epiphytes of a tropical lowland rainforest of Costa Rica. International Society for Microbial Ecology. 2(5): 561-570. doi: 10.1038/ismej.2008.14.

21. Goh C.H., Veliz Vallejos D. F., Nicotra A. B., Mathesius U. (2013) The Impact of Beneficial Plant-Associated Microbes on Plant Phenotypic Plasticity. Journal of Chemical Ecology. 39(7): 826–839. doi: 10.1007/s10886-013-0326-8.

22. Grace J., Malcolm D.C., Bradbury I.K. (1975) Effect of wind and humidity on leaf diffusive resistance in Sitka spruce seedlings. Journal of Applied Ecology. 12(3): 931–940. doi: 10.2307/2402099.

23. Hirano S. S., Upper C. D. (2000) Bacteria in the Leaf Ecosystem with Emphasis on *Pseudomonas syringae*—a Pathogen, Ice Nucleus, and Epiphyte. Microbiology and Molecular Biology Reviews. 64(3): 624–653. doi: 10.1128/MMBR.64.3.624-653.2000.

24. Horton M.W., Hancock A.M., Huang Y.S., Toomajian C., Atwell S., Auton A., Muliyati N.W., Platt A., Sperone F.G., Vilhjálmsson B.J., Nordborg M., Borevitz J.O., Bergelson J. (2012) Genome-wide patterns of genetic variation in worldwide *Arabidopsis thaliana* accessions from the RegMap panel. Nature Genetics. 44: 212–216. doi: 10.1038/ng.1042.

25. Hunter P.J., Hand P., Pink D., Whipps J.M., Bending G.D. (2010) Both leaf properties and microbe–microbe interactions influence within-species variation in bacterial population diversity and structure in the lettuce (*Lactuca* species) phyllosphere. Applied and Environmental Microbiology. 76(24): 8117–8125. doi: 10.1128/AEM.01321-10.

26. Innerebner G., Knief C., Vorholt J.A. (2011) Protection of Arabidopsis thaliana against leaf-pathogenic *Pseudomonas syringae* by *Sphingomonas* strains in a controlled model system. Applied and Environmental Microbiology. 77(10): 3202–3210. doi: 10.1128/AEM.00133-11.

27. Jackson C.R., Denney W.C. (2011) Annual and seasonal variation in the phyllosphere bacterial community associated with leaves of the southern magnolia (*Magnolia grandiflora*). Microbial Ecology. 61(1): 113–122. doi: 10.1007/s00248-010-9742-2.

28. Kadivar H., Stapleton A. E. (2003) Ultraviolet radiation alters maize phyllosphere bacterial diversity. Microbial Ecology. 45: 353–361. doi: 10.1007/s00248-002-1065-5

29. Kembel S.W., Mueller R.C. (2014) Plant traits and taxonomy drive host associations in tropical phyllosphere fungal communities. Botany. 92(4): 303–311. doi: 10.1139/cjb-2013-0194.

30. Kembel S.W., O'Connor T.K., Arnold H.K., Hubbell S.P., Wright S.J., Green J.L. (2014) Relationships between phyllosphere bacterial communities and plant functional traits in a neo tropical forest. Proceedings of the National Academy of Sciences. 111: 13715–13720. doi: 10.1073/pnas.1216057111.

31. Kinkel L.L., Wilson M., Lindow S.E. (2000) Plant species and plant incubation conditions influence variability in epiphytic bacterial population size. Microbial Ecology. 39: 1–11. doi:10.1007/s002489900182.

32. Knief C., Frances L., Vorholt J. A. (2010) Competitiveness of diverse Methylobacterium strains in the phyllosphere of *Arabidopsis thaliana* and identification of representative models, including M. extorquens PA1. Microbial Ecology. 60: 440–452. doi: 10.1007/s00248-010-9725-3.

33. Kozich J.J., Westcott S.L., Baxter N.T., Highlander S.K., Schloss P.D. (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Applied and Environmental Microbiology. 79: 5112-5120. doi:10.1128/AEM.01043-13.

34. Laforest-Lapointe I., Messier C., Kembel S.W. (2016) Host species identity, site and time drive temperate tree phyllosphere bacterial community structure. Microbiome. 4:27. doi: 10.1186/s40168-016-0174-1.

35. Lauber C., Knight R., Hamady M,. Fierer N. (2009) Soil pH as a predictor of soil bacterial community structure at the continental scale: a pyrosequencing-based assessment. Applied and Environmental Microbiology. 75:5111–5120. doi: 10.1128/AEM.00335-09.

36. Leff J.W., Del Tredici P., Friedman W.E., Fierer N. (2015) Spatial structuring of bacterial communities within individual *Ginkgo biloba* trees. Environmental Microbiology. 17(7): 2352–2361. doi: 10.1111/1462-2920.12695.

37. Leveau J.H., Lindow S.E. (2001) Appetite of an epiphyte: quantitative monitoring of bacterial sugar consumption in the phyllosphere. Proceedings of the National Academy of Sciences, USA. 98: 3446–3453. doi: 10.1073/pnas.061629598.

38. Levetin E., K Dorsey (2006) Contribution of leaf surface fungi to the air-spora. Aerobiologia. 22: 3-12. doi: 10.1007/s10453-005-9012-9.

39. Lichtenthaler H.K., Buschmann C., Döll M., Fietz H.J., Bach T., Kozel U., Meier D., Rahmsdorf U. (1981) Photosynthetic activity, chloroplast ultrastructure, and leaf characteristics of high-light and low-light plants and of sun and shade leaves. Photosynthesis Research. 2(2):115–141. doi: 10.1007/BF00028752.

40. Lindow S.E., Andersen G.L. (1996) Influence of immigration on epiphytic bacterial populations on navel orange leaves. Applied and Environmental Microbiology. 62: 2978–2987.

41. Lindow S.E., Brandl M.T. (2003) Microbiology of the phyllosphere. Applied and Environmental Microbiology. 69: 1875-1883. doi: 10.1128/AEM.69.4.1875-1883.2003.

42. Lomolino M.V., Riddle B.R., Brown J.H. (2010) Reconstructing the History of Lineages. Biogeography. Sunderland, Massachusetts, USA: Sinauer Associates. pp. 423–456.

43. Medina-Martínez M.S., Allende A., Barberá G.G., Gil M.I. (2015) Climatic variations influence the dynamic of epiphyte bacteria of baby lettuce. Food Research International. 68: 54–61. doi: 10.1016/j.foodres.2014.06.009.

44. Meyer K.M., Leveau J.H. (2012) Microbiology of the phyllosphere: A playground for testing ecological concepts. Oecologia. 168(3): 621–629. doi: 10.1007/s00442-011-2138-2.

45. Miller W.G., Brandl M.T., Quinones B., Lindow S.E. (2001) Biological sensor for sucrose availability: relative sensitivities of various reporter genes. Applied and Environmental Microbiology. 67: 1308–1317. doi: 10.1128/AEM.67.3.1308-1317.2001.

46. Morris, C. (2001) Phyllosphere. In: Encyclopedia of life sciences. London: Nature Publishing Group. pp. 1–8.

47. Morris C., Kinkel L. (2002) Fifty years of phyllosphere microbiology: significant contributions to research in related fields. In: Phyllosphere Microbiology. Lindow S., Hecht-Poinar E., Elliott V., editors. St. Paul, MN, USA: APS Press. pp. 365-375.

48. O'Brien R.D., Lindow, S.E. (1989) Effect of plant species and environmental conditions on epiphytic population sizes of *Pseudomonas syringae* and other bacteria. Phytopathology. 79: 619–627.

49. Prell J., Poole P. (2006) Metabolic Changes of Rhizobia in Legume Nodules. Trends in Microbiology. 14(4): 161-168. doi: 10.1016/j.tim.2006.02.005.

50. Rastogi G., Coaker G. L., Leveau J. H. (2013). New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. FEMS Microbiology Letters. 348: 1-10. doi: 10.1111/1574-6968.12225.

51. Redford A.J., Fierer N. (2009) Bacterial succession on the leaf surface: a novel system for studying successional dynamics. Microbial Ecology. 58(1): 189–198. doi: 10.1007/s00248-009-9495-y.

52. Redford A.J., Bowers R.M., Knight R., Linhart Y., Fierer N. (2010) The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. Environmental Microbiology. 12(11): 2885–2893. doi: 10.1111/j.1462-2920.2010.02258.x.

53. Reisberg E.E., Hildebrandt U., Riederer M., Hentschel U. (2012) Phyllosphere bacterial communities of trichome-bearing and trichomeless *Arabidopsis*

thaliana leaves. Antonie Van Leeuwenhoek. 101(3): 551–560. doi: 10.1007/s10482-011-9669-8.

54. Reisberg E. E., Hildebrandt U., Riederer M., Hentschel U. (2013). Distinct phyllosphere bacterial communities on *Arabidopsis* wax mutant leaves. PLOS ONE. 8(11):e78613. doi: 10.1371/journal.pone.0078613.

55. Ritpitakphong U, Falquet L, Vimoltust A, Berger A, Métraux JP, L'Haridon F. (2016) The microbiome of the leaf surface of Arabidopsis protects against a fungal pathogen. New Phytologist. 210: 1033–1043. doi: 10.1111/nph.13808.

56. Ruinen J. (1961) The phyllosphere. I. An ecologically neglected milieu. Plant Soil. 15: 81–109. doi: 10.1007/BF01347221.

57. Scheffers B.R., Phillips B.L., Laurance W.F., Sodhi N.S., Diesmos A., Williams S.E. (2013) Increasing arboreality with altitude: a novel biogeographic dimension. Proceedings of the Royal Society of London B: Biological Sciences. 280(1770): 20131581. doi: 10.1098/rspb.2013.1581.

58. Schloss P.D., Gevers D., Westcott S.L. (2011) Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PLOS One. 6(12): e27310. doi: 10.1371/journal.pone.0027310.

59. Schreiber L., Krimm U., Knoll D. (2004). Interactions between epiphyllic microorganisms and leaf cuticles. In: Plant Surface Microbiology. Varma A., Abbott L., Werner D., Hampp R., editors. Springer-Verlag Berlin Heidelberg: pp. 145-156.

60. Schreiber L., Krimm U., Knoll D., Sayed M., Auling G., Kroppenstedt R.M. (2005) Plant–microbe interactions: identification of epiphytic bacteria and their ability to alter leaf surface permeability. New Phytologist. 166(2): 589–594. doi: 10.1111/j.1469-8137.2005.01343.x.

61. Stapleton A.E., Simmons S.J. (2006) Plant control of phyllosphere diversity: genotype interactions with ultraviolet-B radiation. In: Microbial Ecology of the Aerial Plant Surface. Bailey M.J., Lilley A.K., Timms-Wilson P.T.N., Spencer-Phillips P.T.N., editors. Wallingford, UK: Cabi. pp. 223–238.

62. Sternberg G., Wilson James, Wilson Jim. (2004) Native trees for North American landscapes: from the Atlantic to the Rockies. Portland: Timber Press. pp. 268.

63. Stiefel P., Zambelli T., Vorholt J. A. (2013). Isolation of optically targeted single bacteria using FluidFM applied to aerobic anoxygenic phototrophs from the phyllosphere. Applied and Environmental Microbiology. 79: 4895–4905. doi: 10.1128/AEM.01087-13.

64. Stone B.W., Jackson C.R. (2016) Biogeographic patterns between bacterial phylosphere communities of the southern magnolia (*Magnolia grandiflora*) in a small forest. Microbial Ecology. 71: 954–961. doi:10.1007/s00248-016-0738-4.

65. Sundin, G.W. (2002) Ultraviolet radiation on leaves: its influence on microbial communities and their adaptations. In: Phyllosphere Microbiology.

Lindow S.E., Hecht-Poinar E.I., Elliott V.J., editors. St Paul, USA: APS Press. pp. 27–41.

66. Turner T. R., James E. K., Poole P. S. (2013). The plant microbiome. Genome Biology. 14: 209. doi: 10.1186/gb-2013-14-6-209.

67. Vorholt J.A. (2012) Microbial life in the phyllosphere. Nature Reviews Microbiology. 10(12): 828–840. doi: 10.1038/nrmicro2910.

68. Yang C.H., Crowley D.E., Borneman J., Keen N.T. (2001) Microbial phyllosphere populations are more complex than previously realized. Proceedings of the National Academy of Sciences, USA. 98: 3889-3894. doi: 10.1073/pnas.051633898.

69. Yashiro E., Spear R.N., McManus P.S. (2011) Culture-dependent and culture-independent assessment of bacteria in the apple phyllosphere. Journal of Applied Microbiology. 110: 1284–1296. doi: 10.1111/j.1365-2672.2011.04975.x.

70. Zilber-Rosenberg I., Rosenberg E. (2008) Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. FEMS Microbiology Review. 32: 723–735. doi: 10.1111/j.1574-6976.2008.00123.x.