# ELUCIDATION AND BIODIVERSITY OF BACTERIAL COMMUNITIES WITHIN THE PHYLLOSPHERE OF HUMULUS LUPULUS L. S. J. BRITTON<sup>\*1</sup>, M. E. ALLEN<sup>2</sup>, A. J. PIEFER<sup>2</sup>, P. T. BENZINGER<sup>2</sup>, J. M. BALNIS<sup>2</sup>, and L. E. GRIENIESEN<sup>3</sup> Duvel Moortgat, NV<sup>1</sup>, Breendonk, BELGIUM; Hartwick College<sup>2</sup>, Oneonta, NY, USA; University of Notre Dame<sup>3</sup>, Notre Dame, IN, USA

## Abstract

The interaction of microbial communities within the phyllosphere, the airborne portion of plants, has been shown to influence plant wellness, promote plant growth, aid in the suppression of disease, and affect the productivity of agricultural crops. However, a majority of prior investigations have been limited to the microbial communities associated with the leaf, the most prevalent structure within the phyllosphere. Although the flower is of considerable importance to overall reproductive success, the microbiota associated with these anatomical structures remains poorly understood. Therefore, our investigation surveyed the bacterial epiphytes populating the hop cone, an inflorescence, and leaves of mature Humulus lupulus L., and subsequently compared the diversity against those present in the soil at the base of the plant. Characterization of the communities was carried out via the next-generation sequencing of polymerase-chain reaction derived single amplicons (approx. 460 bps) that contain the V3/V4 hyper-variable region of 16S rDNA that were extracted and amplified from each sample. Results indicated that, compared to the soil around the base, the diversity existent on the hop cone and leaf was considerably less. The average number of phyla discovered to populate each sample zone were 28.00 for hop cones, 25.75 for leaves and 47.00 for soils. Analysis revealed the two most dominant taxa populating hop cones and leaves belong to the classes Gammaproteobacteria, predominantly from the family Pseudomonadaceae, and Alphaproteobacteria, primarily from the family Sphingomonadaceae. These account for 56.0% and 22.5% of the sequences in cones and 43.7% and 22.9% of the sequences in leaves, respectively. The most abundant, or most common, taxa identified in this investigation are consistent with the most abundant taxa found to be associated with the flowers and leaves of the tomato (Solanum lycopersicum) and other plants. The intention of this study was to elucidate the microbial ecology associated with Humulus lupulus L., to provide a reference source for bioprospecting that could improve the horticulture and performance of cultivars, and to contribute to the general understanding of the microbial ecology of the phyllosphere.

### Methods

Sample Collection. Cone, leaf, and soil samples were collected from twenty-four individual hops plants (Cascade) located at Brewery Ommegang in Cooperstown, NY (Aug. 2014). Samples were held and stored at -80 °C until DNA isolation was completed.

**Microbial Separation from Plant Matter.** Cold 1 x PBS, 0.15% Tween solution and 6 - 10sterilized 8 mm glass beads were added to cone and leaf samples to detach bacteria from the plant surface. Tubes were horizontally shaken for 20 min. (400 rpm) at -4 °C. PBS solution was subsequently filtered through sterile cheesecloth. Samples were centrifuged (2,000 rpm) at -4 C° for 5 min. Supernatant was removed and centrifuged (5,600 rpm) at -4 C° to pellet bacteria. Cone replicates were pooled and centrifuged (5,600 rpm) for 10 min. Supernatant was discarded and pellets were resuspended in 1-mL cold 1 x PBS, 0.15% tween solution.

**DNA Isolation.** DNA was isolated using the MoBio PowerSoil® kit. Protocol was slightly modifications for cones and leaf samples. Modified Protocol: Beads and solution were removed from PowerSoil® bead tubes and transferred into sterile eppendorf tubes. Resuspended pellets were transferred to the empty bead tubes and centrifuged (10,000 rpm) for one minute. Supernatant was discarded. PowerSoil® beads and solution were transferred back to the PowerSoil bead tubes. DNA isolation proceeded per manufacturer's instructions.

**PCR Amplification.** 16S rRNA gene V3-V4 regions were amplified. Forward primer 5'TCGTCGGCAGCGTCAGATGTGTATAAG AGACAGCCTACGGGNGGCWGCAG and reverse primer 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGG TATCTAATCC.

Illumina MiSeq and Bioinformatics. Final amplicons for sequencing were obtained following the Illumina16S Library Preparation Protocol. QIIME was employed for 16S rDNA amplicon sequencing data analysis



0.8

0.6

Ö.

0.2

0

### Background

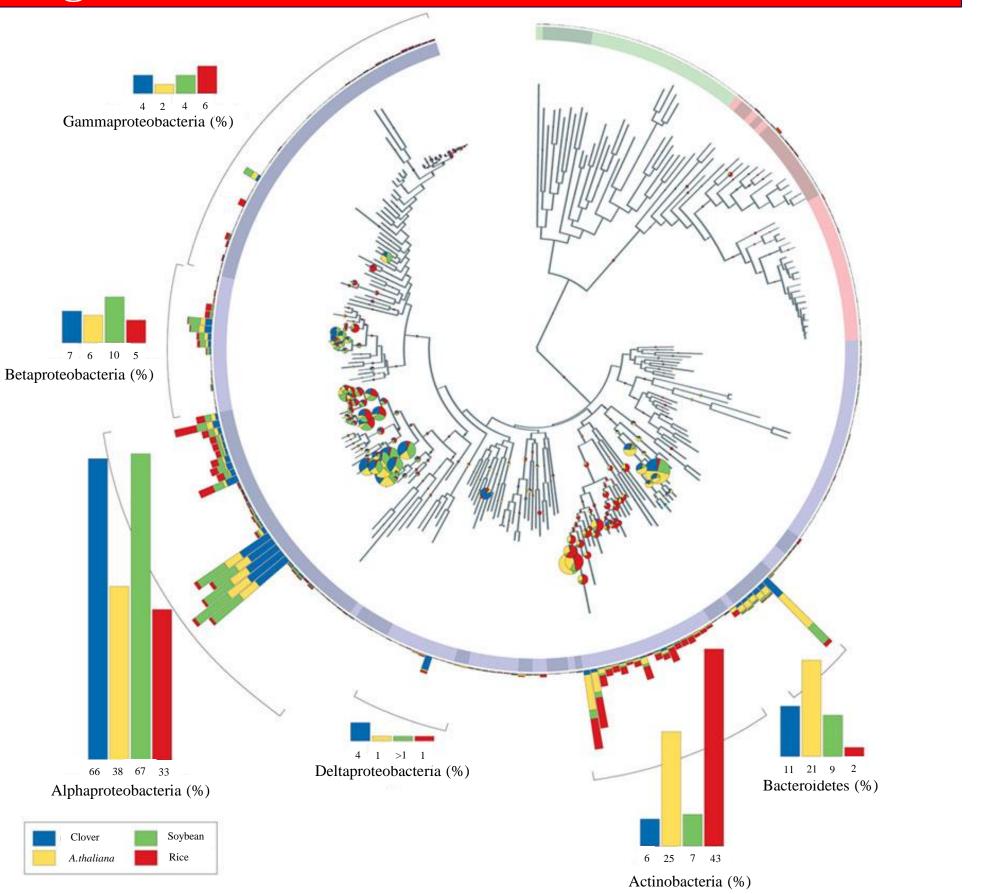
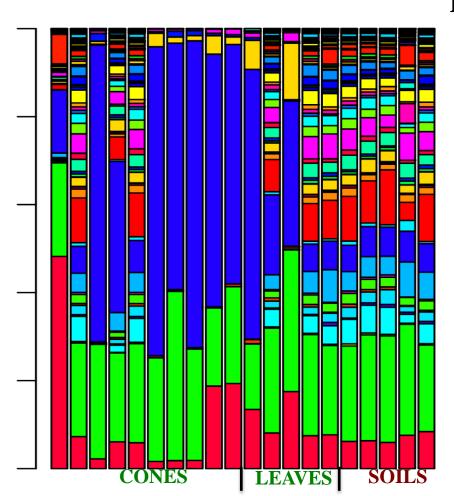
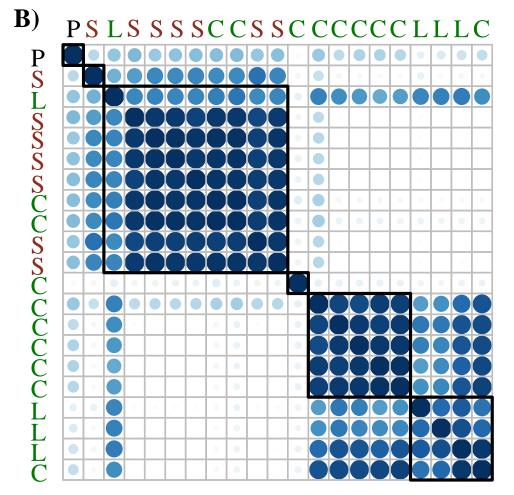
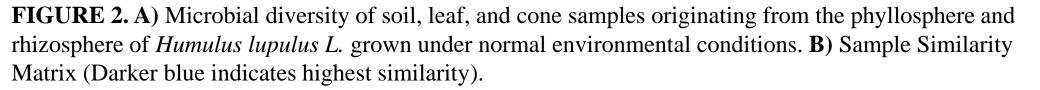


FIGURE 1. Microbial phyllosphere diversity. Microbial community composition of samples obtained from the phyllosphere of Arabidopsis thaliana, soybean, clover and rice grown under environmental conditions via 454 pyrosequencing. (Adapted from Vorholt, 2012).







PHYLLUM
Acidobacteria
Actinobacteria
Bacteroidetes
Chloroflexi
Firmicutes
Gemmatimonadetes
Planctomycetes
Proteobacteria
Verrucomicrobia
FIGURE 3. Microbia that represent ≥1% (MI
CLASS
Alphaproteobacteria

Betaproteobacteria

Deltaproteobacteria

Gammaproteobacteria

are included.

		COTTC		CONES
L	CLASS	SOILS (Pop. %)	LEAVES (Pop.%)	CONES (Pop.%)
	Total	16.6	<1	<1
	Acidobacteria-6	9.6	<1	<1
	Solibacteres	2.1	<1	<1
	Chloracidobacteria	2.2	<1	<1
	Total	9.8	1.7	<1
	Acidimicrobiia	2.6	<1	<1
	Actinobacteria	4.8	1.7	<1
	Thermoleophilia	2.0	<1	<1
	Total	7.0	6.7	3.1
	Cytophagia	2.4	4.3	2.1
	Saprospirae	3.0	<1	<1
	Total	5.7	<1	<1
	Anaerolineae	2.3	<1	<1
	Ellin6529	1.4	<1	<1
	Total	1.0	<1	<1
	Total	1.7	<1	<1
	Total	6.6	<1	<1
	Phycisphaerae	1.6	<1	<1
	Planctomycetia	4.5	<1	<1
	Total	41.6	86.1	94.0
	Alphaproteobacteria	23.0	22.9	22.5
	Betaproteobacteria	7.5	15.5	5.9
	Deltaproteobacteria	4.9	<1	<1
	Gammaproteobacteria	6.5	43.7	56.0
	Total	5.5	<1	1.6
	Pedosphaerae	2.1	<1	<1
	Opitutae	1.5	<1	<1

I Diversity of the Rhizophere and Phyllosphere of Humulus lupulus L. Only classes (EDIAN) of the sample population in at least one sample category are included.

		I				
	ORDER	SOILS (Pop. %)	LEAVES (Pop.%)	CONES (Pop.%)		
	Total	23.0	22.9	22.5		
	Sphingomonadales	1.9	15.3	13.5		
	Rhizobiales	12.4	1.1	2.4		
	Rickettsiales	<1	2.4	4.0		
	Rhodospirillales	3.0	<1	<1		
	Rhodobacterales	1.6	<1	<1		
	Unidentified	1.6	<1	<1		
	Total	7.5	15.5	5.9		
	Burkholderiales	1.8	15.4	2.0		
	Unidentified	1.4	<1	<1		
	Total	4.9	<1	<1		
	Myxococcales	3.4	<1	<1		
ia	Total	6.5	43.7	56.0		
	Pseudomonadales	<1	37.2	47.3		
	Xanthomonadales	4.3	<1	<1		
	Enterobacteriales	<1	5.6	3.2		

FIGURE 4. Microbial Diversity of the Proteobacteria in the Rhizophere and Phyllosphere of *Humulus lupulus* L. Only orders that represent  $\geq 1\%$  (MEDIAN) of the sample population in at least one sample category This investigation represents the first culture-independent analysis of the microbial communities populating the phyllosphere of the common hop (Humulus lupulus L.). Results from this study revealed that the plant leaves and cones are inhabited by a distinctly different, and much less diverse, microbial community than the one populating the soils around the base of the plants. Most studies of the phyllosphere that investigate flowers describe microbial communities associated with individual flower parts (e.g. nectar, petals, pollen, etc) and very few include next generation DNA sequencing (e.g. Illumina) methods, which provide better detection of rare taxa and quantitative measures of diversity than earlier sequencing methods. As such, the data exhibited here represent a rare, in depth analysis of the microbial community associated with whole flowers. This microbial community is dominated by Proteobacteria, a group also commonly detected in other whole flowers and flower parts. Some striking dissimilarities were also detected between the common hop and other flowers. In particular, the phylum of Actinobacteria was far less abundant in hops than in other plant species. Also, in soybeans, rice, clover, and A. thaliana a majority of the Proteobacteria belong to the class Alphaproteobacteria, while the constituents of *Humulus lupulus* L. overwhelmingly belong to the class Gammaproteobacteria and more specifically the order Pseudomonadales.

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