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HPLC-MS Analysis of Secondary Metabolites in Leaves from Orange Trees Infected with Huanglongbing: A 9-Month Time Series Study

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Huanglongbing (HLB) disease, presumably caused by *Candidatus* Liberibacter asiaticus (CLas), is threatening one million acres of commercial citrus groves that have an annual production value of approximately $3 billion across the United States. The objectives of this study were to identify the earliest significant difference in the metabolome of leaves from citrus affected with HLB, and to characterize the evolution of differences in metabolite profiles as related to bacteria titer and HLB symptom development in planta. Twenty each of 8-month-old 'Valencia' and 'Hamlin' sweet orange trees were graft inoculated with budwood from a PCR-positive HLB source tree. Leaves from five inoculated trees of each variety and three control trees were sampled biweekly and analyzed by HPLC-MS and PCR. Fourteen weeks after inoculation, CLas was detected in newly growing flushes in 55% and 42% of the inoculated 'Valencia' and 'Hamlin' trees, respectively. Inoculated trees remained visibly asymptomatic in the first 20 weeks but HLB symptoms were evident 30 weeks after grafting. No metabolomic differences were detected in leaves from HLB-infected trees 24 weeks after inoculation. However, 28 weeks after inoculation, just prior to the appearance of visible symptoms, metabolomic differences between control leaves and those from HLB-infected trees were clear. The abundance of 27 out of the 38 detected metabolites in leaves from infected 'Valencia' trees increased with time, two metabolites decreased with time, and nine did not change significantly. The response of 'Hamlin' metabolites to HLB was similar to 'Valencia'; 24 out of the 38 detected metabolites increased with symptoms development, five metabolites decreased as symptoms increased, and the rest did not change significantly.

Citrus greening disease, or Huanglongbing (HLB) is the most destructive citrus disease worldwide. It is believed to be caused by the bacteria *Candidatus* Liberibacter spp., which is transmitted by the Asian citrus psyllids (ACP) (Gottwald, 2010). HLB symptoms in the leaves are characterized by a yellow blotchy mottle, or asymmetrical chlorosis; affected fruit are underdeveloped, lopsided, and green in color with aborted or stained seeds (Batool et al., 2007). As disease severity increases, yield is reduced and fruit quality degrades. According to the U.S. Department of Agriculture (USDA), citrus greening is threatening nearly one million commercial citrus acres that have an annual production value of approximately $3 billion across the nation, and losses could reach $10 billion if citrus greening is not controlled (http://www.usda.gov/wps/portal/usda/!ut/p/c4/04_SB8K8xLLM9MSSzPy8xBz9CP0os_gAC9wMJ8QY0MDpxBDA09nXw9DFxcXw2ALU_2CbEdFAFsoRU!/?printable=true&contentidonly=true&contentid=2011%2F07%2F0299.xml).

The incubation period in planta for HLB ranges from a few months to one or more years (Gottwald, 2010). Folimonova and Achor (2010) showed that 1 and 2 months after graft-inoculating sweet oranges and grapefruit seedlings, plants remained asymptomatic, and *Candidatus* Liberibacter asiaticus (CLas) was not detected by qPCR in any of the plants. At about 3 months after inoculation, the bacterium was detected in 70% of inoculated trees; however, no visual leaf symptoms were observed at that time. Severe symptoms were observed 5 to 6 months after grafting. Thus, there was an apparent 2-month lag time between inoculation and infection and a 2- to 3-month lag time between infection and symptom development. Transmission electron micrographs showed a large number of bacteria-like cells in several sieve elements in tissues of young, asymptomatic leaves. In contrast, no bacteria-like cells were observed in samples of highly symptomatic leaves. Folimonova and Achor (2010) hypothesized that the majority of the pathogen population is present as live bacteria in asymptomatic tissues, and as symptoms develop, most of the bacteria become nonviable. Tatineni et al. (2008) found that CLas was distributed in bark tissue, leaf midrib, roots, and different floral and fruit parts, but not in seed endosperm and embryos of infected citrus trees. Quantification analysis of the bacterium showed that it was distributed unevenly in planta and ranged from 14 to 137,031 cells/µg DNA in different tissues.

The effect of HLB on the profile of secondary metabolites in citrus leaves has not been extensively studied. Early studies targeted specific metabolites such as starch (Takushi et al., 2007) and gentisic acid (Hooker et al., 2003). Takushi et al. (2007) showed that the starch test was a rapid and simple diagnostic method (scratch method) for citrus HLB, and they reported up to 90% agreement between PCR analysis and starch tests with iodine. Recent studies have been done to characterize the differences in the metabolomes of leaves from HLB-affected and healthy citrus trees using HPLC-MS (Cevallos-Cevallos et al., 2008; Manthey,

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2008), capillary electrophoresis with photo diode array detection (Cevallos-Cevallos et al., 2009), and GC-MS (Cevallos-Cevallos et al., 2011). However, leaves analyzed in these studies were obtained from commercial groves for which it was not possible to determine the time of initial infection and where, most likely, multiple inoculations by psyllids occurred. Moreover, all of these studies focused on metabolic differences between symptomatic and healthy leaves. Cevallos-Cevallos et al. (2012) also used GC-MS metabolite profiles to differentiate HLB-tolerant citrus varieties from HLB-sensitive varieties. In a parallel experiment, Jones et al. (2012) sought to identify potential early biomarkers for HLB trees in newly graft-inoculated trees using GC-MS and statistical methods. The objectives of this current study are to identify the earliest significant difference in the metabolomes of leaves from citrus inoculated with HLB and healthy leaves using HPLC-MS, and to determine any correlations in the timelines of differences in metabolite profile, bacteria titer, and symptom development in planta.

Materials and Methods

Tree inoculation with *Candidatus* Liberibacter and sampling procedures. Twenty 8-month-old 'Valencia' and 'Hamlin' sweet orange trees on Volkamer rootstock were grafted with four pieces of budwood from PCR-positive HLB source trees. The grafts were 10 cm apart and the lowest graft was at about 15 cm aboveground. Inoculated trees were kept in a USDA-APHIS approved secure greenhouse with temperature control (28 to 32 °C). Eight control trees from each variety were grafted with disease-free budwood. Five inoculated trees of each variety and three control trees were sampled after 3 and 13 weeks, and then biweekly up to 38 weeks. From each tree, two leaves were randomly harvested from one shoot, bagged, immediately placed on ice for transportation, frozen, and stored at –80 °C.

Metabolomic analyses. One leaf from each sampled tree was removed from the –80 °C storage and a weighed portion (≈0.5 g) was ground with a mortar and pestle to a fine powder under liquid nitrogen. A 10-mL aliquot of methanol and dimethylsulfoxide (1:1; Sigma, St. Louis, MO) was added and the sample was extracted by shaking overnight at 200 rpm using an Innova 2100 platform shaker from New Brunswick Science (Edison, NJ) at 0 °C. The next morning the samples were equilibrated to room temperature and filtered using 0.45-µm Titan 2 HPLC filters from Thermo Fisher Scientific (Pittsburgh, PA). A 0.9-mL aliquot of the filtrate was spiked with 0.1 mL of 2500 ppm of 5-hydroxy-4-7-dimethoxyflavone as an internal standard.

HPLC-MS analyses. Compound profiles were analyzed by HPLC-MS, using Varian ProStar 210 pumps and a ProStar 410 autosampler controlled by Star software (ver. 6.41). Compound separations were achieved with a Waters XBridge C8 column (4.6 × 150 mm). The mobile phase composition and the flow rate are given in Table 1.

MS peak detection was achieved with a Leco atmospheric pressure chemical ionization (APCI) source coupled with a LECO Unique HT MS analyzer (LECO, St. Joseph, MI). MS parameters were as follows: interface temperature 99 °C, nebulizer pressure 300 kPa, desolvation temperature 350 °C, desolvation gas flow 2.0 $L \cdot min^{-1}$, nozzle voltage 100 V, skimmer voltage 52 V, and quad RF voltage 165 V. Data processing occurred with LECO ChromaTOF ver. 4.0.

Peak identifications were achieved by comparing retention times and mass spectra of sample peaks with those of authentic standards. Data were manually aligned using retention time and mass values. Data from infected and non-infected trees were normalized by dividing the area of each peak on the area of the internal standard and analyzed using principal component analysis (PCA) followed by analysis of variance (ANOVA) using Unscrambler® X (www.camo.com).

PCR analyses and visual leaf symptoms. The second stored leaf from each sampled tree was used for PCR analysis for the presence of the CLas bacterium as described by Tatineni et al. (2008). Briefly, 250 mg leaf tissue was extracted in 2.5 mL extraction buffer (100 mM Tris-HCL pH 8.0; 50 mM EDTA; 500 mM NaCl; 10 mM dithiothreitol). Fifteen hundred microliters were transferred to a 1.5-mL Eppendorf tube, and 100 µL 20% SDS was added and incubated at 65 °C for 30 min. A 500-µL aliquot of 5M potassium acetate was added, mixed thoroughly, and incubated on ice for 20 min. DNA was recovered by centrifugation and precipitation with isopropanol and kept at –20 °C overnight. Re-suspended DNA was analyzed by PCR. All reactions were done in triplicate with positive, healthy, and water controls. Control and CLas inoculated trees were photographed before sampling and sampled leaves were also photographed before storage to document any leaf symptoms.

Results and Discussion

PCR analyses and visual leaf symptoms. Thirteen weeks after inoculation, CLas bacteria were detected by PCR analysis in more than 55% and 33% of the inoculated 'Valencia' and 'Hamlin' trees, respectively (Table 2). All of the tested 'Valencia' and

Table 1. Mobile phase composition and flow rate.

Time (min)	% A (0.5% formic acid in distilled water)	% B (acetonitrile)	Flow mL/min
0	86	14	0.3
16	72	28	0.3
21	62	38	0.3
28	50	50	0.3
43	45	65	0.5
48	30	70	0.75
53	30	70	0.75
58	86	14	0.75
63	86	14	0.75
64	86	14	0.3
70	86	14	0.3

Table 2. PCR results for inoculated 'Hamlin' and 'Valencia' trees.

	Hamlin		Valencia	
Week	% positive	n	% positive	n
13	33	9	55.5	9
19	50	4	60	5
21	0	4	20	5
23	29	7	40	5
25	60	5	60	5
27	40	5	20	5
29	0	5	20	5
31	80	5	60	5
33	80	5	100	5
35	80	5	75	4
37	80	5	80	4

80% of the tested 'Hamlin' trees were PCR positive at 33 weeks post inoculation (Table 2). Some of the trees were PCR positive 13 weeks after inoculation, while others did not show any PCR positive before 35 weeks after inoculation. The PCR results also showed that the bacterium was unevenly distributed in inoculated trees. In certain instances, leaves from some inoculated trees were PCR positive 13 weeks after inoculation, while leaves from the same trees, but at a different location, were PCR negative 21 and 29 weeks post inoculation. Leaves from the same trees were PCR positive 45 weeks post inoculation.

The leaves of the grafted 'Valencia' trees remained visually asymptomatic in the first 19 weeks (Fig. 1A–B). HLB leaf symptoms started to develop in shoots of grafted plants in subsequent weeks and were notably symptomatic 29 weeks post inoculation (Fig. 1C–F). Leaves from 'Hamlin' trees followed a similar trend. In a manner similar to the results obtained from the detection of CLas by PCR analysis, HLB leaf symptoms were not evenly distributed within individual plants; some leaves were highly symptomatic while others did not show any symptoms. According to Batool et al. (2007), HLB-symptoms may appear on a single shoot or "yellow shoot" branch or on different parts of the plants. The PCR analyses (Table 2) and visual observations (Fig. 1) of leaf symptoms showed that the CLas bacterium in inoculated citrus trees can be detected before the development of visual leaf symptoms. However, the probability of detecting CLas was higher in symptomatic leaves (31–37 weeks post inoculation).

Results of PCR analysis and leaf symptom development reported in this study are in close agreement with the results of previous studies on the distribution and detection of the CLas bacteria in inoculated citrus trees. Three months after inoculation Folimonova and Achor (2010) were able to detect CLas bacteria in 71% of inoculated sweet oranges and grapefruit seedlings, and severe asymmetrical yellowing was clear after 5 to 6 months after grafting. In their study, a large number of bacteria cells were also detected in phloem sieve tubes in tissues from presymptomatic young flushes of infected leaves using electron microscopy. Cevallos-Cevallos et al. (2012) showed that sensitive varieties like 'Madam Vinous' sweet orange (MV) and 'Duncan' grapefruit (DG) develop more pronounced visual leaf symptoms earlier than HLB-tolerant varieties like *Poncirus trifoliata* (TR) and 'Carrizo' citrange (CAR). HLB-symptoms in MV and DG began to appear around 12–14 weeks after graft-inoculation and their severity progressed with time. PCR testing showed high levels of the CLas bacterium in MV and DG 14 weeks after graft-inoculation. In contrast, the levels of HLB bacterium in HLB-tolerant varieties were low.

No visual leaf symptoms were observed in our study before the detection of the CLas bacteria, suggesting that the presence of the bacteria in the leaves is necessary for the development of the visual symptoms. In our study, we were also able to detect the CLas bacterium in the leaves of young flushes (2 weeks of age), suggesting again that the presence of the bacterium is linked to the development of the symptoms. However, even though most symptomatic leaves were PCR positive, it remains uncertain that the bacteria must be present in the leaves for visual leaf symptom development. Rather, it is possible that the bacterium could alternatively block the phloem of lower stems, thus producing chlorosis in the upper leaves (Tatineni et al., 2008).

Metabolites analysis by HPLC-MS. The influence of graft inoculation of the CLas bacterium on disease symptoms development was further explored by analyses of changes in the leaf metabolome. These analyses were conducted with a LECO

Fig. 1. Progression of HLB-related symptoms in 'Candidatus Liberibacter asiaticus'-inoculated 'Valencia' sweet orange seedling: (**A**) leaves from control healthy plants 19 weeks after inoculation; (**B**) leaves from HLB-grafted plants 19 weeks after inoculation; (**C**) leaves from control healthy plants 29 weeks after inoculation; (**D**) leaves from HLB-grafted plants 29 weeks after inoculation; (**E**) leaves from control healthy plants 35 weeks after inoculation; (**F**) leaves from HLB-grafted trees 35 weeks after inoculation.

Unique HT MS analyzer with ChromaTof software capable of peak detection and deconvolution in the highly overlapping HPLC chromatograms of orange leaf extracts. Particular emphasis was placed on the analysis of the numerous phenolic compounds in the orange leaf extracts (Table 3).

Principal component analysis (PCA) and *t*-tests were carried out on each set of samples gathered at each sampling date to compare control and HLB samples and to identify compounds whose concentrations were significantly influenced by the progression of the HLB disease. No group clustering was observed in the profiles of 'Valencia' leaf metabolites during the first 23 weeks (Fig. 2A). However, the PCA of the 'Valencia' leaf compounds provided evidence of clustering into two groups (HLB and healthy) at 27 weeks, and the clustering was well defined by 38 weeks (Fig. 2C). The compounds responsible for clustering were Unknowns 5, 6, 7, diosmin 1, doismin 2, hesperidin, sinensetin, and Unknown 22. Three weeks after grafting, the concentrations of the above listed peaks in leaves from CLas-inoculated trees were either similar or less than those in healthy leaves (Fig. 3). Twenty-three weeks after grafting, none of the detected peaks in HLB-infected leaves were significantly different from those of healthy leaves. However, at 27 weeks after grafting, Unknowns 5 and 6 were dramatically more abundant in leaves from HLB-affected trees than in leaves from control trees (Fig. 3).

Figure 3 also shows that after 35 weeks from grafting the mean increase in the quantity of hesperidin in leaves from the CLas-infected 'Valencia' seedlings was more than 700% above that from healthy trees. Cevallos-Cevallos et al. (2009) used CE-DAD to detect potential biomarkers for HLB in citrus leaves collected from infected trees 4 weeks after symptoms were discovered. Six compounds were present in significantly higher concentrations in HLB-infected samples. Three of these compounds were identified as hesperidin, naringenin, and quercetin. The levels of these six compounds in HLB-affected leaves were 154% to 1300%

Table 3. Summary of ANOVA results.

Peak no.	RT (min)	m/z	Compound	'Valencia' time	*P*-value group	'Hamlin' time	*P*-value group
1	5.2	313.8	Unknown 1	0.3940	0.0380	0.989	0.090
2	5.3	268	Unknown 2	0.0060	0.2010	0.005	0.730
3	5.4	151	Unknown 3	0.3780	0.0290	0.0149	0.1673
4	10.2	265	Feruloyl putrescine[z]	0.0820	0.0056	0.021	0.0137
5	10.5	177	Unknown 4	0.1280	0.0003	0.0187	0.0059
6	12.5	177	Unknown 5	0.0056	<0.0001	0.0128	0.0006
7	14.1	177	Unknown 6	0.0007	<0.0001	0.0007	0.0001
8	14.4	594.5	Apigenin 6,8-diglucoside[z]	0.2000	<0.0001	0.4298	0.0007
9	16.1	177	Unknown 7	0.0064	<0.0001	0.0183	0.0001
10	19.4	573	Unknown 8	0.0087	0.0003	0.018	0.0005
11	20.5	303	Unknown 9	0.0200	0.7039	0.0018	0.152
12	18.5	595	Apigenin 6,8-diglucoside[y]	0.0206	0.0078	0.0907	0.0132
13	19.5	565	Apigenin-glu-Rah[y]	0.0381	0.0147	0.066	0.0194
14	20.2	565	Unknown 10	0.0104	0.0008	0.0936	0.0123
15	20.3	595	Apigenin 6,8-diglucoside[y]	0.0513	0.0009	0.428	0.0084
16	21.2	595	Apigenin 6,8-diglucoside[y]	0.0413	0.0383	0.0952	0.0327
17	22.1	463	Unknown 11	0.612	0.0002	0.860	0.0017
18	24.4	609	Diosmin 1[y]	0.254	0.0058	0.0605	0.0047
19	25.8	609	Diosmin 2[y]	0.926	0.0020	0.5138	0.0035
20	26.3	303	Hesperdin[z]	0.186	0.0006	0.521	0.0008
21	29.1	303	Unknown 12	0.484	0.1179	0.797	0.0018
22	32.2	287	Isosakuranetin rutioside[z]	0.2003	<0.0001	0.480	0.735
23	33.5	728	Unknown 13	0.1849	0.0024	0.003	0.001
24	34.1	713	Unknown 14	0.884	0.0003	0.0932	0.0001
25	33.5	359	Unknown 15	0.0001	0.0095	0.001	0.0039
26	34.4	359	Unknown 16	0.0520	0.0041	0.0724	0.2864
27	35.2	359	Unknown 17	0.1740	0.1055	0.6949	0.9887
28	35.3	331	Unknown 18	0.3660	0.0002	0.2536	0.0006
29	36.1[x]	373	Pentamethoxyflavone[y]	0.445	0.0029	0.0951	0.001
30	37.1	389	Unknown 19	0.654	0.5315	0.1669	0.4856
31	37.6	373	Sinensetin[z]	0.0001	0.0037	0.001	0.0027
32	38.4	345	Unknown 20	0.0019	0.0030	0.0003	0.0003
33	39.4[w]	403	Nobelitin[z]	0.251	0.468	0.967	0.003
34	40.1	343	Tetramethyl-o-scutellarin[y]	0.0223	0.0082	0.0003	0.0023
35	40.2	375	Unknown 21	0.458	0.7399	0.611	0.991
36	40.5[w]	433	Heptamethoxyflavone[z]	0.189	0.2531	0.996	0.0014
37	41.3	359	Unknown 22	0.0136	0.0003	0.0043	0.001
38	41.5[w]	373	Tangeretin[z]	0.234	0.107	0.531	0.0013
39	42.3[x]	359	Unknown 23	0.2018	0.0117	0.110	0.0001
40	43.2	389	5-Desmethylnobletin[y]	0.259	0.838	0.153	0.026

[z]Identified by matching their retention time, mass spectra, and UV spectra with known standard.
[y]Tentatively identified using MS fragmentation patterns.
[x]Lower in HLB-infected 'Hamlin' and 'Valencia'
[w]Lower in HLB-infected 'Hamlin' only.

above that in healthy leaves. Manthey et al. (2008) also reported an increase in hesperidin levels in orange leaves during blight-induced zinc deficiency, suggesting that hesperidin participates in plant response to stress.

'Hamlin' leaves started to cluster into two groups (HLB and control) 35 weeks after grafting and the clustering was obvious 38 weeks after grafting (Fig. 2D). Similar to the changes that occurred in the 'Valencia' leaves, the compounds that were responsible for clustering were Unknowns 5, 6, 7, diosmin 1, doismin 2, hesperidin, sinensetin, and Unknown 22. Three weeks after grafting, the abundance of some metabolites in leaves from HLB-affected trees was significantly higher than those of the control (Fig. 4). Although significantly higher, the levels of these metabolites were only 1- to 2-fold higher than the levels of these compounds in the control leaves, and at 23 weeks after inoculation, none of the analyzed compounds in the HLB-affected trees were significantly different from those from uninfected trees (Fig. 4). Twenty-seven weeks after grafting, the mean concentrations of a number of the above listed metabolites in leaves from CLas-inoculated trees were numerically higher than those in control leaves, however their concentrations were not significantly different. The same observations were made for the leaves sampled at 29 and 35 weeks after grafting. Yet, after 38 weeks from grafting, the levels of some metabolites such as Unknowns 5 and 6 were more than 10-fold higher in leaves from HLB-affected trees compared to those from uninfected trees (Fig. 4).

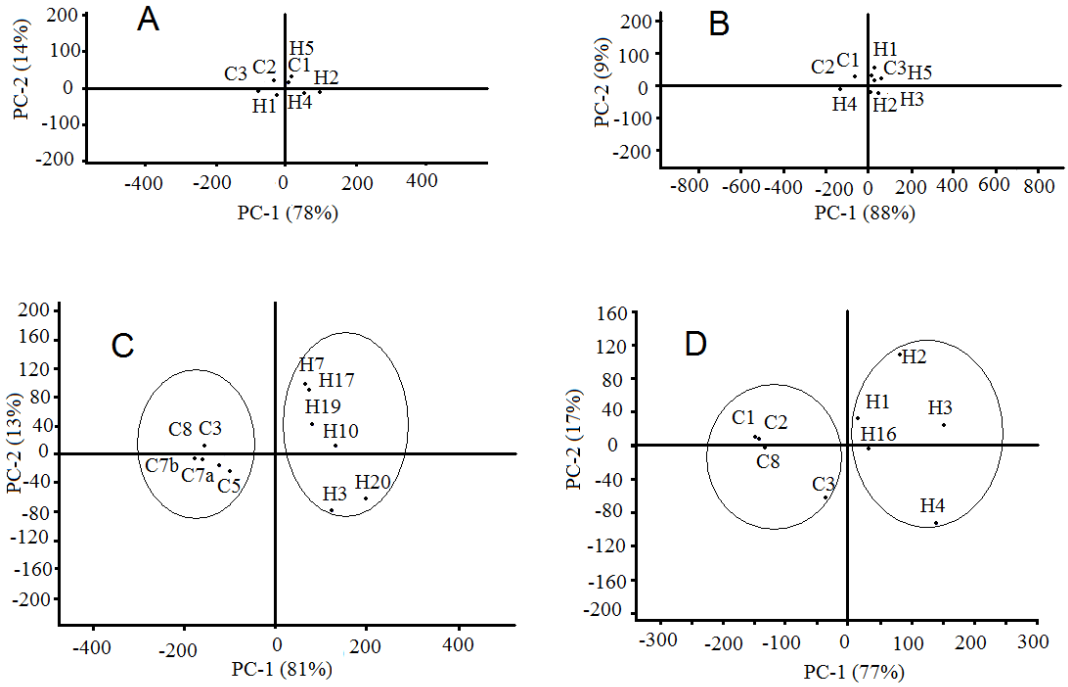

Fig. 2. Principal component analysis of HPLC-MS leaves metabolites from controls (C) and HLB-affected (H) 'Valencia' and 'Hamlin' trees. Score plot of leaves metabolites from (**A**) 'Valencia' trees 3 weeks after inoculation; (**B**) 'Hamlin' trees 3 weeks after inoculation; (**C**) 'Valencia' trees 38 weeks after inoculation; and (**D**) 'Hamlin' trees 38 weeks after inoculation.

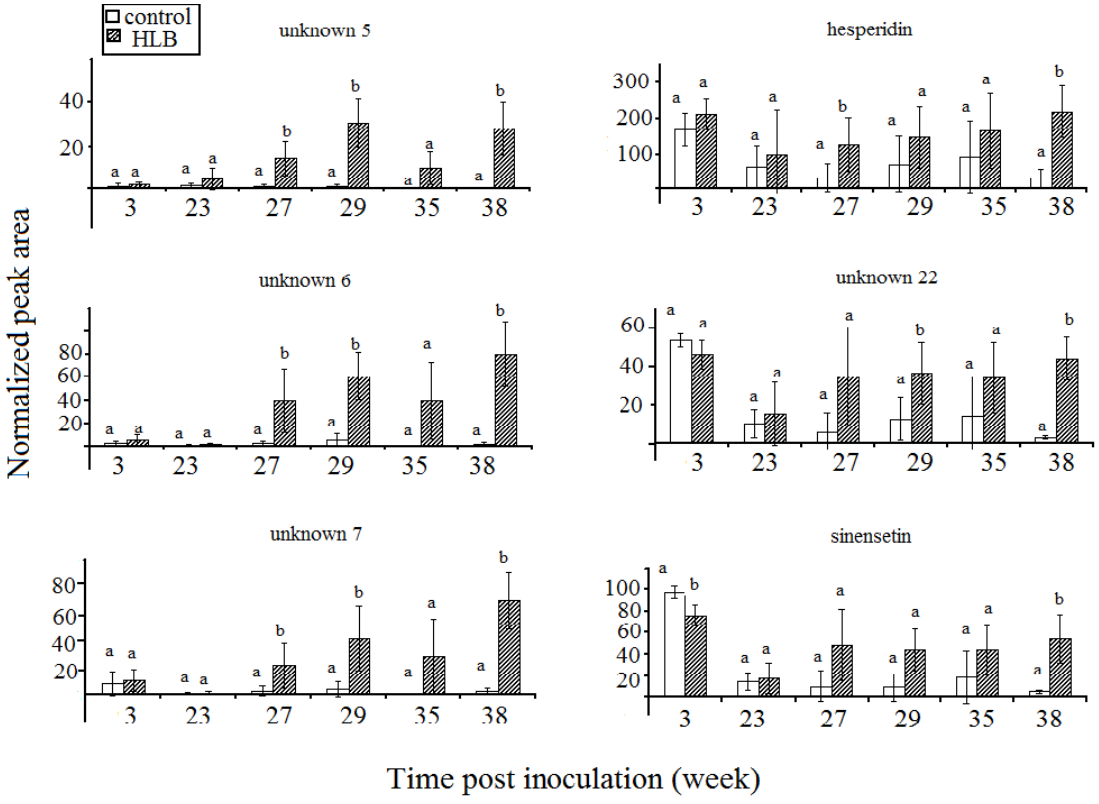

Fig. 3. Mean (±SE) quantity of different metabolites in leaves from controls (C, open bars) and HLB-affected (H, closed bars) 'Valencia' seedlings 3, 23, 27, 29, 35, and 38 weeks after inoculation.

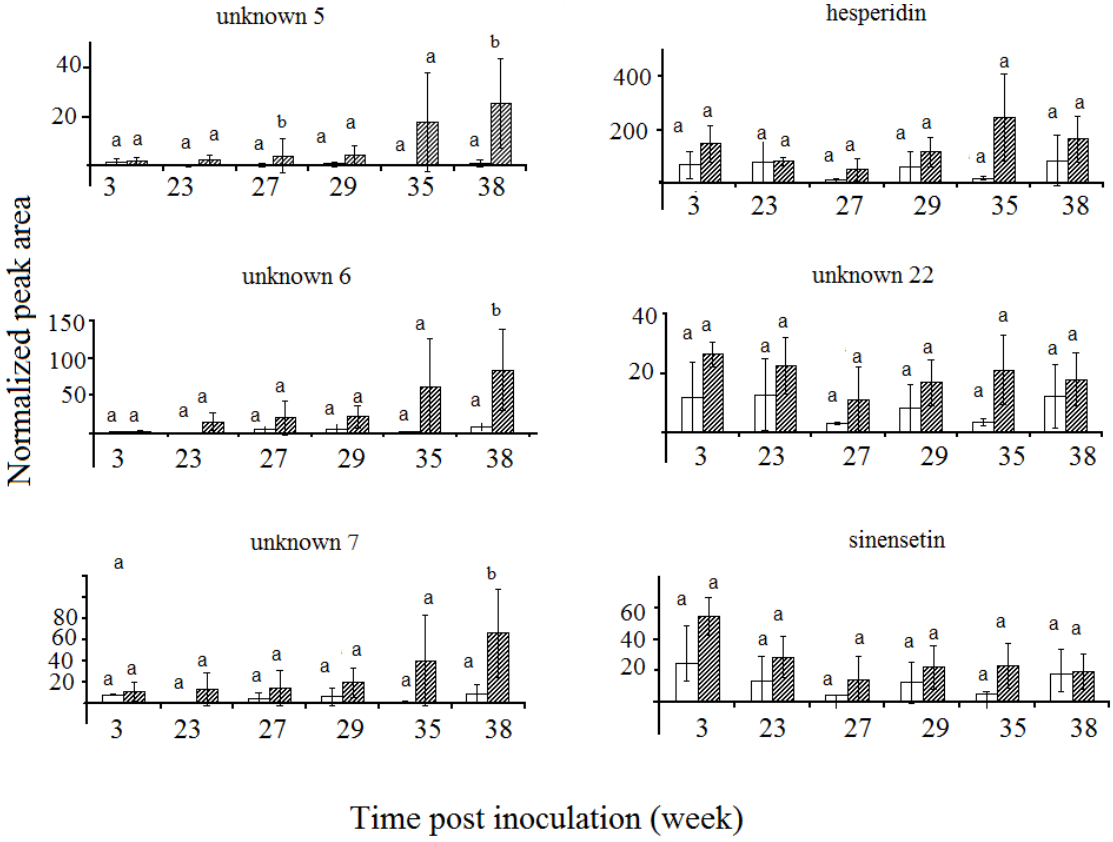

Fig. 4. Mean (±SE) quantity of different metabolites in leaves from controls (open bars) and HLB-affected (closed bars) 'Hamlin' seedlings 3, 23, 27, 29, 35, and 38 weeks after inoculation.

ANOVA was also performed on all of the combined samples (all sampling dates together) to study the effect of CLas inoculation on leaf metabolites with time. ANOVA results (Table 3) showed that most of the detected metabolites were significantly affected by HLB and only 10 compounds in 'Valencia' and 8 compounds in 'Hamlin' leaves were not affected by HLB. Most of the detected metabolites were higher in leaves from HLB-affected trees and only two metabolites (pentamethoxyflavone and Unknown 23) were lower in leaves from HLB-affected 'Valencia' trees and five metabolites (pentamethoxyflavone, nobelitin, hexamethoxyflavone, tangeretin, and Unknown 23) were lower in leaves from HLB-affected 'Hamlin' leaves. ANOVA results confirmed our finding in the PCA analysis. For example, Table 3 showed that Unknown 5, 6, and 7 were significantly higher in leaves from CLas inoculated 'Valencia' trees (P value < 0.0001) and their levels increased with time (P value < 0.01).

Ferulic acid containing hydroxycinnamats (Unknowns 5, 6, and 7) were higher in leaves from the CLas-infected 'Valencia' and 'Hamlin' trees and their levels increased with time and symptoms progression. Although the levels of these metabolites were higher in most of the leaves from HLB-affected trees, there were cases where the levels of these metabolites in some leaves from HLB-affected trees were similar to the uninfected trees (Fig. 6A–F). This uneven distribution of metabolite concentrations within replicates in leaves from the HLB-affected trees produced non-significant differences among means from t-test. The uneven distribution of metabolites in leaves from HLB-affected trees may have resulted from an uneven distribution of the CLas bacterium in inoculated plants which lead to uneven distribution of symptoms in leaves and different latency periods for the onset of the disease in each individual tree.

In conclusion, a number of secondary metabolites in the leaves of 'Valencia' and 'Hamlin' seedlings were significantly affected by HLB. 'Valencia' seedlings appeared more sensitive to HLB than 'Hamlin' based on the observations that 'Valencia' leaves developed visual HLB symptoms before 'Hamlin' leaves, 'Valencia' leaves showed significant differences in metabolite profiles earlier than 'Hamlin' leaves, and the differences in metabolites between healthy and infected leaves was more significant in 'Valencia' leaves. The changes in the profiles of leaf metabolites from CLas inoculated citrus trees related to those of healthy leaves first appeared at 28 weeks after inoculation. Increasingly notable profile differences were evident 38 weeks after inoculation. The change in metabolites of leaves from CLas inoculated trees was in parallel with the development of visual symptoms. However, the responses in leaves of HLB-affected seedlings were not consistent and are believed to be attributable to the uneven spread of the bacterium and symptoms in CLas inoculated trees. The results of our study suggest that the latency period in sweet orange is different for individual plants, and that the HLB symptoms are not evenly distributed within the same tree. This weakens the validity of the comparison among means at any particular time. Because the variations in metabolite concentrations were largely observed in samples from HLB-affected trees and not in control trees, it is

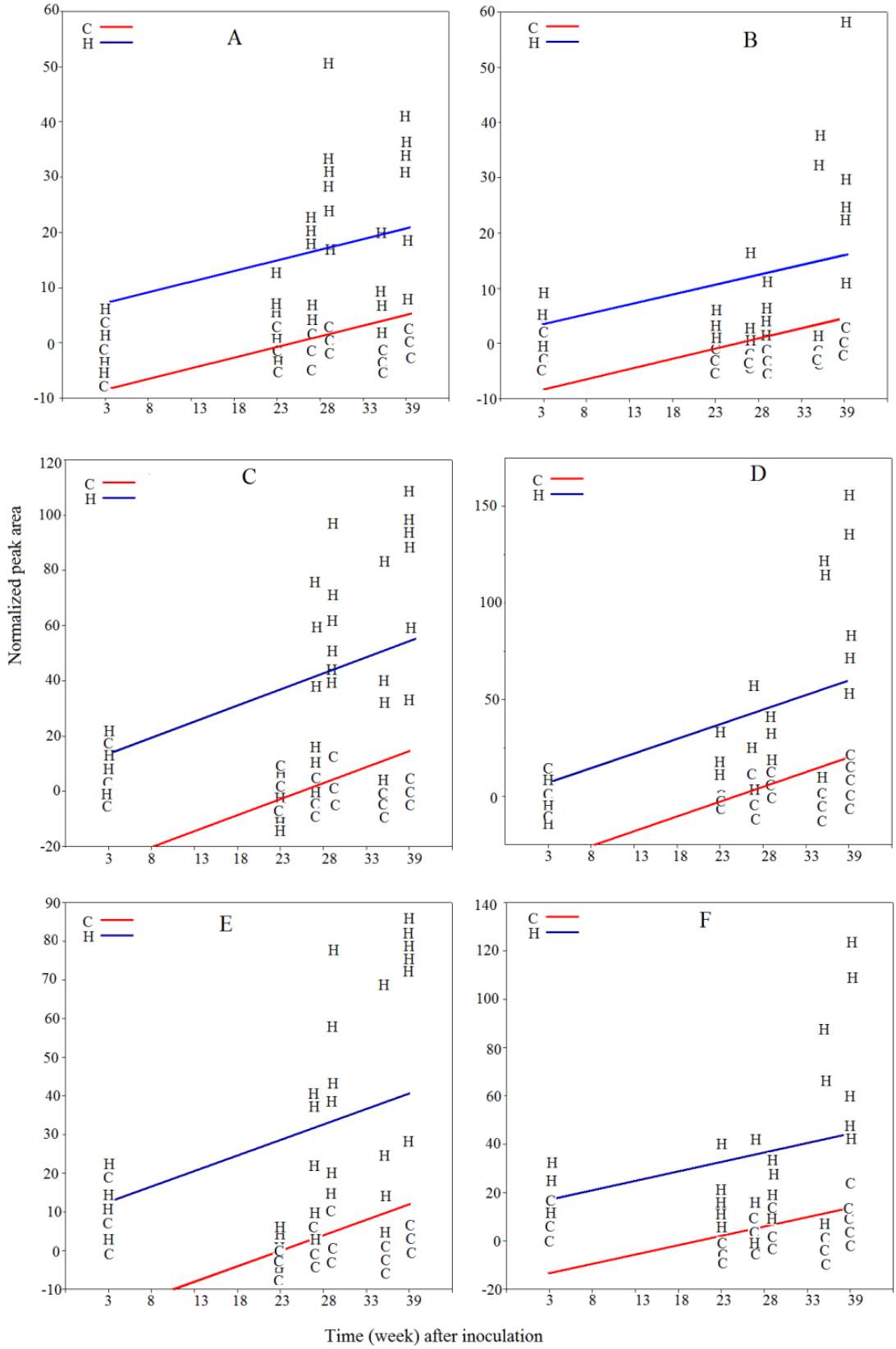

Fig. 5. Abundance vs. time (week) after inoculation of different citrus leaf metabolites from controls (C) and HLB-inoculated (H) trees: (**A**) unknown 5 in 'Valencia' leaves; (**B**) unknown 5 in 'Hamlin' leaves; (**C**) unknown 6 in 'Valencia' leaves; (**D**) unknown 6 in 'Hamlin' leaves; (**E**) unknown 7 in 'Valencia' leaves; (**F**) unknown 7 in 'Hamlin' leaves.

reasonable to infer that these differences indeed exist and might be used as biomarkers if infection levels could be standardized.

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