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Secondary Metabolite Profiles of Leaves of Healthy and Huanglongbing-infected Orange (*Citrus sinensis* L.) Seedlings Measured by HPLC-Fluorescence Detection

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Leaves of greenhouse-grown 'Hamlin' and 'Valencia' orange (*Citrus sinensis* L.) seedlings were analyzed by high performance liquid chromatography in a study of the progression of changes in secondary metabolite profiles resulting from infection by *Candidatus* Liberibacter asiaticus (CLas), the Huanglongbing causative pathogenic bacterium. Following graft transmission of CLas into the young trees, infection was monitored in new growth by PCR and visual symptoms. Leaves from healthy non-infected trees and from infected trees were analyzed principally for their profiles of fluorescent secondary metabolites. Two distinct profiles of compounds were observed at later collection dates, one predominantly in leaves of non-infected trees, and the other in visually symptomatic infected leaves. Characterizations of these peaks were made using high performance liquid chromatography coupled with photodiode array, fluorescence, and mass spectrometry. These characterizations showed that fluorescent compounds in the orange leaf extracts included polymethoxylated flavones, coumarins, and conjugated hydroxycinnamates, although other unknowns were also detected. The fluorescence detection of these compounds was optimized by measuring the emission and excitation spectra of purified standards of structurally related citrus leaf compounds.

Huanglongbing (HLB) is one of the most devastating diseases in worldwide citriculture, and since its introduction into Florida in 2005, it has been the primary concern of Florida citrus growers (Gotwald 2010). The recent detection of the transmittal agent of HLB in California indicates that this disease will soon impact the entire U.S. citrus crop. In the United States the primary HLB causative agent is the gram-negative bacterium *Candidatus* Liberibacter asiaticus (CLas) (Tyler et al., 2009). Although it is known that this bacterium is phloem limited, there remain many questions about the movement and distribution of this bacterium within infected trees, and the resulting expression of disease symptoms throughout the plant. Previous capillary electrophoresis and HPLC studies using UV/VIS photodiode array (PDA) analysis have provided preliminary characterizations of the changes caused by HLB on the secondary metabolites in visually symptomatic leaves (Cevallos et al., 2008; Manthey, 2008). Yet, no HLB-specific marker compounds indicative of early stages of CLas infection have thus far been detected by these techniques. To expand this search, studies of leaves of CLas-infected greenhouse-grown trees were conducted using HPLC coupled with fluorescence detection. This latter technique has been widely used in studies of coumarins and psoralens in citrus oils and oil residues (Dugo and McHale, 2002) and of phenolic alkaloids in bitter orange (*Citrus aurantium* L.) (Pellati et al., 2002; Putzbach et al., 2007). HPLC-fluorescence detection has also been used in studies of the highly fluorescent coumarin phytoalexins induced in common commercial citrus cultivars following fungal and bacterial attacks. These plant defense compounds include in part, scoparone (6,7-dimethoxycoumarin) (Afek et al., 1986; Kim et al., 1991; Ortuño et al., 1997; Sulistyowati et al., 1990), scopolin (7-hydroxy-6-methoxycoumarin-7-glucoside) (Runkel et al., 1997) and umbelliferone (7-hydroxycoumarin) (Afek et al., 1999). In consideration of this tendency in citrus to respond to environmental stresses and pathogen attacks by the production of such fluorescent compounds, HPLC coupled with fluorescence spectrophotometry appeared to be a potentially useful analytical technique to detect biomarkers of HLB in orange leaves, especially at early points of infection. In this study, time dependent changes in the profiles of diverse sets of fluorescent compounds were measured in CLas-infected 'Valencia' and 'Hamlin' orange leaves over a 9-month period following initial graft inoculation of greenhouse-grown seedlings.

Methods and Materials

Plants, greenhouse culture, and CLas inoculations. Twenty 8-month-old 'Valencia' and 'Hamlin' sweet orange seedlings grown under greenhouse conditions were grafted with four pieces of budwood from PCR-positive HLB source trees.

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The grafts were 10 cm apart and the lowest graft was at about 15 cm above ground. Inoculated plants were kept in a USDA-APHIS approved secure greenhouse with temperature control (28 to 32 °C). Eight plants of each variety were grafted with disease-free budwood to serve as controls and were grown under the same conditions. Five inoculated trees of each variety and three control trees were sampled biweekly over a 9-month period. From each tree two leaves were randomly harvested from one shoot. Leaves were bagged, immediately placed in ice and stored at –80 °C. One leaf per pair was submitted for PCR analysis for the detection of the CLas bacterium (Tatineni et al., 2008), and the other was used for chemical analysis. Trees ranged from 0.5 m to 1 m tall at the beginning of the study and were planted two per gallon-sized pot. All control and HLB-inoculated trees received water and standard nutrition with liquid and solid fertilizers on a regular basis.

Leaf extractions. One leaf from each sampled tree per sampling date was weighed, and 0.5 g FW was ground to a fine powder in liquid nitrogen using a mortar and pestle. A 10-mL aliquot of methanol/dimethylsulfoxide (1:1, v/v) was added and the sample was shaken overnight at 200 rpm at 4 °C. Samples were equilibrated to room temperature and filtered with 0.45 micron Titan 2 HPLC filters (Sun Sri, Rockwood, TN).

HPLC-fluorescence analyses. Compound profiles in extracts of healthy uninfected (CON) and infected (HLB) 'Hamlin' and 'Valencia' leaves, were analyzed by HPLC-fluorescence, 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 5-µm column (150 × 4.6 mm i.d.). Elution conditions included gradients of aqueous 0.5% formic acid/acetonitrile, initially at (90/10, v/v), and subsequently changed in linear gradients to 80/20, 75/25, 60/40, and 30/70 (v/v), at 10, 15, 23, and 40 min, and then remained at 30/70 until 45 min at a flow rate of 0.75 mL·min^{-1}. Peak detection was achieved with a JASCO FP 2020 Plus fluorescence detector. Fluorescence peak detection occurred at emission wavelengths of either 400 or 480 nm, with an excitation wavelength of 335 nm. Fluorescence data were recorded and processed with JASCO ChromNav software ver. 1.17.01. Additional peak analyses were made using a Varian 335 ProStar UV/VIS PDA detector. Chromatograms were monitored at 275 and 330 nm, and spectra were scanned between 240 and 500 nm. A flow splitter was used to simultaneously monitor the UV/Vis and fluorescence spectra of the HPLC eluting peaks.

HPLC-MS analyses. Compound profiles were additionally analyzed by HPLC-MS, using the above described Varian HPLC coupled with a Leco Unique HT mass spectrometer (Leco, St. Joseph, MI). MS instrumental parameters were as follows: positive ion atmospheric pressure chemical ionization (APCI), interface temperature 99 °C, nebulizer pressure 300 kPa, desolvation temperature 350 °C, desolvation gas flow 2.0 L·min^{-1}, nozzle voltage 100 V, skimmer voltage 52 V, quad RF voltage 165 V. Data were processed with the Leco ChromaTOF ver. 4.0 software. HPLC eluent was split into two portions to simultaneously pass through the MS and fluorescence detectors in order to obtain MS information about co-eluting fluorescent peaks.

Results

To investigate changes in the metabolic profiles of 'Valencia' and 'Hamlin' orange leaves following initial CLas graft inoculation (November), HPLC-fluorescence and HPLC-UV/Vis PDA analyzes were conducted on greenhouse-grown trees sampled over a 9-month period. Results of this survey showed that at the later sampling dates (June–August), consistent differences occurred in the CON and HLB leaf profiles for both varieties of orange. Figures 1 and 2 show the typical profiles of fluorescent compounds in 'Hamlin' and 'Valencia' leaves, respectively, where peaks were labeled **A**–**Z**. The HPLC chromatograms of the 'Hamlin' and 'Valencia' CON leaf extracts exhibited two highly fluorescent peaks at 8.5 min (**H**) and 22.5 min (**U**), and several other smaller peaks (**S**, **T**, and **V**). Numerous other peaks jointly occurred in the CON and HLB metabolite profiles, the most prominent included peaks **E**, **F**, **G**, **W**, **X**, **Y**, and **Z**. The most prominent peak that uniquely occurred in the HLB leaf profile for both varieties was peak **I** (11.5 min).

Profiles of CON and HLB leaves were monitored from the earliest sampling time point (3 weeks), and were then monitored throughout an additional 36-week period. Distinctions between the CON or HLB leaves remained largely undetected up to 24 weeks post inoculation, but became increasingly apparent at later sampling dates. Plots of the intensities of the most prominent peaks throughout the sampling period are shown in Figure 3 for the 'Valencia' leaves. Nearly identical observations for these compounds were made in the 'Hamlin' leaf samples (data not shown). These results show that for the compounds that occurred at higher levels in the HLB leaves, i.e., **F**, **G**, and **I**, there were gradually increasing concentrations of these compounds at later sampling dates. This was also true for compounds **S** and **T** in

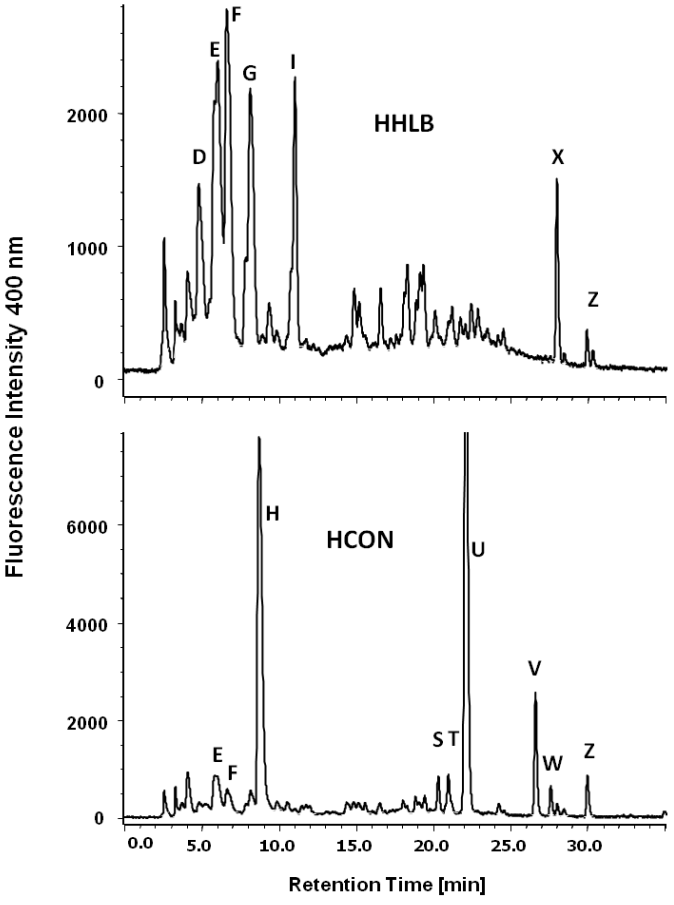

Fig. 1. HPLC-fluorescence chromatograms of Huanglongbing-inoculated (HLB) and control (CON) 'Hamlin' leaf extracts measured at 335 nm excitation and 450 nm emission.

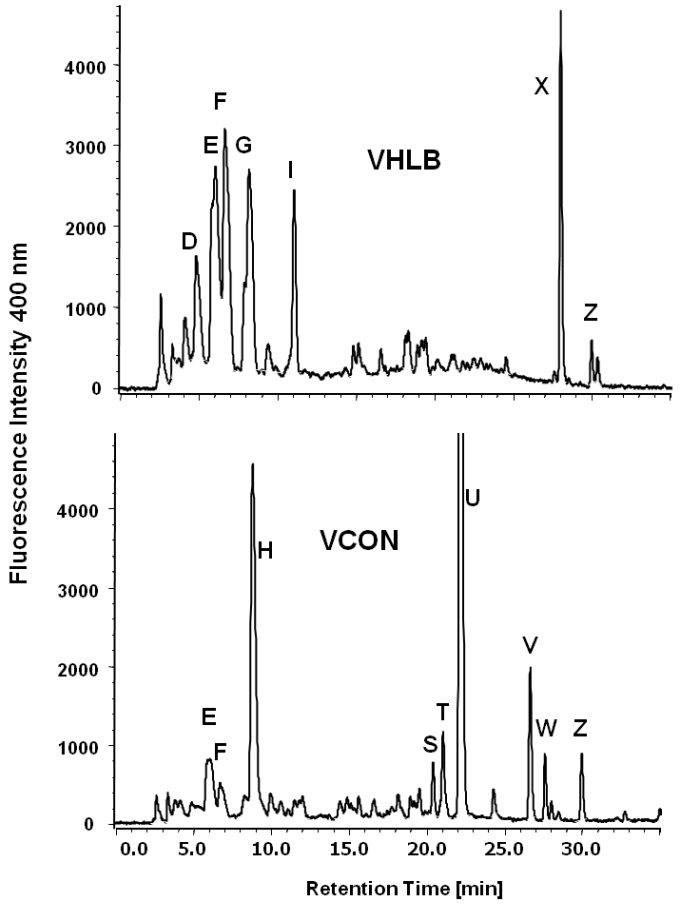

Fig. 2. HPLC-fluorescence chromatograms of Huanglongbing-inoculated (HLB) and control (CON) 'Valencia' leaf extracts measured at 335 nm excitation and 450 nm emission.

the CON leaves. In contrast, compounds **U** and **H**, as well as minor peaks **V**, **V1**, and **X** (data not shown) failed to show such increases in concentrations.

The identities of the peaks responsible for peaks **E**–**Z** (Table 1) are largely unknown, but the spectroscopic analyses of these peaks by fluorescence, UV/VS, and APCI-MS enable us to propose reasonable structural classifications for these compounds. Compounds **E**–**G** exhibited UV and fluorescence emission spectra nearly identical to ferulic acid. Furthermore, the APCI-MS of each of these peaks exhibited fragment ions at *m/z* 177 indicative of [ferulic acid+H-H_2O]$^+$. Analyses of these compounds by negative electrospray ionization-MS provided evidence that these compounds were also adducts of aldaric acids (data not shown), and hence suggested that these compounds occurred as a set of related ferulic acid-aldaric acid hydroxycinnamates (HCAs). In contrast, both peaks **I** and **H** exhibited UV and MS different from these latter compounds, and the structural classifications of these compounds remain unknown. The UV spectra of **S** and **T** were similar to *p*-coumaric and ferulic acids, respectively, and these associations were further supported by the 147 (*p*-coumaric

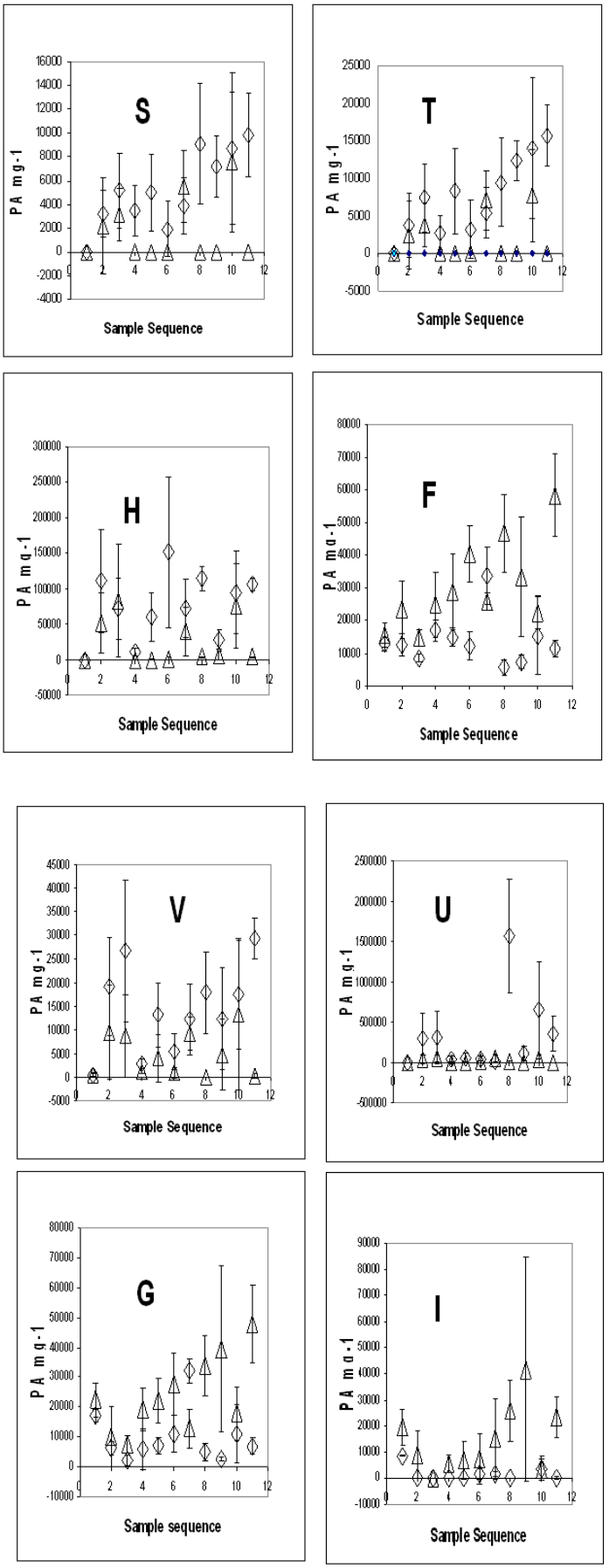

Fig. 3 (*right*). Time courses for the relative concentrations of fluorescent compounds in Huanglongbing-inoculated (HLB) (**triangles**) and control (CON) (**diamonds**) 'Valencia' orange leaf extracts. Dates associated with sample sequence (X-axis) are: 1, Nov. 5; 2, Mar. 13; 3, Mar. 30; 4, Apr. 13; 5, Apr. 27; 6, May 6; 7, May 25; 8, June 8; 9, June 22; 10, July 6; 11, July 12.

Table 1. Spectroscopic properties of fluorescent compounds in 'Hamlin' and 'Valencia' leaf extracts.

Compound	UV/VIS	Fluorescence	Mass spec	Structure class
E	Ferulic acid	NM	177 *m/z*	Hydroxycinnamate
F	Ferulic acid	NM	177 *m/z*	Hydroxycinnamate
G	Ferulic acid	NM	177 *m/z*	Hydroxycinnamate
H	313 nm	NM	217/137 *m/z*	Unknown
I	---	NM	---	Unknown
S	*p*-Coumaric acid	NM	147 *m/z*	Hydroxycinnamate
T	Ferulic acid	NM	177 *m/z*	Hydroxycinnamate
U	Coumarin	NM	177 *m/z*	Coumarin
Z	Flavone	NM	403 *m/z*	Flavone

acid) and 177 (ferulic acid) *m/z* fragment ions exhibited by these compounds. These compounds represent additional HCAs in the leaf extracts, but are likely structurally distinct from **E**–**G**. Although **U** also exhibits a 177 *m/z* fragment ion, the fluorescence emission and excitation spectra of this compound contrast sharply from those of HCAs, and in fact, more closely resemble those of simple coumarins (Murray et al., 1982). Coumarins have high intrinsic fluorescence, and this would explain the high fluorescence intensity of **U** relative to its weak UV absorbance. The identities of peaks **V1**–**Z** remain to be determined, although several appear to correspond to polymethoxylated flavones.

Discussion

The results of this study show a progression of changes in the secondary metabolite profiles in healthy 'Valencia' and 'Hamlin' leaves which appear different from changes occurring in HLB-affected trees. Our initial expectations were that there would be compounds newly formed in response to the presence of the invading CLas bacterium. Such responses to the infection of grapefruit leaves by the citrus canker-forming bacterium, *Xanthomonas citri* citri were recently reported (Manthey and Narciso, 2011). Interestingly though, this is not the response elicited by the CLas bacterium. Rather, over a 9-month period of investigation, the most notable changes in leaf phytochemical profiles occurred in the CON samples. Compounds **H**, **S**, **T**, **U**, and **V** were largely absent in CON leaves obtained at the early collection dates, but were prominent at the later dates. Only trace levels of these compounds occurred in the HLB leaves throughout the 9-month investigation period, and these results suggest that the changes that occurred in the CON leaves may have been blocked by the presence of HLB in the young seedlings. These findings suggest that the CLas exerts influences on phytochemical production and response mechanisms that contrast sharply with those elicited by other invading bacterial pathogens, particularly those recently described in canker-infected sweet orange and grapefruit leaves (Manthey and Narciso, 2011).

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