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Metabolomic Analysis of Citrus Infection by '*Candidatus* Liberibacter' Reveals Insight into Pathogenicity

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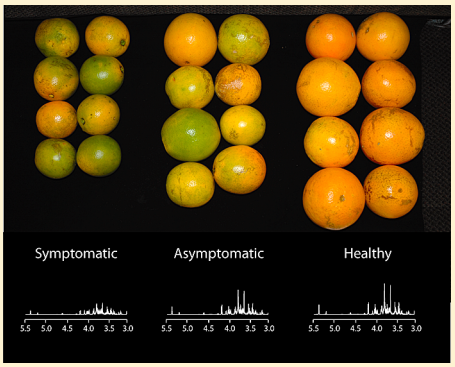
Supporting Information

ABSTRACT: Huanglongbing (HLB), considered the most serious citrus disease in the world, is associated with the nonculturable bacterium '*Candidatus* Liberibacter asiaticus' (Las). Infection of citrus by this pathogen leads to reduced plant vigor and productivity, ultimately resulting in death of the infected tree. It can take up to two years following initial infection before outward symptoms become apparent, making detection difficult. The existing knowledge gap in our understanding of Las and its pathogenesis leading to HLB has stymied development of treatments and methods to mitigate the pathogen's influence. To evaluate the influence of Las on fruit quality in both symptomatic and asymptomatic fruit, and gain further insight into the pathogenesis of the disease, a ^{1}H NMR metabolomics investigation, complemented with physicochemical and analyte-specific analyses, was undertaken. Comparison of the juice obtained from oranges gathered from Las^{+} (symptomatic and asymptomatic) and Las^{-} (healthy) trees revealed significant differences in the concentrations of sugars, amino and organic acids, limonin glucoside, and limonin. This study demonstrates differing metabolic profiles in the juice of oranges from Las^{+} and Las^{-} and proposes how Las may be able to evade citrus defense responses.



KEYWORDS: *citrus, Citrus sinensis, Huanglongbing, metabolomics, NMR, Valencia oranges*

INTRODUCTION

Huanglongbing (HLB) is considered one of the most serious citrus diseases and has emerged as a major threat to citrus production worldwide. To date, there are no known cures, treatments, or varieties resistant to this disease. Infected trees not only succumb to the disease within a few years of infection, but also produce fruit that are not suitable for fresh consumption or juice production due to a significant increase in bitter and acidic flavor in these oranges.[1]

HLB is the result of an infection by the phloem-limited plant pathogenic bacteria '*Candidatus* Liberibacter'. Three species of this bacterium are known to cause the disease: asiaticus, africanus, and americanus, and insect vectors transmit the disease from tree to tree. In the United States, only the asiaticus species (Las) has been detected. The Asian citrus psyllid, *Diaphorina citri*, is the vector responsible for spreading the bacterium in Asia and the Americas, while the bacteria in Africa is transmitted by another psyllid species known as *Trioza erytreae*.[2] Psyllids carrying any of these bacteria can infect healthy trees by feeding on them. As it feeds on the tree, the psyllid injects the bacterium into the phloem, allowing it to become a part of the vascular system of the plant. Regardless of cultivar, symptoms of HLB are similar, and telltale signs include blotchy mottling of leaves, twig dieback, and fruit drop. Fruit produced by severely HLB-affected (Las^{+}) trees may be lopsided, remain green at the stylar end, contain aborted seed and discolored vascular bundles, and have a bitter taste,[3] or may show no phenotypic difference from fruit collected from healthy, Las^{-} trees.

For decades, HLB has caused devastation to citrus producing regions in Asia and Africa, and in recent years has spread to the Americas, where it has been discovered in São Paulo, Brazil, Florida, and, most recently, Texas and California in the United States.[2−5] The main reason for orchard devastation is the long latency period between infection and manifestation of symptoms, and the fact that the symptoms may resemble other citrus disorders. Moreover, detection of the pathogen requires PCR techniques, which demand significant bacterial titer for a positive identification. For the 2009−2010 season,

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the top orange producing states in the U.S. were Florida (65% of total production), California (31%), as well as Texas and Arizona (4% combined).[6] In addition, Valencia oranges accounted for approximately 50% (47.45%) of citrus produced in Florida.[7] The Las infection of citrus has been seriously damaging Florida's citrus industry and, as a result, Florida's overall agricultural economy.

Plant metabolomics is becoming an important tool for understanding how plant metabolism is affected by various conditions. Metabolomics attempts to measure all of the metabolites present in a chosen tissue or fluid at a specific point in time, providing scientists with a tool suitable for determining plants' responses to various environmental conditions.[8] To determine the effect of Las infection on the sweet orange fruit's metabolic signature, a ^{1}H nuclear magnetic resonance (NMR) metabolomics study was undertaken. Previous studies have shown ^{1}H NMR spectroscopy coupled with multivariate analysis as an appropriate and applicable tool for quantifying metabolites in orange juice.[9−13] The application of NMR spectroscopy for this study proved beneficial for providing an understanding of how the Las bacterium is able to evade plant defenses, and providing clues as to the basis of the effect of infection on fruit juice quality.

EXPERIMENTAL SECTION

Plant Materials

Citrus sinensis cultivar Valencia fruit samples used in this study were collected from five Las$^-$ and five symptomatic Las$^+$ trees grown in a commercial orchard in Florida. The trees were mature, fruit-bearing trees, grown in two adjacent rows with no more than 10 trees separating experimental trees, and all were approximately the same age: 10−12 years. Initial sampling was conducted in May 2007 and a replicate harvest was made in June of the same year. For each harvest, a minimum of 15 asymptomatic (i.e., fruit from Las$^+$ trees that were indistinguishable from healthy fruit) or healthy fruit (i.e., fruit from healthy, Las$^-$ trees) were collected per tree and juiced by hand. The resulting juice was pooled, aliquoted, and stored at −20 °C until used for analysis. At the June harvest, a single juice sample was prepared from symptomatic fruit gathered from a number of trees. The presence of the Las bacteria was confirmed by PCR,[14] and healthy trees were shown to be negative for the bacterium by the same assay.

Measurement of Physicochemical Properties

°Brix, defined as the grams of sucrose in 100 mL of water, was approximated by measuring Total Soluble Solids (TSS) using a Rudolph Research model J257 Automatic Refractometer (Hackettstown, NJ). Since sucrose represents approximately 90% of the TSS in orange juice, it is common practice to use °Brix when referring to the TSS content of citrus juice. Total Titratable Acidity (TTA) was determined with a Metrohm 751GPD Titrino (Westbury, NY) calibrated with pH 7 and 10 buffers used in conjunction with a 730 Sample Changer. The juice sample was diluted 10:1 with water and 100 mL of the test solution was titrated to a pH of 8.2 using 0.156 M NaOH. The acid level was calculated from amount of NaOH (mL) × 0.1 and expressed as percent citric acid.

Determination of Limonin by High-Performance Liquid Chromatography (HPLC)

Frozen juice samples were thawed to 4 °C and incubated at the same for 48 h to convert any limoninoate A-ring lactone present into limonin.[15] Each sample was extracted three times by combining a 1.0 mL aliquot of sample with 2 mL of chloroform, followed by centrifugation for 2 min at 200*g*, and collection of the chloroform layer. The three repeat extracts were combined and evaporated to dryness at 40 °C. Prior to analysis, the sample was reconstituted in 30% acetonitrile (ACN) in 10 mM formic acid (500 μL). The entire extraction procedure was performed in triplicate for each sample. HPLC analysis was conducted as previously described,[16] using a 50 × 2.0 mm Phenomenex Phenosphere-Next-5 μm Phenyl Column, equipped with a guard column of the same material and maintained at 30 °C. The flow rate was 1.0 mL/min and an isocratic solvent composition of 70% of 10 mM formic acid, 30% ACN was used. Results are presented as the average of three replicate analyses ± SD.

NMR Data Collection

For NMR spectroscopy, samples were thawed and centrifuged at 4 °C for 15 min at 14 000*g* to remove particulate matter. The supernatant was filtered through Omega-3 3000 molecular weight cutoff filters to remove pectin. To each filtrate, an internal standard containing 3-(trimethylsilyl)-1-propanesulfonic acid-d_6 (DSS-d_6), NaN_3, and D_2O was added such that the final concentration of each was 0.5 mM DSS-d_6, 0.02% NaN_3, and 10% D_2O. The pH for each sample was adjusted to 6.8 ± 0.1 by adding small amounts of NaOH. A total of 600 μL was transferred to 5 mm NMR tubes, and samples were stored at 4 °C until NMR data acquisition (within 24 h of sample preparation).

NMR spectra were acquired using the Bruker "noesyprld" experiment on a Bruker Avance 600 MHz NMR spectrometer equipped with a SampleJet. Acquisition parameters were the following: 12 ppm sweep width, 2.5 s acquisition time, 2.5 s relaxation delay, and 100 ms mixing time. Water saturation was applied during the 2.5 s relaxation delay and the 100 ms mixing time. The resulting data 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 manually baseline corrected using a spline fit within the Processor module of Chenomx NMRSuite (Chenomx, Inc., Edmonton, Alberta, Canada).

Data Analysis

Metabolite identification and quantification was achieved through targeted profiling using Chenomx NMRSuite 6.1 (Chenomx, Inc., Edmonton, Alberta, Canada).[17] A total of 29 compounds were assigned and quantified. All metabolites measured by NMR were clearly distinguishable from one another (see Supporting Information Figure S1), and subtraction of the fit line from the spectral line resulted in a flat baseline for those metabolites within the Chenomx library (Supporting Information Figure S2). Concentrations are reported to the nearest micromolar (μM) for low concentration metabolites (<1 mM), and to the nearest 0.1 mM for higher concentration metabolites (>1 mM). These significant figures are based on a previous study which illustrated the accuracy and precision of metabolite measurement in urine (a more complex medium).[18] Metabolite concentrations were corrected for dilution, and subjected to $\log_{10}$ transformation to ensure normal distribution as previously described.[13] Multivariate statistical data analyses, including principal component (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA), were performed using SIMCA-P with mean centering and unit variance scaling. Significance testing, using

Table 1. Average Metabolite Concentrations (μM) and Coefficient of Variation (CV) (Expressed as a Percent) in Healthy, Asymptomatic, And Symptomatic Fruit Obtained from Valencia Orange Trees[a]

	May harvest				June harvest				
metabolite	healthy	CV	asymptomatic	CV	healthy	CV	asymptomatic	CV	symptomatic[b]
				Sugars					
Fructose	94.6×10^3	9	87.3×10^3	20	94.6×10^3	8	91.3×10^3	10	81.8×10^3
Glucose	76.8×10^3	7	72.3×10^3	16	76.2×10^3	7	68.7×10^3	13	62.1×10^3
Sucrose	120.7×10^3	4	108.5×10^3	15	135.6×10^3	5	$113.8 \times 10^{3***}$	4	44.9×10^3
myo-Inositol	5.2×10^3	8	5.0×10^3	8	6.1×10^3	4	$5.6 \times 10^{3*}$	6	6.1×10^3
				Amino Acids					
Alanine	1.2×10^3	11	1.1×10^3	14	1.9×10^3	15	1.7×10^3	17	860
Arginine	3.8×10^3	3	3.6×10^3	10	4.2×10^3	6	$3.6 \times 10^{3*}$	11	3.3×10^3
Asparagine	2.8×10^3	12	2.6×10^3	27	2.6×10^3	20	2.8×10^3	17	3.7×10^3
Aspartate	2.0×10^3	7	1.8×10^3	7	2.4×10^3	12	2.1×10^3	7	2.2×10^3
Histidine	73	35	92	20	97	17	86	34	167
Isoleucine	50	6	52	16	79	8	70*	6	51
Leucine	48	9	52	23	75	18	70	14	47
Phenylalanine	133	13	171	25	168	11	186	8	389
Proline	4.4×10^3	25	$2.9 \times 10^{3*}$	18	5.1×10^3	14	$3.1 \times 10^{3**}$	20	1.7×10^3
Threonine	167	10	165	19	240	13	219	11	163
Valine	189	10	182	13	276	9	234*	11	162
				Organic Acids					
Ascorbate	1.3×10^3	13	1.2×10^3	16	1.0×10^3	9	978	17	1.8×10^3
Citrate	34.0×10^3	11	30.0×10^3	11	25.0×10^3	10	20.0×10^3	20	48.1×10^3
Formate	62	10	87*	13	45	12	48	13	66
Malate	20.0×10^3	14	17.2×10^3	9	19.4×10^3	8	$16.4 \times 10^{3*}$	11	13.8×10^3
Succinate	57	28	56	27	78*	9	87*	4	81
GABA	2.1×10^3	9	2.1×10^3	14	3.3×10^3	10	2.8×10^3	12	3.1×10^3
				Others					
Adenosine	38	8	34*	5	39	6	34	12	30
Ethanol	7.4×10^3	18	8.1×10^3	10	9.6×10^3	25	8.3×10^3	18	8.9×10^3
Synephrine	22	12	24	10	26	23	25	21	46
Limonin[c]	7	28	10*	16	6	21	11**	20	16
Limonin glucoside	814	15	$1.1 \times 10^{3**}$	17	1.1×10^3	28	1.4×10^3	27	1.5×10^3
Methanol	982	13	971	20	1.3×10^3	21	1.5×10^3	23	1.1×10^3
Proline betaine	5.3×10^3	3	5.3×10^3	9	5.9×10^3	6	5.9×10^3	10	6.2×10^3
Trigonelline	91	7	89	7	113	10	103	9	98
Choline	280	15	257	22	391	5	366	8	245

[a] *P*-values represent comparisons within harvests: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. [b] Concentration reflects determination from one sample compiled from several oranges. [c] Concentration determined by HPLC.

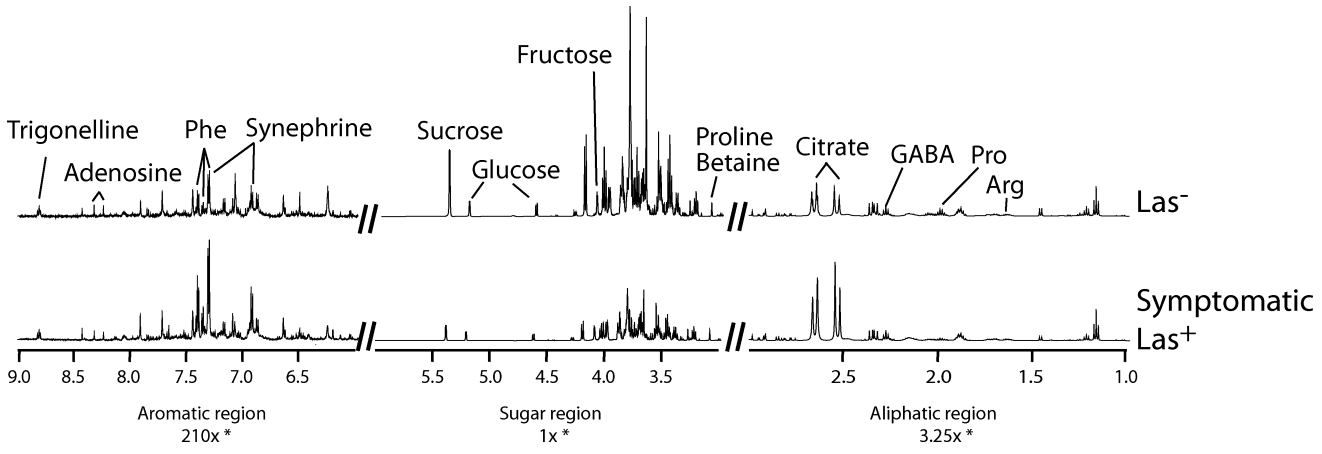

Figure 1. ^{1}H NMR spectral comparison of Valencia orange juice collected from (A) healthy fruit from Las$^-$ trees, and (B) symptomatic fruit Las$^+$ trees. For ease of visualization, the vertical scale was increased approximately 210 times for the aromatic region and 3.25 times for the aliphatic region.

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Student's t test, was performed using Microsoft Excel, and set at $\alpha = 0.05$.

RESULTS

In a previous study, the effects of elevation, rootstock, and soil depth on the nutritional quality of mandarin oranges were investigated to highlight how ^{1}H NMR metabolomics could be used to understand how unique microenvironments affect the nutrient composition of citrus.[13] Using a similar methodology for this study, the same compounds were identified and quantified from ^{1}H NMR spectra of Valencia orange juice samples obtained from Las$^-$ and Las$^+$ trees (Table 1). Sugars, amino acids, organic acids, and other metabolites were included in this collection of compounds. Figure 1 provides an NMR spectral comparison of juice samples from oranges collected from symptomatic fruit from a Las$^+$ tree versus fruit collected from a healthy, Las$^-$ tree. Clear differences can be seen within each region of the spectra. In particular, a 3-fold decrease in sucrose concentration, a 3-fold increase in phenylalanine concentration, and a nearly 2-fold increase in citrate concentration are apparent. Other changes include decreased fructose and glucose, decreased amino acids (alanine, arginine, isoleucine, leucine, proline, threonine, and valine), as well as decreased choline, malate, and adeonsine. Other metabolites higher in concentration in the symptomatic fruit included asparagine, histidine, ascorbate, formate, synepherine, limonin, and limonin glucoside.

To determine which metabolites present in the orange juice could differentiate asymptomatic fruit from Las$^+$ trees and healthy fruit from Las$^-$ trees, metabolite concentrations were measured, and multivariate statistical analysis was applied. Figure 2A shows a PCA plot of all samples showing discrimination between harvest date in the first component, and infection status in the second component. To further understand the contribution and determine which metabolite variables are most important for differentiating asymptomatic fruit from Las$^+$ trees and fruit from healthy trees, OPLS-DA was applied (Figure 2B). R^2 and Q^2 were 0.988, and 0.761, respectively, and validation by random permutation (100 permutations) of the PLS-DA model revealed significantly positive slopes with y-intercepts of 0.788 and −0.27 for R^2 and Q^2 respectively, indicating a valid model. Interestingly, tight clustering along component 1 and spread along component 2 was observed in the OPLS-DA plot. Spread along component 2 is explained by harvest date. The fruit harvested in May are located in the lower half of the plot, and those harvested in June in the upper half of the plot. This spread could also be observed in the first component of the PCA plot where those fruit harvested in May were located to the left of the plot, and those in June to the right of the plot. The tight clustering along component 1 and the clear separation of groups is likely due to the major differences in metabolite concentrations between groups, coupled with low CVs of many of the most important metabolites responsible for separating healthy and asymptomatic fruit. The loadings plot revealed several metabolites that were higher in the juice collected from fruit from healthy trees, including proline, malate, sucrose, adenosine, arginine, aspartate, citrate, threonine, and fructose. Metabolites higher in asymptomatic fruit from Las$^+$ trees included limonin, limonin glucoside, phenylalanine, and formate. Supporting Information Figure S3 shows an overlay of spectra from fruit from healthy and asymptomatic trees obtained from the May harvest illustrating clear differences in the spectral integral of several

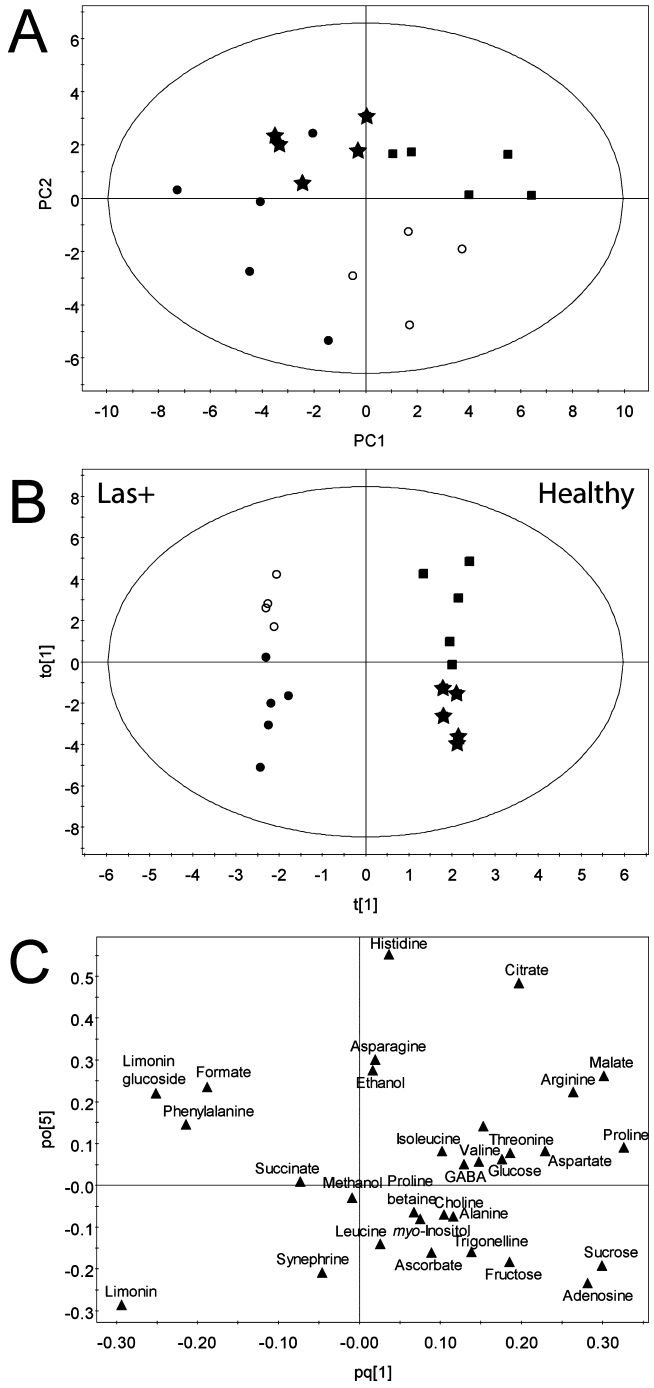

Figure 2. Comparison of metabolite composition of Valencia orange juice samples obtained from Las$^+$ ($N = 9$) and Las$^-$ ($N = 10$) trees. (A) PCA plot comparing juice from asymptomatic (circles) and healthy (squares and stars) fruit. Black circles and stars represent May harvest, and white circles and black squares represent June harvest. (B) OPLS-DA scores plot comparing juice from asymptomatic and healthy trees. Designation for harvest was done after multivariate analysis by hand. (C) OPLS-DA loadings plot.

metabolites including proline, arginine, phenylalanine, and sucrose.

To determine metabolite differences between asymptomatic and healthy fruit depending on harvest, fruit collected from the May harvest were compared with fruit collected in June (Figure 3). For Las$^-$ trees (Figure 3A), most metabolites were in higher concentration in the June harvest when compared to the May

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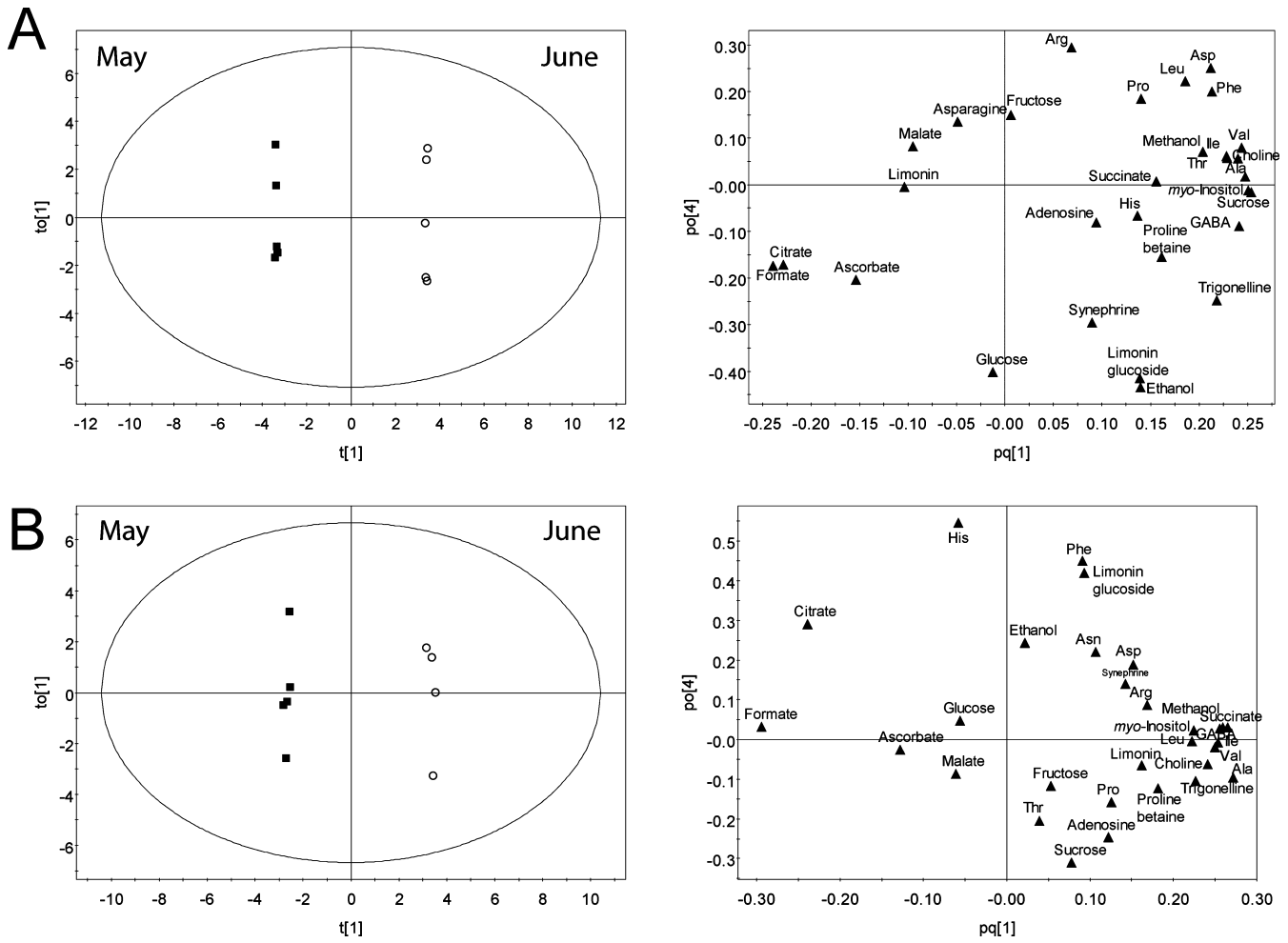

Figure 3. OPLS-DA comparison of juice from Valencia oranges obtained from the May and June harvest of the same year. (A) Fruit from Las$^-$ trees harvested in May (squares) (N = 5) and June (circles) (N = 5). (B) Fruit from Las$^+$ trees harvested in May (squares) (N = 5) and June (circles) (N = 4).

harvest, and included sucrose, *myo*-inositol, alanine, valine, choline, threonine, isoleucine, GABA (γ-amino butyrate), trigonelline, phenylalanine, aspartate, methanol, and leucine. In the May harvest, formate, citrate, and ascorbate were higher. Fruit collected from Las$^+$ trees in May and June revealed similar differences (Figure 3B), with higher concentrations of *myo*-inositol, alanine, valine, isoleucine, leucine, choline, GABA, trigonelline, and methanol in the June harvest, whereas formate and citrate were higher in the May harvest. Interestingly, sucrose, phenylalanine and aspartate did not change as much from the May to June harvest in the fruit from Las$^+$ trees as compared to fruit from healthy trees.

Comparison of juice from asymptomatic fruit with juice obtained from symptomatic fruit revealed more pronounced differences (Table 1). The asymptomatic fruit had approximately 2.5 times the concentration of sucrose found in the symptomatic sample. Additionally, many amino acids including alanine, arginine, isoleucine, leucine, proline, threonine, and valine were lower in concentration in the symptomatic samples with the exception of phenylalanine, asparagine, and histidine, which were higher in concentration. Ascorbate and citrate concentrations were nearly double in the symptomatic sample as compared to asymptomatic samples, and limonin and limonin glucoside concentrations were higher as well. Comparison of sugar/acid ratios revealed a higher °Brix/TTA for juice collected from fruit from healthy trees compared to juice collected from asymptomatic or symptomatic fruit from Las$^+$ trees (Table 2).

Table 2. Physicochemical Measurements of Orange Juice Samples from Las$^+$ and Las$^-$ Trees

	May		June		
	Las$^-$	Las$^+$	Las$^-$	Las$^+$	Las + Sym.
°Brix	10.56	9.63	10.84	9.68	6.91
TTA	0.54	0.52	0.40	0.38	0.69
°Brix/TTA	19.48	18.54	27.31	25.68	10.07

To understand the variation in metabolite concentration of fruit collected from healthy and Las$^+$ trees, coefficients of variation (CV) were calculated (Table 1). Most of the metabolites in the asymptomatic fruit have higher CVs compared to the metabolites measured in healthy fruit. Interestingly, in the June harvest, the CVs for sucrose and *myo*-inositol are similar between healthy and asymptomatic fruit, but fructose and glucose concentrations in the asymptomatic fruit have CVs approximately 2 times greater than those of the healthy fruit. The CVs of the organic acids appeared to be consistent regardless of harvest date or infection status, except for citrate, which doubled between the May and

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June harvests for asymptomatic fruit, and was double for asymptomatic fruit as compared to healthy samples obtained in the June harvest. Interestingly, there was more variation in metabolite concentration in the May harvest of asymptomatic fruit than in the June harvest.

DISCUSSION

The Asian citrus psyllid, which is the vector of the '*Ca.* Liberibacter' bacterium, infects a tree by injecting the bacterium into the phloem. The phloem is the conduit by which organic nutrients created by photosynthesis in the leaves are transported throughout the plant, including the fruit. For the bacterium to thrive in phloem, it needs to utilize nutrients found there for its own growth and reproduction in addition to inhibiting the plant's natural defense mechanisms. Sequencing of the '*Ca.* Liberbacter asiaticus' bacterium genome found in the Kyoto Encyclopedia of Genes and Genomes (KEGG) online database has revealed a functional TCA cycle allowing the bacterium to utilize a wide range of amino acids as energy sources including glutamate, alanine, aspartate, glycine, serine, threonine, methionine, cysteine, arginine, proline, histidine, tyrosine, phenylalanine, and tryptophan,[19] in addition to glucose.

Interestingly, adenosine, as well as several amino acids (proline, arginine, and the branched-chain amino acids (BCAA)), was among the metabolites with the most significant concentration differences between fruit collected from Las^+ trees and those from Las^- trees. The amino acid with the largest difference in concentration was proline, which was found to be significantly lower in asymptomatic and symptomatic orange juice samples collected from Las^+ trees. This is in contrast to what was expected. Indeed, proline accumulation has been shown in stressed plants of many species, and it is thought to have a protective function, as increased concentrations have been observed during conditions of droughts, high salinity, high light, UV irradiation, heavy metals, oxidative stress, and in response to biotic stresses.[20] Moreover, previous studies have shown an increase of proline in citrus leaves under physiological and biological stresses, including leaves from Las^+ trees.[21−23]

Arginine concentrations were also significantly lower in orange juice samples from Las^+ trees compared to those from Las^- trees. Similar to proline accumulation, studies have shown that arginine accumulation appears to occur to citrus during periods of stress.[24−27] Hanks and Feldman performed multiple studies focusing on the effect of infection by the nematode *Radopholus similus* on amino acid levels in citrus roots and leaves. They determined that roots from infected trees had higher levels of amino acids than healthy roots, with proline, asparagine, and arginine accounting for more than 50% of the total amount of amino acids measured. In addition, they also found the total free amino acid content increased as the infection period continued,[24,25] and that arginine constituted a much greater proportion of the free amino acids in the leaves of the infected plants.[26] Nemec and Meredith found similar results when they studied amino acid content of citrus leaves exposed to mineral deficiencies, observing an increase in arginine, proline, and lysine.[27]

At present, it is not clear why proline and arginine would be lower in concentration in the fruit than in vegetative tissues. A possible explanation for these findings could be related to the distribution of '*Ca.* Liberibacter' in the plant. Indeed, its distribution has been found throughout the plant, including all fruit parts except the endosperm and embryo.[28] However, this distribution is uneven, with the highest concentrations of the bacterium in the fruit peduncle.[28] Evidence for an uneven distribution of the bacteria within the tree is indicated by the higher CVs observed in the asymptomatic fruit as compared with the healthy fruit. Differing bacterial titers close to the fruit may alter the extent of the effect the bacterium has on fruit metabolism, and thus cause variation in the measured metabolite concentrations. Moreover, decreased proline and arginine concentrations suggest that the bacteria may suppress plant defense mechanisms (such as a defense-related H_2O_2 burst) in both asymptomatic and symptomatic fruit that could in turn contribute to bacterial pathogenicity. In plants, H_2O_2 has been shown to decrease the activity of proline dehydrogenase and simultaneously activate the glutamate and ornithine pathways of proline biosynthesis ultimately resulting in higher levels of proline.[29] Decreased levels of arginine in asymptomatic and symptomatic fruit may also reflect a lower ability to create nitric oxide (NO), important in plant signaling pathways and defense.[30]

Another interesting difference between fruit from Las^- and Las^+ trees was a higher concentration of phenylalanine in the asymptomatic and symptomatic fruit from Las^+ trees. The higher concentration of phenylalanine could be due to inhibition of the phenylpropenoid biosynthetic pathway by the Las bacteria. Phenylpropenoids are alleochemicals whose biosynthesis is induced in response to biotic and abiotic stresses. The initial step in the phenylpropenoid biosynthetic pathway is the conversion of phenylalanine to cinnamic acid by the action of phenylalanine ammonia-lyase (PAL). Another citrus pathogen, the fungus *Penicillium digitatum*, has been shown to suppress host defenses by inhibiting PAL.[31]

Other amino acids affected by infection are the BCAAs and threonine. Concentrations of isoleucine, leucine, and valine were very similar between the healthy and asymptomatic samples from the May harvest, but lower in the asymptomatic samples compared to the healthy samples from the June harvest. As with proline, BCAAs have been shown to accumulate in response to stress, and may act as signaling molecules to regulate gene expression.[32] Interestingly, in humans, BCAAs provide energy to the immune system, and are precursors for the synthesis of new cells, effector molecules, and protective molecules.[33] Similarly, BCAAs in plants appear to be important for plant defense.[34] Threonine, an amino acid related to isoleucine synthesis, was also negatively impacted with '*Ca.* Liberibacter' infection.

Concentrations of adenosine were also significantly lower in samples from Las^+ trees. Lower adenosine concentrations may be related to increased citrate in fruit from the Las^+ trees. ATP levels in citrus juice have been shown to be directly correlated with citrate concentration potentially due to the regulatory action of ATP on citrate synthetase as the fruit reaches maturity.[35] The lower concentration of adenosine in fruit from Las^+ trees may partially explain the higher citrate concentrations characteristic of infection. Citrate accumulation may also be bacterially produced, as has been observed with another citrus pathogen, *P. digitatum*. *P. digitatum* compromises citrus fruit defense by acidifying host tissue through production of citric and gluconic acids.[36] This acidification may be important for pathogenicity and virulence of '*Ca.* Liberibacter'.

Analysis based on time of harvest indicated a greater effect of the bacterium on fruit metabolism as it matures. Normally, sugar concentrations increase while acid concentrations

decrease as the fruit approaches maturity, as may be observed by comparing the sugar and acid concentrations of the fruit from Las$^-$ trees between the May and June harvests. In contrast, the asymptomatic fruit from Las$^+$ trees experienced a *decrease* in sugar and an *increase* in acid concentrations from May to June. Similar results have been reported elsewhere.[37] The more pronounced effect of the infection on the juice profile as the fruit matures may provide additional evidence that high concentrations of '*Ca.* Liberibacter' are present in the peduncle, and may suggest the possibility that the organism reproduces more rapidly during fruit development and maturation, partially explaining why infection takes so long to be detected. This hypothesis will need to be tested in subsequent studies.

CONCLUSION

The objective of this study was to determine the effect of '*Ca.* Liberibacter' infection on citrus fruit metabolism using ^{1}H NMR spectroscopy. Concentration differences between fruit obtained from Las$^-$ and Las$^+$ trees were observed for sugars, amino acids, organic acids, adenosine, limonin glucoside, and limonin which may be attributed to the effect of '*Ca.* Liberibacter' on plant defense mechanisms. This study is the first to show the ability of this pathogen to hinder the plant's natural defense mechanisms, and is the first to provide a complete analysis of the alterations in nutrient composition with infection. These results may be useful for developing quality control assays that could be used in juice processing to ensure consistent products, and may be helpful in designing new assays to detect infection earlier.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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