Metabolome and Microbiome Signatures in the Roots of Citrus Affected by Huanglongbing

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Accepted for publication 4 August 2019.

ABSTRACT

Huanglongbing (HLB) is a severe, incurable citrus disease caused by the bacterium ‘*Candidatus Liberibacter asiaticus*’ (CLAs). Although citrus leaves serve as the site of initial infection, CLAs is known to migrate to and colonize the root system; however, little is known about the impact of CLAs infection on root metabolism and resident microbial communities. Scions of ‘Lisbon’ lemon and ‘Washington Navel’ orange grafted onto ‘Carrizo’ rootstock were grafted with either CLAs-infected citrus budwood or uninfected budwood. Roots were obtained from trees 46 weeks after grafting and analyzed via ¹H nuclear magnetic resonance spectroscopy to identify water-soluble root metabolites and high-throughput sequencing of 16S rRNA and ITS gene amplicons to determine the relative abundance of bacterial and fungal taxa in the root rhizosphere and

endosphere. In both citrus varieties, 27 metabolites were identified, of which several were significantly different between CLAs(+) and control plants. CLAs infection also appeared to alter the microbial community structure near and inside the roots of citrus plants. Nonmetric multidimensional scaling (NMDS) and a principal coordinate analysis (PCoA) revealed distinct metabolite and microbial profiles, demonstrating that CLAs impacts the root metabolome and microbiome in a manner that is variety-specific.

Keywords: bioinformatics, ‘*Candidatus Liberibacter asiaticus*’, *citrus limon*, *citrus sinensis*, huanglongbing, metabolomics, microbiome, plant–microbe interactions, plant pathogen interactions, roots

Huanglongbing (HLB), or citrus greening disease, is an infectious disease that is associated with the bacterium ‘*Candidatus Liberibacter asiaticus*’ (CLAs) and is a serious threat to the sustainability of the citrus industry (McCollum and Baldwin 2016). CLAs is transported systemically throughout the nutrient-rich phloem tissue, where it colonizes sieve tubes and taps into resources meant for the plant. This eventually leads to phloem collapse, which severely disrupts nutrient transport (Kim et al. 2009). A key emerging component in HLB management is preserving root health as the pathogen is detected in root tissue as early as 2 months after infection, causing substantial degradation of the root system well before foliar symptoms appear (Johnson et al. 2014). Current management practices limit root loss by altering watering and fertilization methods; however, correctly

diagnosing and removing infected trees is the most effective way to limit disease spread. The root system releases primary and secondary metabolites (termed ‘root exudates’) into the rhizosphere to serve as carbon/nitrogen substrates, signaling molecules, and chemoattractants for surrounding microbes (Hu et al. 2018), and plants shape the root microbiome by adjusting the root exudate composition, attracting beneficial microbes while deterring harmful ones (Lareen et al. 2016).

Metabolomics is an emerging technology that is able to examine changes in metabolism as an organism responds to an external stimulus, such as pathogen attack. Metabolomics has been used to quantify juice and fruit metabolites from HLB-affects citrus (Chin et al. 2014; Slisz et al. 2012), to examine the effects of Asian citrus psyllid (ACP) feeding on the citrus leaf metabolome (Chin et al. 2017), and to identify a metabolite fingerprint in CLAs-infected citrus leaves (Cevallos-Cevallos et al. 2009) and root tissues (dos Santos Freitas et al. 2015). Similarly, microbiome studies of citrus have identified HLB-induced changes in bacterial community composition (Wang et al. 2017), including a shift toward bulk soil community composition with reductions in the populations of *Burkholderia*, *Pseudomonas*, *Rhodanobacter*, *Sphingobium*, and *Sphingomonas* (Trivedi et al. 2012). Others have reported similar results, with the depletion of *Burkholderia* and *Bradyrhizobium* observed in HLB-impacted sweet orange trees compared with healthy ones (Zhang et al. 2017). Conversely, *Comamonadaceae*, *Hyphomicrobiaceae*, and *Rhizobiales* appear to be enriched on the roots of infected trees (Blaustein et al. 2017). However, little is known regarding the metabolic response of citrus roots to CLAs and the impact on surrounding microbial communities, and a combined analysis of the root metabolome and microbiome in response to a challenge by CLAs has not been performed. Furthermore, no other research has compared how this response varies by cultivar. Therefore, the aim of this work was to characterize the response

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Funding: This study was supported by the California Citrus Research Board grant no. 5300-150. The salary for Shin-Yi Lin was provided by the Ministry of Science and Technology, Taiwan, Republic of China (NSC-102-2911-I-005-301, NSC-103-2911-I-005-301) and the Ministry of Education, Taiwan, ROC, under the ATU plan. C.S. also acknowledges support through the U.S. Department of Agriculture National Institute of Food and Agriculture Hatch Project 1005945. Support for the Bruker Advance 600 MHz NMR came from NIH grant RR011973.

*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures and six supplementary tables are published online.

The author(s) declare no conflict of interest.

Modified: 19 Feb 2020.

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of two distinct varieties of citrus, 'Lisbon' lemon (*Citrus limon* L. Burm. f.) and 'Washington Navel' orange (*Citrus sinensis* (L.) Osbeck), grafted onto 'Carriizo' rootstock in terms of primary metabolites, bacteria, and fungi in the root system to ascertain the impact of host genotype and HLB infection on root-associated metabolism and microbial communities. We hypothesized that a distinct metabolite and microbial fingerprint would emerge for each variety based on health status, with different varietal responses to HLB.

MATERIALS AND METHODS

Plant materials. Roots were sampled from citrus trees ($n = 34$) that were part of a longitudinal experiment measuring metabolomics, microbiome, transcriptomics, and proteomics changes in the leaves of citrus trees affected by CLAs (E. L. Chin, J. Ramsey, S. Saha, D. Mishchuk, J. Chaves, K. Howe, X. F. Zhong, M. Flores-Gonzalez, E. Mitrovic, M. L. Polek, K. Godfrey, L. A. Mueller, J. Bruce, M. Heck, and C. Slupsky, *unpublished data*). Trees were grown and maintained in an insect-free greenhouse in 2-gallon pots containing a 2:1 mixture of UC mix no. 2 and peat moss at the University of California (UC) Davis Contained Research Facility (Davis, CA, USA). The greenhouse was maintained at 27°C ($\pm 1.5^\circ\text{C}$) with supplemental lighting (high-pressure sodium lights) and a photoperiod of 16 h of light and 8 h of darkness. Humidity ranged from 30 to 50%. Plants were watered as needed and fertilized each time with complete fertilizer (5-12-26) containing magnesium (31 ppm), sulfate (SO_4 125 ppm), iron (3 ppm), manganese (0.50 ppm), zinc (0.15 ppm), copper (0.15 ppm), boron (0.50 ppm), molybdenum (0.10 ppm), and calcium (116 ppm). All experimental trees were scions of 'Lisbon' lemon (*Citrus limon* L. Burm. f.) or 'Washington Navel' sweet orange (*Citrus sinensis* (L.) Osbeck) grafted onto 'Carriizo' rootstock (*C. sinensis* (L.) $\times$ *P. trifoliata*) that were grown from certified seed. Scions were grafted when rootstock were 6 months old, and they were allowed to grow for an additional 6 months. Seventeen trees (lemon, $n = 11$; navel, $n = 6$) were randomly assigned to be graft-inoculated onto the varietal scion with three pieces of lemon or navel budwood of CLAs(+) lemon or navel trees that were originally sourced from Hacienda Heights, CA. Seventeen trees (lemon, $n = 11$; navel, $n = 6$; control group) were grafted with uninfected budwood (lemon or navel). All trees were grown in the same soil and maintained under the same conditions.

Prior to grafting, the abundance of CLAs in infected source trees was confirmed by a quantitative PCR (qPCR), as previously described (Li et al. 2006), and expressed as a cycles to threshold (Ct) value for each sample. Similarly, the presence of the pathogen in experimental trees was quantified by performing qPCR for leaf samples that were randomly sampled throughout the canopy starting 10 weeks after grafting. For this, six to eight leaves per tree were collected, of which 200 mg (fresh weight) of midrib and petiole tissue was excised and used for qPCR as per the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) Plant Protection and Quarantine (PPQ) program protocol using the Qiagen MagAttract plant DNA extraction kit (Valencia, CA). Leaf sampling for metabolomics in the parent study commenced 10 weeks after exposure and was performed approximately every 6 weeks thereafter for a total of seven times between February 2014 and January 2015 (E. L. Chin, J. Ramsey, S. Saha, D. Mishchuk, J. Chaves, K. Howe, X. F. Zhong, M. Flores-Gonzalez, E. Mitrovic, M. L. Polek, K. Godfrey, L. A. Mueller, J. Bruce, M. Heck, and C. Slupsky, *unpublished data*). Visual symptoms of HLB on the canopy of experimental plants were monitored throughout the study, and visual symptoms of the roots were recorded at the final time point (January 2015; 46 weeks after exposure). A tree with a Ct value <37 at least once during the study was considered to be infected with CLAs (USDA-APHIS 2013). Root samples were collected by snipping feeder roots from each quadrant of the bulk root mass of each individual tree using stainless steel surgical scissors that were

sterilized with 70% ethanol between sample collections. These subsamples were then pooled into a single sample.

Sample preparation and data collection. To perform a microbial analysis of the pooled root samples, roots were shaken to remove loosely adhering soil particles. Up to 3 g of root tissue was transferred to a 50 ml conical tube and filled with wash buffer (20 mM Tris-HCl, 10 mM EDTA, and 0.024% Triton) (Rastogi et al. 2010) to an adjusted volume of 45 ml. The tube was vortexed for 20 s, sonicated in a Branson water bath for 7 min, and vortexed again for 20 s. The root material was removed, and the root wash was centrifuged for 7 min at $7,197 \times g$. The supernatant was discarded and the pellet was used for DNA extraction. The root endosphere community was extracted by washing the roots twice more and grinding them in liquid nitrogen into a fine powder using a mortar and pestle. Rhizosphere soil DNA extractions were performed using the PowerSoil DNA Isolation Kit (cat. #12888; Mo Bio Labs) using approximately 250 mg of soil per extraction. Root endosphere DNA extractions were performed using the PowerPlant Pro DNA Isolation Kit (cat. #13400-50; Mo Bio Labs) using approximately 60 mg of tissue per extraction. Microbial DNA extracts from rhizosphere soils or root tissues were diluted 10-fold and used as template during PCR to amplify the V5-V7 region of the bacterial 16S ribosomal gene using primers 799F and 1193R (Bodenhausen et al. 2013; Chelius and Triplett 2001) or the ITS1 (intergenic spacer region 1) region using primers BITS1 and B58S3 (Bokulich and Mills 2013). The 799F and BITS forward primers contained built-in barcodes (Alekklett et al. 2015; Hamady et al. 2008). PCRs were performed using GoTaq Green Master Mix (cat. #M7122; Promega) in 15- μl reactions containing 7.5 μl of 2X GoTaq Green master mix, 3.9 μl of water, 0.3 μl of reverse primer (10 μM), 0.3 μl of bovine serum albumin (BSA) (20 mg/ μl ; NEB cat. #B9000S), 1.5 μl of forward primer (2 μM), and 1.5 μl of template DNA. Cycling conditions were as follows: initial denaturation at 94°C (bacterial) or 95°C (fungal) for 2 min, then 35 cycles of denaturation at 94°C (bacterial) or 95°C (fungal) for 30 s, primer annealing at 55°C for 30 s, product extension at 72°C for 1 min, and final extension at 72°C for 10 min (bacterial) or 5 min (fungal). Amplicons were visualized on a 1% agarose gel, pooled, and, in the case of bacterial amplicons, run again on a 0.6% gel to achieve separation of the 430-bp nonmitochondrial band that was excised from gel and purified using the Zymoclean Gel DNA Recovery Kit (cat. #D4007; Zymo Research). The mitochondrial product was ≈ 800 bp. The fungal amplicon pool was purified using the QIAquick PCR Purification Kit from Qiagen (cat. #28104). Bacterial and fungal amplicon pools were submitted for library preparation and sequencing at the UC Davis DNA Technologies core, where sequencing was performed using the Illumina MiSeq platform with the 300 base pair (bp) paired-end read platform.

For metabolomics, root samples were washed with deionized water to remove attached soil, blotted dry with paper towels, and stored in sealable plastic bags at -80°C . Approximately 1 g of frozen root sample was lyophilized in a Falcon 15 ml conical centrifuge tube using a Labconco FreeZone Plus 4.5-liter freeze-dry system (Kansas City, MO, USA) for 48 h. Tubes containing dry roots were subsequently placed in a resealable bag and stored at room temperature and protected from light until metabolite extraction. Dried root samples were cut into small pieces using stainless steel surgical scissors, and ≈ 75 mg was transferred to 2-ml screw-cap microcentrifuge tubes. One 3.5-mm-diameter glass bead (BioSpec Products Inc., Bartlesville, OK, USA) was placed in each tube and ground for 2 min using a BioSpec Mini-Beadbeater-16 cell disrupter (BioSpec Products Inc., Bartlesville, OK, USA), after which 10 mM of potassium phosphate buffer (pH 6.8 ± 0.1) was added to each tube at a ratio of 1:20 wt/vol. Samples were then incubated in an Eppendorf Thermomixer (15 min, 90°C, 1000 rpm) and centrifuged (15 min, 4°C, 14,000 RCF) using an Eppendorf Model 5415R microcentrifuge (Eppendorf North America,

Hauppauge, NY, USA). An aliquot (1000 µl) of the supernatant was transferred to a new tube, to which 100 mg of BioRad Chelex 100 resin in sodium form (BioRad, Hercules, CA, USA) was added; the mixture was allowed to stand for 15 min (4°C). Samples were centrifuged (15 min, 4°C, 14,000 RCF), and 400 µl of supernatant was transferred to an Amicon Ultra 0.5-ml centrifugal filter unit with an Ultracel-3 membrane (EMD Millipore, Billerica, MA, USA) that was prewashed (4×) with purified water and centrifuged again (30 min, 4°C, 14,000 RCF). From this, 207 µl of filtrate was combined with 23 µl of internal standard containing 5 mM 3-(trimethylsilyl)-1-propanesulfonic acid-d6 (DSS-d6), NaN₃, and D₂O (Chenomx, Edmonton, AB, Canada). The final concentrations were 0.5 mM DSS-d6, 0.02% NaN₃, and ≈10% D₂O. The pH of each sample was adjusted to 6.8 ± 0.1 using small amounts of 1 N HCl or NaOH, and 180 µl was transferred to 3-mm nuclear magnetic resonance (NMR) tubes that were stored at 4°C until NMR data were acquired (within 24 h of sample preparation).

Metabolomics analysis. NMR spectra were acquired at 298 K using the Bruker ‘noesypr1d’ experiment on a Bruker Avance 600 MHz NMR spectrometer equipped with a SampleJet. The acquisition parameters were as follows: sweep width, 12 ppm; acquisition time, 2.5 s; relaxation delay, 2.5 s; and mixing time, 100 ms. Water saturation was applied during the relaxation delay and mixing time. The resulting spectra were zero-filled to 128,000 data points, and an exponential apodization function corresponding to a line-broadening of 0.5 Hz was applied. Spectra were processed for metabolite identification and quantification using the Chenomx Inc. NMR Suite Processor version 8.2 (Edmonton, AB, Canada).

Microbiome analysis. Illumina DNA sequences from bacteria and fungi were processed and analyzed using QIIME version 1.9.1 (Caporaso et al. 2010). For the bacterial community, paired reads were joined using join_paired_ends.py; barcodes were extracted using extract_barcodes.py; reads were quality-filtered and demultiplexed using split_libraries_fastq.py -q 19; OTUs (operational taxonomic units) picked at 97% similarity using pick_open_reference_otus.py -s 0.1; the OTU table was filtered to remove OTUs that constituted <0.005% of all reads using filter_otus_from_otu_table.py -min_count_fraction 0.00005; and the OTU table was filtered again to remove chloroplast OTUs using filter_taxa_from_otu_table.py -n c_Chloroplast,f_mitochondria. For the fungal community, sequence processing was performed as previously described with the following exceptions: the paired ends were not joined and taxonomy was assigned using the RDP classifier with the UNITE database (ver. 6, 2014-09-10, developer files) (Kõljalg et al. 2013). Beta diversity comparisons were performed using jackknifed_beta_diversity.py with the Bray-Curtis dissimilarity and the weighted and unweighted unifracs (bacteria only) metrics. Cluster significance was tested using the Adonis function, an implementation of Permutational MANOVA (Anderson 2001), in compare_categories.py. Core OTUs were calculated using compute_core_microbiome.py.

Statistics and data analysis. Root metabolite concentrations and microbial abundance data in roots were evaluated for normality using histograms and the Shapiro-Wilk test. Metabolite concentrations were corrected for dilution and converted to nmol/g of dry leaf weight. Differences in metabolite concentration and microbial abundance between healthy and CLas(+) plants were compared using the Mann-Whitney *U* test, and results were considered significant if *P* < 0.05. The application of a false discovery rate (FDR) resulted in nonsignificant findings for all microbial data. Therefore, significant unadjusted *P* values, in combination with large effect sizes, were used to interpret the results. Absolute median log₂ fold-change values in metabolites between CLas(+) and control groups were calculated, with negative fold changes representing a reduction in metabolite concentrations with exposure to CLas. Effect sizes were estimated with Cliff’s delta (*d*) using the following calculation:

$$ABS(2*U)/n_a n_b - 1$$

where *U* represents the calculated Mann-Whitney *U* statistic and *n_an_b* represents the product of the control and CLas+ samples sizes (Goedhart 2016). Effect sizes were interpreted as follows: *d* < 0.147, negligible; *d* < 0.33, small; *d* < 0.474, medium; and *d* > 0.474, large (Romano et al. 2006). Associations between metabolites or microbes and qPCR Ct values from leaves were examined using the Spearman rank correlation (‘Hmisc’ package; R), and results were considered significant if the FDR-adjusted (Benjamin-Hochberg) results had *P* < 0.05. Shifts in the root metabolome and microbiome were compared using a permutational multivariate analysis of variance (PERMANOVA) of cluster centers (termed centroids) on the Bray-Curtis distance matrix created via nonmetric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA), respectively (‘vegan’ and ‘phyloseq’ packages; R). Graphs were produced and statistical procedures were performed using R version 3.5.1 (R Core Team 2012).

Accession numbers. Sequencing data will be deposited in the European Nucleotide Archive.

RESULTS

Metabolic and microbiome signatures of healthy control rootstocks grafted with lemon or navel scions. A comparison of roots from control (healthy) trees revealed significant differences in a small number of metabolites in lemon roots compared with navel (Supplementary Table S1); however, the NMDS analysis of the centroids of each group revealed that the metabolomes of lemon and navel controls were not significantly different (Supplementary Fig. S1). NMDS is an ordination method that configures points in two-dimensional or three-dimensional space, where each point represents individuals characterized by the data collected, and in which the distances between individual points are calculated dissimilarity values (Cox and Ferry 1993). PERMANOVA utilizes a pseudo *F* statistic to test the null hypothesis that there is no difference in the position of the group centroids (the center of the clusters) given by the selected dissimilarity measure (Anderson 2001). Differences were not expected because samples were collected ≈18 months after scions were fused onto rootstock, allowing sufficient time for vascular tissues to fuse (Melnik 2017). This was in contrast to differences observed in the bacterial and fungal communities measured in and around the root system of healthy lemon and navel plants, which suggested unique microbial structures are formed in the root systems of citrus plants and are shaped by the varietal scion (Supplementary Table S2). These differences were reflected in varietal clustering apparent in PCoA plots (Supplementary Fig. S2). At the phylum level, roots of healthy lemons harbored more *Proteobacteria* and *Acidobacteria* than roots of healthy navels, less *Actinobacteria*, and more of the fungal phylum *Basidiomycota*. At the family level, lemons were dominated by *Burkholderiaceae* (represented largely by *Burkholderia*). In contrast, navels contained significantly more *Microbacteriaceae*, *Patulibacteriaceae*, and *Rhizobiaceae* compared with lemons. Fungal signatures distinguishing lemon and navel roots were also observed; in order of greatest abundance, *Herpotrichiellaceae*, *Nectriaceae*, and *Entolomataceae* dominated in lemons, and *Nectriaceae*, *Trichocomaceae*, *Pleosporaceae*, *Microascaceae*, and *Herpotrichiellaceae* were dominant in navels.

Infection status and visual symptoms of CLas graft-inoculated trees. Among 17 citrus trees graft-inoculated with budwood originating from CLas(+) trees, 6 of 11 lemon and 4 of 6 navel trees were confirmed by qPCR to contain a sufficient bacterial titer to warrant a positive CLas classification (i.e., Ct < 37). The remaining plants in the graft-inoculated group (5 lemon, 2 navel) did not reach this threshold; therefore, these plants were considered CLas(−) and were removed from the analysis (Supplementary Table S3). The Ct values of infected lemon plants at week 46 ranged from

23.30 to 25.65 and from 19.38 to 22.63 among infected navel plants. All control trees tested negative for CLAs, with the exception of two navel controls, which had Ct <37 at one time point during the study period. After retesting, the same samples were determined to have a Ct of 40; however, we did not include these two trees in the final statistical analyses because of uncertainty. Because of a limitation during sample collection, root materials for microbial analyses were randomly collected for only 6 of 11 lemon control plants, resulting in a total of 20 trees for microbial community analysis: 10 CLas(+) trees (6 lemon, 4 navel) and 10 CLas(−) (control) trees (6 lemon, 4 navel). Roots obtained from CLas(+) plants at 46 weeks after exposure were visually distinguishable from control plants, especially in terms of bulk root mass, which was substantially less in CLas(+) plants. Other visual symptoms of HLB including mottling, yellowing, and curling of the plant leaves (Fig. 1). Trees that were CLas(+) in leaf tissue also presented with detectable CLas in the roots.

Metabolite signature of CLas(+) citrus roots. Of approximately 320 compounds in the Chenomx metabolomics database, 27 water-soluble metabolites were identified in citrus roots, including sugars, amino acids, organic acids, and other compounds related to plant metabolism and defense. This method enabled us to examine changes in several primary metabolites that are central to metabolism, including amino acids, sugars, and organic acids. The NMDS analysis of citrus root metabolites revealed four clusters related to the variety and infection status (Fig. 2). CLas-induced changes in citrus roots included a significant reduction in the concentration of 15 metabolites in lemons and 3 metabolites in navels, which were accompanied by large effect sizes for each significant metabolite. Additionally, several metabolites in navel roots (sucrose, asparagine, malic acid, quinic acid, choline, trigonelline) did not reach statistical significance, but they did have a large effect size. Compared with standard null hypothesis significance testing, which indicated whether an experimental effect is caused by chance (Middlemis Maher et al. 2013), effect sizes allowed the magnitude of the effect to be examined, thereby providing an indicator of biological significance (Nakagawa and Cuthill 2007).

Overlap in the plant response to CLas was observed between varieties, including a reduction in all sugars measured, the amino acids proline and asparagine, the metabolites choline and uridine, and an increase in trigonelline. Interestingly, there were some differences in the varietal responses to disease. For example, the metabolites malic acid and quinic acid were significantly reduced in

lemon roots but appeared to increase in navel roots (nonsignificant results, but large effect sizes) (Fig. 3). Correlation analyses were conducted to examine relationships between bacterial titers of CLas (lowest reported Ct value in leaf tissue; Supplementary Table S4) and root metabolites. Lower Ct values represent greater proliferation of the bacterium in planta. In lemon roots, higher concentrations of sugars (glucose, maltose, and sucrose) and some amino acids (alanine, asparagine, proline, threonine, and GABA) correlated with higher CLas Ct values. Additionally, as the Ct value decreased, there appeared to be a trend toward an increase in the trigonelline concentration (results not significant after Benjamini-Hochberg FDR correction). These findings agreed with the difference testing conducted during this study that showed higher concentrations of these key metabolites in CLas(−) lemon plants relative to CLas(+) lemon plants.

Microbial signature of CLas(+) in citrus roots. Overall, 1,002 bacterial and 409 fungal OTUs were identified in root soil (rhizosphere) and root tissue (endosphere) samples combined. The core bacterial and fungal microbiomes of the rhizosphere and endosphere of Lisbon lemon and Washington Navel orange trees were summarized at the family and genus levels and were defined as at least one sequencing read in ≥75% of samples in a group (Blaustein et al. 2017) [i.e., present in the roots of 5 of 6 plants in the lemon control and CLas(+) groups and in 3 of 4 plants belonging to the navel control and CLas(+) groups]. Figure 4 shows the 10 most abundant dominant bacteria and fungi (described at the family and genus levels). The majority of dominant bacteria were also members of the core bacterial microbiome (9 of 10 families, 8 of 10 genera). Several differences in bacterial and fungal community compositions were observed in CLas(+) plants compared with controls; however, interestingly, there was no overlap in these difference between citrus varieties.

The navel response to CLas was characterized by large increases in the relative abundance of *Burkholderiaceae* and *Burkholderia* in both the endosphere and rhizosphere, a dramatic decrease in *Enterobacteriaceae* in the rhizosphere, an increase in representatives from the fungal genus *Exophiala*, and changes in the relative abundance of several other less dominant microbes (Fig. 5, Supplementary Table S5). Conversely, the lemon response to CLas was characterized by large changes in fewer microbes. A large increase in the bacterial family *Xanthomonadaceae* was observed in both the endosphere and rhizosphere of lemons, along with a

Fig. 1. Images of the bulk root mass and sample leaves from healthy and ‘*Candidatus Liberibacter asiaticus*’ lemon and navel plants.

dramatic decrease in the fungal genus *Exophiala* and an increase in the bacteria *Scytaldium* and *Penicillium*. Similar to navel roots, *Burkholderia* species were dominant, but the abundance of this genus did not change with infection in lemons. The fungi *Hypocrea* increased significantly in both the endosphere and rhizosphere of lemons, and a large effect size was observed in the navel rhizosphere. The PCoA of microbial communities in the lemon and navel endosphere and rhizosphere showed a significant separation of samples based on the infection status (Fig. 6). Correlation analyses revealed several significant correlations between bacterial titers of the least reported CLAs Ct value in leaf tissue and microbial communities (Supplementary Table S6), but these findings were mostly nonsignificant after FDR correction at all sites measured except the navel rhizosphere. Here, strongly significant negative correlations were observed between CLAs Ct values of leaf tissue and the bacterial classes *Betaproteobacteria* and *Solibacteres*, the family *Methylocystaceae*, and the genus *Burkholderia*, and a strongly positive correlation was observed for the class *Gammaproteobacteria*. Additionally, fungi in *Ascomycota* were strongly and positively correlated with CLAs Ct values, whereas a strong negative correlation was observed with *Basidiomycota*. No significant correlations were observed in lemon roots.

DISCUSSION

Summary of findings. The aim of this research was to characterize metabolic and microbial shifts in lemon and navel roots affected by HLB. Differences in key metabolites, bacteria, and fungi were evident between healthy and diseased plants. Interestingly, host responses to CLAs were strongly influenced by variety, despite the use of identical ‘Carrizo’ rootstock. A recent study showed that alterations in root metabolite concentrations of citrus occur when scions are grafted onto different rootstock, but that the extent of these alterations depends on the variety of citrus examined (Albrecht et al. 2019). Here, we found that the scion variety did not significantly alter the root metabolome in healthy plants 18 months after grafting. The opposite was observed in microbial communities; significant differences were found between lemon and navel controls. This suggested that the scion variety strongly influences root microbial communities, but not root metabolites in citrus. If root exudates are primary drivers of the microbial community composition (Hu et al. 2018), then our findings suggest transient changes in metabolites are sufficient to alter community structure. Alternatively, these findings could suggest that the microbial structure is not impacted by water-soluble

Fig. 2. Nonmetric multidimensional scaling (NMDS) ordinations based on Bray-Curtis dissimilarity distances of metabolite concentrations in citrus roots. Plots show variations in the root metabolome by variety and infection status (*Candidatus Liberibacter asiaticus* [CLAs] versus healthy). Each small circle represents a single tree. The large circles represent the centroid (the weighted average location) of each sample type. Points plotted closer together indicate similar metabolic compositions. Low stress values (<0.20) indicate the model is representative of sample distances in two dimensions. **A**, Greenhouse-grown citrus trees (PERMANOVA, $R^2 = 0.42$, $P = 0.0010$, stress = 0.100). **B**, Lisbon lemon (PERMANOVA, $R^2 = 0.33$, $P = 0.0040$, stress = 0.045). **C**, Washington Navel orange (PERMANOVA, $R^2 = 0.26$, $P = 0.0600$, stress = 0.138). Grey = lemon control ($n = 11$); black = CLAs(+) lemon ($n = 6$); light blue = navel control ($n = 4$); dark blue = CLAs(+) navel ($n = 4$).

metabolites, or that, perhaps, the metabolome is not the primary driver of the community structure. To our knowledge, no other study has compared the root microbiome of different citrus varieties with an identical rootstock. This finding is supported by recent work involving apple fruit trees, which showed endophytic microbial community composition diverging as the distance in the pedigree grows (Liu et al. 2018). A limitation to our study was the relatively small sample size, particularly for navel, which likely offered insufficient power to detect statistically significant differences. This was underscored by the greater number of significant findings in the lemon group compared with navel. Nevertheless, significant effects were observed in both the metabolome and microbiome, and these results, coupled with the calculated effect sizes, were used to interpret the findings of this study. Another limitation was the lack of data regarding root exudate metabolites, which would have enabled stronger conclusions regarding plant crosstalk with microbial communities.

Response of Lisbon lemon to CLAs. Few studies have examined the response of *Citrus limon* to CLAs, and none has combined metabolomics with an analysis of the microbiome. Unique features of the lemon root metabolome included reductions in quinic acid, malic acid, GABA, methanol, ethanol, cytidine, synephrine, and several amino acids. GABA is a nonprotein amino acid that accumulates in plant tissue under stressed conditions, and it is synthesized in plants, animals, bacteria, and fungi via the GABA shunt pathway (Roberts 2007). Although its role in plant defense is unclear, it may act as a signaling molecule between plants and other organisms (Roberts 2007), and it has been shown to

modulate plant growth by regulating the aluminum-activated malic acid transporter (ALMT) protein complex (Ramesh et al. 2015). Interestingly, reduced GABA concentrations in lemon occurred in concert with a reduction in malic acid. A similar effect was not observed for navel. Ethanol can increase in diseased plants because of the increasingly hypoxic conditions created by accumulating necrotic plant tissue (Kelsey and Joseph 1998); however, ethanol is also metabolized by plant tissues to synthesize organic acids, amino acids, lipids, and sugars to be utilized by the host (Cossins and Beevers 1963). Alterations in the microbial communities of lemon roots were primarily driven by a large significant reduction in *Exophiala*, which includes fungi that are endophytic and frequently colonize plant roots, as either a mutualist or a pathogen (Jumpponen 2001), possessing enzymes capable of breaking down polysaccharides (Caldwell et al. 2000).

Response of Washington Navel orange to CLAs. Compared with lemon roots, fewer metabolites in navel roots were significantly altered in response to CLAs, and fewer significant correlations between metabolite concentrations and CLAs Ct values were observed. Interestingly, a greater number of bacterial and fungal genera appeared to be altered with CLAs infection in navel compared with lemons. Despite the small sample size, the observed differences were sufficient to distinguish the root metabolome and microbiome of healthy and HLB-affected navel roots. Previously, it was shown that the metabolite signature of midribs from symptomatic 6-year-old CLAs(+) Valencia orange leaves were distinguishable from asymptomatic CLAs(+) trees, but symptomatic

Fig. 3. Log basis twofold change in metabolite concentrations of *Candidatus Liberibacter asiaticus* (CLAs) relative to healthy controls in **A**, Lisbon lemon and **B**, Washington Navel orange roots. Metabolites are color-coded by category (sugars = black; amino acids = red; organic acids = green; other metabolites = purple). Positive values correspond to levels higher in CLAs(+) plants relative to control. **C**, Metabolite signatures in a Venn diagram summarize the differences and overlap in the varietal response to CLAs. Symbols indicate differences that were not significant but were associated with a large effect size. ◆ = navel; ■ = lemon.

Fig. 4. Stacked bar graph showing the 10 most dominant bacteria (top left and top right) and fungi (bottom left and bottom right) in roots obtained from greenhouse-grown citrus trees. Values are median % relative abundance. ‘*Candidatus Liberibacter asiaticus*’ trees are denoted as qPCR+.

and asymptomatic root tissue were not (dos Santos Freitas et al. 2015). In the current study, *Burkholderia* was the dominant genus in both citrus varieties and increased in navel roots, but not lemon roots, in response to CLAs. This contrasted with previous reports indicating that *Burkholderia* is associated with healthy sweet orange. For example, *Burkholderia* was observed to be more abundant in healthy 11-year-old mid-sweet orange trees compared with trees with HLB (Zhang et al. 2017), and in Valencia orange field trees affected by HLB (Trivedi et al. 2010). *Burkholderiaceae* has been identified as a key taxon in the root microbiome of healthy citrus (Zhang et al. 2017). However, Blaustein et al. (2017) reported higher abundances of *Burkholderia* in HLB-symptomatic compared with asymptomatic trees (Blaustein et al. 2017), which supported our findings of higher abundance in the roots of infected compared with healthy navels.

Trivedi et al. (2010) found that the relative abundance of *Methylobacterium* and *Sphingobacterium* genera increased in roots

obtained from CLAs(+) Valencia orange trees grown in a heavily infected citrus grove compared with uninfected trees. In contrast, *Methylobacterium* and *Sphingobacterium* were detected in the current study at low levels ($\leq 0.05\%$) in both healthy and CLAs(+) lemon and navel roots and did not change with infection. *Methylobacterium* has been identified as a common source of microbial DNA contamination during sample preparation (Salter et al. 2014); therefore, it is possible that previous reports were spurious. Trivedi et al. (2010) also observed that several bacterial genera appeared to be associated with uninfected field-grown Valencia orange root samples (e.g., *Caulobacter*, *Burkholderia*, *Lysobacter*, *Pantoea*, *Pseudomonas*, *Stenotrophomonas*, *Bacillus*, *Paenibacillus*). Similar observations were not made in the current study of greenhouse-grown Washington Navel oranges, except for *Pseudomonas*, which was higher in healthy controls, and *Bacillus*, which increased in the rhizosphere of both varieties in response to CLAs.

Fig. 5. Venn diagrams show bacterial and fungal signatures associated with ‘*Candidatus Liberibacter asiaticus*’ in the endosphere and rhizosphere of Lisbon lemon and Washington Navel orange roots. Symbols indicate differences that were not significant but were associated with a large effect size. ◆ = navel; ■ = lemon.

Fig. 6. Principal coordinate analysis (PCoA) plots showing differences in **A**, bacterial and **B**, fungal communities in Lisbon lemon and Washington Navel orange roots (PERMANOVA). Each point represents a single tree. Light circles are the control; dark circles are CLAs(+). Clustering was driven by infection status, particularly in navel samples despite a relatively smaller sample size.

Similarities and differences in the responses of lemons and navels to CLAs. There was overlap in the varietal response to CLAs, including reductions in all sugars measured and in the amino acids proline and asparagine, and an increase in trigonelline. Furthermore, overlapping microbial shifts included decreases in *Pseudomonas* and *Rhizobium*, increases in *Bacillus* and *Kaistobacter*, and an increase in the fungi *Hypocrea*. Moreover, a dramatic decrease in *Enterobacteriaceae* was observed in both varieties. Recently, *Enterobacteriaceae* was shown to dominate the phyllosphere of arugula, thereby improving plant resistance to pathogen attacks through its antibiotic capabilities (Cernava et al. 2019). Altered sucrose concentrations in citrus roots were consistent with reports that CLAs influence the carbohydrate gene expression in citrus host plants (Kim et al. 2009). Furthermore, starch accumulation in the leaf tissue and an extensive loss of starch reserves in the root system have been observed (Ettxeberria et al. 2009).

Stress caused by pathogenic microorganisms alters the plant source/sink relationship (Lemoine et al. 2013); therefore, lower metabolite concentrations in the root system ('sink') are consistent with higher concentrations in citrus leaves ('source'). Proline is implicated in the plant defense response, and proline accumulation in source organs during a pathogen attack has been documented in several plant models (Qamar et al. 2015). Conversely, HLB appears to lower the concentrations of the amino acids alanine, proline, and threonine in fruit, the other dominant 'sink' organ of citrus (Slisz et al. 2012). Asparagine is involved in the transport and storage of nitrogen in plants. One study showed that asparagine concentrations in the roots of white lupin plants decrease rapidly when nitrogen availability in the surrounding environment becomes limited (Lea et al. 2007). Trigonelline is a secondary metabolite in niacin metabolism that accumulates and acts as an osmoprotectant during stress (Rajasekaran et al. 2001). Recently, the *tnaA* gene cluster was identified in the degradation pathway of trigonelline to the end products succinate and methylamine, which are utilized by bacteria such as *Agrobacterium*, *Burkholderia*, and *Pseudomonas* as sources of carbon and nitrogen (Perchat et al. 2018).

Xu et al. (2018) showed that *Burkholderia*, *Pseudomonas*, and *Rhizobium* form part of the core citrus rhizosphere, and both *Pseudomonas* and *Rhizobacterium* are known to suppress plant pathogens and promote root growth (Preston 2004; Datta et al. 2015). In the current study, both lemons and navels responded to CLAs infection with decreases in *Pseudomonas* and *Rhizobium*, whereas an increase in *Burkholderia* was observed only in navels. It is unclear why *Burkholderia* increased in navels in response to CLAs, because others have associated this genus with beneficial effects in citrus (Trivedi et al. 2012; Zhang et al. 2017). However, *Burkholderia* is a large genus comprising many species, some of which are beneficial and others that are pathogenic to plants (Eberl and Vandamme 2016).

Populations of *Bacillus* and *Kaistobacter* increased in both lemon and navel roots, whereas the fungus *Hypocrea* increased significantly in lemons. Members of *Bacillus* have been shown to colonize the roots and promote growth in tomato plants and *Arabidopsis thaliana*; interestingly, malic acid appears to act as a chemo-attractant for these microbes (Lugtenberg and Kamilova 2009). Curiously, malic acid concentrations increased in navel roots and decreased in lemons, but large effect sizes corresponding to increased *Bacillus* in the rhizosphere of both citrus varieties were observed. *Kaistobacter* is associated with disease suppression in tobacco plants (Liu et al. 2016). Increases in these two potentially beneficial microbes suggested that *Bacillus* and *Kaistobacter* may form part of the global response to CLAs in citrus. *Hypocrea* species have been shown to have biocontrol activity but also opportunistic pathogens that are often associated with rotting wood (Kubicek et al. 2008). Furthermore, it is possible that the abundance of *Hypocrea* increased in response to the advanced diseased state of HLB-affected plants, as observed in their decayed root mass relative to controls.

Other differences in the varietal response to CLAs were evident. For instance, the concentrations of malic acid and quinic acid increased in navel roots, but they were decreased in lemons. Furthermore, several more metabolites in lemon were significantly different compared with navel. Quinic acid is an intermediate metabolite formed in the shikimate pathway, which plants and microbes utilize to synthesize the aromatic amino acids phenylalanine, tyrosine, and tryptophan, and it is important for protein biosynthesis and the production of plant hormones and secondary metabolites (Guo et al. 2014). No differences in the concentrations of phenylalanine and tyrosine in navel roots were observed (tryptophan was not detected), which suggested disruptions to the shikimate pathway did not occur, at least in terms of downstream products. However, bacteria, such as *Bacillus*, which increased in the rhizosphere of lemon and navel roots, are capable of metabolizing quinic acid into shikimic acid (Ghosh et al. 2014). Interestingly, large effect sizes for the metabolites 2-oxoglutarate, lysine, and formate were observed in navel but not lemon roots, despite the relatively smaller sample size in the former. This perhaps warrants further investigations to confirm these findings.

Dominant bacteria and fungi varied by root compartment (i.e., endosphere versus rhizosphere) and by variety. For example, *Herpotrichiellaceae* was the most abundant fungal family in the lemon endosphere, and it was reduced with CLAs infection, but a similar observation was not made in the navel endosphere. A recent study identified a consortium of phloem-inhabiting bacteria in leaf tissue that appear integral to the survival of CLAs, including *Comamonadaceae*, *Flavobacteriaceae*, *Microbacteriaceae*, and *Pseudomonadaceae* (Fujiwara et al. 2018). In the current study, *Comamonadaceae* was reduced in the endosphere and significantly increased in the rhizosphere of infected navel roots, which agreed with the results of Blaustein et al. (2017), who showed that a lower abundance of *Comamonadaceae* was associated with HLB-symptomatic compared with asymptomatic trees. Differences in *Flavobacteriaceae* were not observed, and the abundance of *Microbacteriaceae* was different in lemons compared with navels.

Tolerant versus susceptible varieties. A growing body of evidence points to a delineation among citrus varieties based on their tolerance or susceptibility to CLAs. For example, varieties of sweet orange and grapefruit were shown to be highly sensitive to CLAs infection, whereas Mexican limes and Eureka lemons appeared to be more tolerant (Folimonova et al. 2009), and some accessions of *C. limon* were shown to fare much better than sweet orange and mandarin in a published field study (Miles et al. 2017). Emerging evidence has suggested multiple genes involved in defense are upregulated to a greater extent in rough lemon compared with sweet orange, which may account for differences in the observed tolerance between the citrus species (Yu et al. 2017). Although some varieties of citrus within the citron pedigree appear to be more HLB-tolerant, *Citrus sinensis* and *Citrus limon* have been categorized as having approximately the same degree of susceptibility to CLAs (Ramadugu et al. 2016); therefore, distinction based on tolerance and susceptibility may not be appropriate in the current study. However, Chin et al. (E. L. Chin, J. Ramsey, S. Saha, D. Mishchuk, J. Chaves, K. Howe, X. F. Zhong, M. Flores-Gonzalez, E. Mitrovic, M. L. Polek, K. Godfrey, L. A. Mueller, J. Bruce, M. Heck, and C. Slupsky, unpublished data) recently showed that the rate of CLAs transmission was lower in *C. limon*, and that photosynthesis was less affected in presymptomatic plants compared with *C. sinensis*. In the root system, we observed varietal differences in the response to CLAs characterized by larger changes in lemons in terms of the root metabolite composition, but greater differences in navels in terms of root microbial communities, which translated into distinct metabolite and microbial fingerprints emerging for each variety. The implication is that treatment targeting the health of the root system likely requires consideration for the varietal response to disease as opposed to a global approach to HLB management.

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This article was modified on 19 Feb 2020.

ERRATUM / Volume 109, Number 12, 2019 / PHYTO-03-19-0103-R

In the article “Metabolome and Microbiome Signatures in the Roots of Citrus Affected by Huanglongbing” by E. M. T. Padhi, N. Maharaj, S.-Y. Lin, D. O. Mishchuk, E. Chin, K. Godfrey, E. Foster, M. Polek, J. H. J. Leveau, and C. M. Slupsky, Figure 4 incorrectly indicates a drop in *Hyphomicrobacteriaceae* and an increase in *Enterobacteriaceae* instead of a dramatic drop in the bacterial family *Enterobacteriaceae*. The color legend for the fungal genera pertaining to *Conlarium* and *Clitopilus* has also been corrected due to a similar error in the color scheme. The corrected Figure 4 is shown below.

Revised Figure 4

(Continued on next page)

Fig. 4. Stacked bar graph showing the 10 most dominant bacteria (top left and top right) and fungi (bottom left and bottom right) in roots obtained from greenhouse-grown citrus trees. Values are median % relative abundance. ‘*Candidatus Liberibacter asiaticus*’ trees are denoted as qPCR+.

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