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Differences in Secondary Metabolites in Leaves from Orange (*Citrus sinensis* L.) Trees Affected with Greening Disease (Huanglongbing) (HLB)

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HPLC analyses of methanol extracts of two sets of orange leaves that were symptomatic of HLB showed differences in concentrations of hydroxycinnamates and flavonoids compared to unaffected, healthy leaves. Other differences were also detected in selected total ion currents (TICs) of HPLC–mass spectral (HPLC–MS) analyses of the leaf extracts. One main difference was the higher levels of limonin glucoside in the HLB-symptomatic leaves compared to the healthy leaves. Another difference was an elevated concentration in HLB symptomatic leaves of a compound, possibly an alkaloid, exhibiting a protonated *m/z* ion at 188 amu, and a violet-colored Ehrlich Reagent spot on TLC.

The effects of HLB (citrus greening disease) on the profiles of natural products in the leaves and juice of common citrus varieties remain largely uncharacterized, yet the importance of such changes to juice quality and to leaf physiology is obvious. The occurrence of changes in plant secondary metabolites can be reasonably anticipated in light of the influence of HLB on phloem transport and nutrient storage in citrus tissues (Schneider, 1968; Taba et al., 2006). Early chemical analyses of HLB-affected citrus bark and fruit albedo showed a fluorescent compound (Schwartz, 1965), later identified as gentisoyl glucoside (Feldman and Hanks, 1969). The elevated concentrations of this compound occurred in a large percentage of HLB-affected trees as well as in other sets of trees exhibiting stubborn disease, stem pitting (tristeza), and leaf mottle. However, gentisoyl glucoside was not detected in the bark or albedo from trees afflicted with a number of other citrus diseases that produce similar visual symptoms to HLB, including exocortis, citrus blight, and young citrus decline (Feldman and Hanks, 1969).

Our preliminary studies have dealt with the analysis of leaves of HLB-symptomatic sweet orange (*Citrus sinensis* L.) trees. Three classes of compounds were initially studied: the flavonoids, hydroxycinnamates, and limonoids. Citrus flavonoids occur in several main groups, including the flavone-*C*-glycosides, flavone-*O*-glycosides, flavanone-*O*-glycosides, and the polymethoxylated flavones (Horowitz and Gentili, 1977). The hydroxycinnamates occur as a complex set of conjugates of ferulic, *p*-coumaric, and sinapic acid (Risch and Herrmann, 1988). The third class, the limonoids, were of interest because their biosynthesis occurs in the phloem of stems (Ou et al., 1988), and thus, are presumably reliant on tissue transport for their occurrence in leaves. For all of these classes of compounds two methods of analysis, including thin layer chromatography (TLC), and HPLC–MS, provided evidence of significant changes in mature, HLB-symptomatic leaves. Significant differences in the levels of a putative alkaloid in HLB-symptomatic leaves were also detected.

Materials and Methods

PLANT MATERIAL. Sweet orange trees were identified as HLB-symptomatic by the visual appearances of their foliage (Bové, 2006). Mature, fully-developed leaves (30) that exhibited HLB "blotchy mottle" symptoms and healthy, nonsymptomatic leaves (30) were harvested from 'Valencia' and 'Midsweet' orange trees in Nov. 2006 and Feb. 2007, respectively. HLB in the symptomatic trees was subsequently confirmed by PCR analysis.

SAMPLE EXTRACTION AND THIN LAYER CHROMATOGRAPHY. Leaves were dried overnight under vacuum at 55 °C, then ground into a fine powder with a coffee grinder. Powdered leaf samples (~300 mg) were mixed with 10 mL methanol and shaken overnight. Extracts were analyzed by analytical HPLC and silica gel TLC. Separations of polar compounds by TLC [200 micron silica gel plates with fluorescence indicator (Analtech, Newark, DE)] were achieved with *n*-butanol/acetic acid/water/methanol (180/30/60/50 v/v/v/v). Visualization was achieved with the Ehrlich Reagent as described by Fong et al. (1989) with slight modification. The TLC plate was sprayed with a solution of 4 g *p*-dimethylaminobenzaldehyde in 200 mL 95% ethanol, then subsequently placed into a chamber containing hydrochloric acid vapors generated by the addition of 5 mL concentrated sulfuric acid to a beaker with 20 g ammonium chloride.

HIGH PRESSURE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY (HPLC–MS). An Alliance 2695 (Waters, Milford, MA) liquid chromatography system was used with a Waters 996 PDA detector. Spectra were scanned between 600 and 200 nm. Chromatograms were monitored at 280 nm. A Waters ZQ single quadrupole mass spectrometer with an electrospray interface was used in conjunction with the above HPLC system. Interface parameters were: source temperature 100 °C, desolvation temperature 255 °C, capillary voltage 3.2 kV, cone voltage 20 V, extractor voltage 3 V, RF lens voltage 0.3 V, desolvation gas flow 650 $L \cdot h^{-1}$ and cone gas flow 0 $L \cdot h^{-1}$.

Chromatographic separations were obtained with a C8 X-

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Table 1. HLB/CON peak area ratios of hydroxycinnamates (HCA) and flavonoid glycosides in 'Midsweet' and 'Valencia' sweet orange leaves. The fragment ions at 271, 287, 301, and 303 are due to apigenin (5,7,4′-trihydroxyflavone), luteolin (5,7,3′,4′-tetrahydroxyflavone), diosmetin (5,7,3′-trihydroxy-4′-methoxyflavone), and hesperetin (5,7,3′-trihydroxy-4′-methoxyflavanone) and are indicative of flavone- and flavanone-*O*-glycosides. The MS of flavone-*C*-glycosides (i.e., elution times 8.3, 9.4,13.2, 13.7) do not exhibit similar aglycone fragment ions (Chopin and Bouillant, 1975). The HLB/CON peak area ratios are calculated from averages of peak areas measured in HLB and CON samples prepared in triplicate.

Elution time	MS fragment	Structure	Midsweet	Valencia
5.3	---	HCA	1.84	1.95
6.3	---	HCA	1.2	1.43
7.2	---	HCA	1.78	2.06
8.3	595/577/559/457	6,8-di-*C*-glucosyl apigenin	0.50	0.67
8.8	---	HCA	1.30	1.92
9.4	---	flavone glycoside	0.83	0.87
13.2	565/433	apigenin-*C*-glucosyl-*O*-xyloside	0.69	0.66
13.7	565/433	2″-xylosylvitexin	0.63	0.63
14.7	595/449/287	luteolin rutinoside	0.70	0.80
18.3	579/433/271	isorhoifolin (apigenin-7-*O*-rutinoside)	0.42	0.59
19.1	609/463/301	diosmin (diosmetin-7-*O*-rutinoside)	0.99	0.97
19.9	611/465/303	hesperidin	1.39	1.34

Bridge column (15 cm × 4.6 mm, 5 um) (Waters). Initial solvent conditions were water/acetonitrile/2% formic acid (85/10/5 v/v/v). Liner gradients were run to (75/20/5), (70/25/5), (55/40/5), (25/70/5), (25/70/5), (85/10/5), and (85/10/5) at 10, 15, 23, 40, 45, 53, 60 min, respectively, at a flow rate of 0.75 mL·min^{-1}. Data acquisition was done with Mass Lynx v. 4.1.

Results

Changes in profiles of hydroxycinnamates and flavonoids in HLB-affected leaves. Methanol extracts of leaves from healthy (nonsymptomatic) trees and trees with HLB-like leaf mottling symptoms were analyzed by reversed-phase HPLC. Eight of the major flavonoid glycosides in the leaf samples were also analyzed by HPLC coupled with positive electrospray mass spectrometry (MS). Molecular weights and major fragment ions of these compounds are listed in Table 1, and together with the UV data, provide preliminary structural classifications of these compounds. Use of authentic standards (R.M. Horowitz, Pasadena, CA) facilitated further identification of five compounds (hesperidin, diosmin, isorhoifolin, 2″-xylosylvitexin, and 6,8-di-*C*-glucosylapigenin). In addition to the flavone glycosides, a complex set of hydroxycinnamates (HCAs) were also located by their characteristic UV spectra. Ratios of the integrated peak areas (HLB/CON) for these compounds are listed in Table 1. For some of the hydroxycinnamates the amounts were nearly 2 times higher in the HLB-symptomatic leaves than in the healthy leaves. In contrast, there were no differences in the levels of diosmin (diosmetin-7-rutinoside), and only modest differences in the levels of hesperidin (hesperetin-7-rutinoside) in the HLB-symptomatic and healthy (control) leaves for both orange varieties. Different from these two flavonoids, levels of the four apigenin glycosides (elution times 8.3, 13.2, 13.7, and 18.3) were dramatically lower in the HLB-symptomatic leaves (HLB/CON ratios 0.50 to 0.67). The luteolin rutinoside (14.7 min) also occurred at lower concentrations (HLB/CON ratios 0.70 to 0.80) for the two orange varieties.

In addition to the analysis of the flavonoid glycosides, levels of the polymethoxylated flavones were also compared in the HLB-symptomatic and healthy leaves. As shown in Table 2, the levels of most of the polymethoxylated flavones in the HLB-symptomatic leaves were moderately lower than in the healthy leaves (HLB/CON ratios of ~0.6–0.9), with the exception of 3,5,6,7,8,3′,4′-heptamethoxyflavone (HMF), which occurred at 1.6 to 4.3 times higher in the HLB-symptomatic leaves compared to the healthy leaves.

Differences in limonoid glucoside in HLB-symptomatic leaves. The leaf extracts were also analyzed for their limonoid glucoside contents. Authentic standards of limonin glucoside, nomilin glucoside, and nomilinic acid glucoside were resolved on TLC with *n*-butanol/acetic acid/water/methanol (180/30/60/50 v/v/v/v), then visualized with the Ehrlich reagent. Results showed that the concentrations of these compounds in the leaf extracts were too low for detection by TLC. Subsequent analyses by HPLC–MS showed far better detection. With this latter technique, 300 ± 22 ppm (n=3) limonin glucoside was measured in the HLB-affected 'Valencia' leaves (Fig. 1), while only trace levels were detected in the healthy leaves. Similar findings (approximately 70 ppm (n=3) limonin glucoside in the HLB-symptomatic leaves) were made with the 'Midsweet' orange leaves (data not shown).

HPLC–MS detection of a 188 *m/z* compound in the HLB-affected leaf tissues. Although the TLC analyses failed to provide detection of the trace-occurring limonoids, an Ehrlich reagent-positive compound was observed, and was distinct from the limonoid glucoside standards. In addition to having a differ-

Table 2. HLB/CON peak area ratios of the main polymethoxylated flavones in 'Midsweet' and 'Valencia' sweet orange leaves. Peak areas of the four identified compounds [sinensetin (SIN), tangeretin (TAN), (nobiletin) NOB, and heptamethoxyflavone (HMF)] and the one unknown compound (UK) were measured as mass extracted chromatograms from the total ion currents (TICs) recorded during the HPLC chromatographic runs. Values in parentheses are the mass ions (*m/z*) at which the TICs were recorded. Ratios are calculated from averages of triplicate values obtained for the HLB-symptomatic and healthy (control) leaves.

Variety	UK(373)	SIN(373)	TAN(373)	NOB(403)	HMF(433)
Midsweet	0.74	0.79	0.90	0.74	4.82
Valencia	0.70	0.64	0.63	0.57	1.66

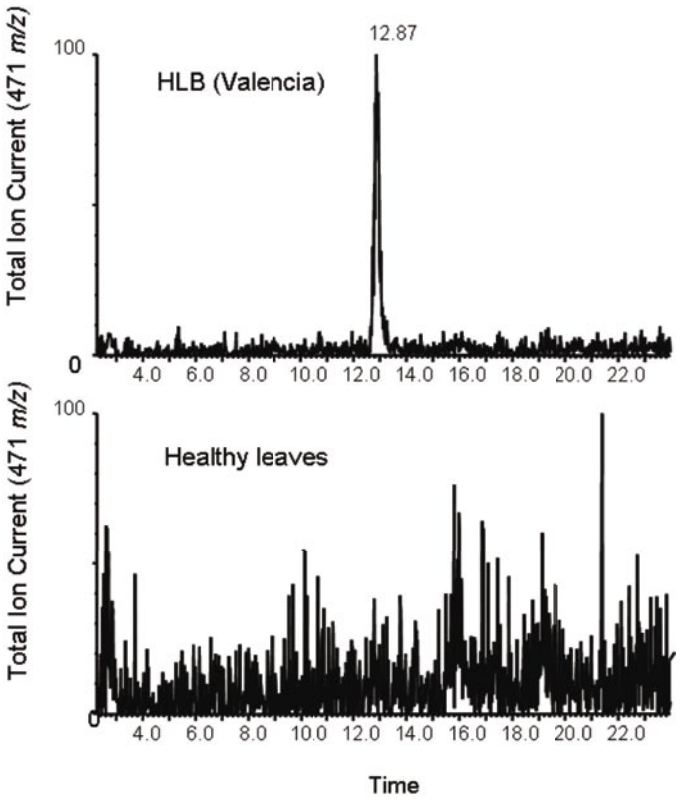

Fig. 1. Mass extracted (471 amu) total ion chromatograms of 'Valencia' HLB-symptomatic (upper) and healthy (lower) leaves. Limonin glucoside eluted at 12.87 min.

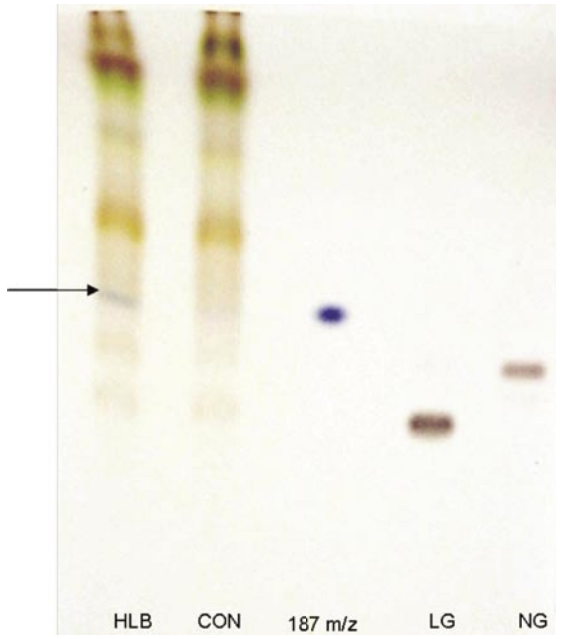

Fig. 2. TLC of 'Valencia' leaf extracts. HLB denotes HLB-symptomatic and CON denotes healthy leaf extracts. The middle spot shows the 187 *m/z* compound recovered after separation by semi-preparative HPLC. LG denotes limonin glucoside and NG denotes nomilin glucoside standards. The arrow shows the Ehrlich reagent positive spot that co-migrates with the 187 *m/z* compound enriched in the HLB-symptomatic 'Valencia' orange leaves.

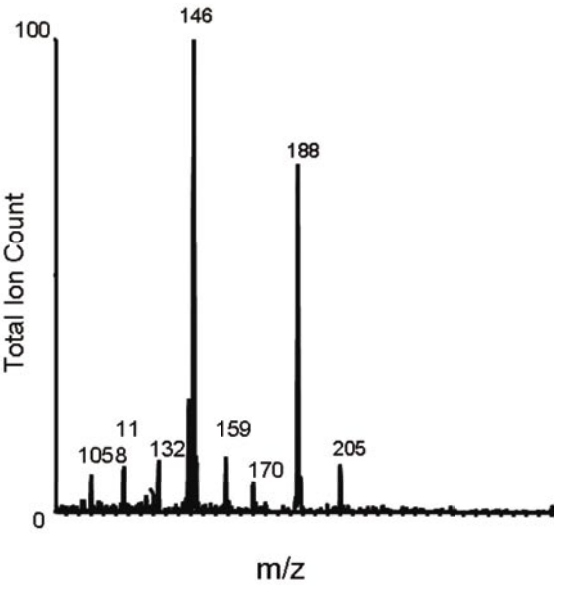

Fig. 3. Electrospray ionization mass spectrum of the 188 *m/z* compound (elution time 5.82) in orange leaf extracts.

ent R_f value from the limonoid standards, this compound formed a violet color with the Ehrlich reagent, whereas the limonoid glucosides formed reddish-color spots. This new Ehrlich reagent-positive compound visually appeared to occur at 2–4 times higher in concentration in the HLB-affected leaves compared to the healthy leaves (Fig. 2). Analyses using HPLC–MS additionally provided evidence of an early-eluting compound that occurred at higher concentrations in the HLB-affected leaf extracts. The mass spectrum of this compound (Fig. 3) exhibited a protonated molecular weight ion $(M+H)^{+1}$ *m/z* ion at 188 amu. Peak integrations of the 188 *m/z* compound in the mass-extracted Total Ion Currents showed that the HLB-symptomatic leaves of 'Valencia' contained 4.0 times higher levels (n=3) of this compound than the healthy leaves (Fig. 4). Similarly, HLB-symptomatic 'Midsweet' orange leaves contained 2.3 times higher concentrations (n=3) of the 188 *m/z* compound than the healthy leaves (data not shown). The leaf extracts were subsequently run on a semi-preparative C18 reversed-phase column, and the fractions containing the 188 *m/z* compound were collected. The early-eluting column fraction containing mainly the 188 *m/z* compound produced the same Ehrlich reagent positive spot when developed on normal phase TLC (Fig. 2), thus providing an indication that the Ehrlich reagent positive spot detected by TLC is the same 188 *m/z* compound detected by HPLC–MS.

Discussion

HLB causes severe stem phloem necrosis, and thus is anticipated to affect leaf and fruit tissue nutrient transport and accumulation (Schneider 1968), and this is illustrated by the dramatic build-up of starch in the leaves of HLB-affected trees. The detection of nutrient or secondary metabolite accumulation specific to HLB-affected citrus leaf tissue has been the focus of much previous research, with the aim of providing a means of rapid and economical detection of HLB in commercial groves. In addition to the discovery of the starch build-up, this earlier work established the occurrence of a fluorescent compound in the albedo and bark of HLB-affected sweet orange, and the detection of this compound

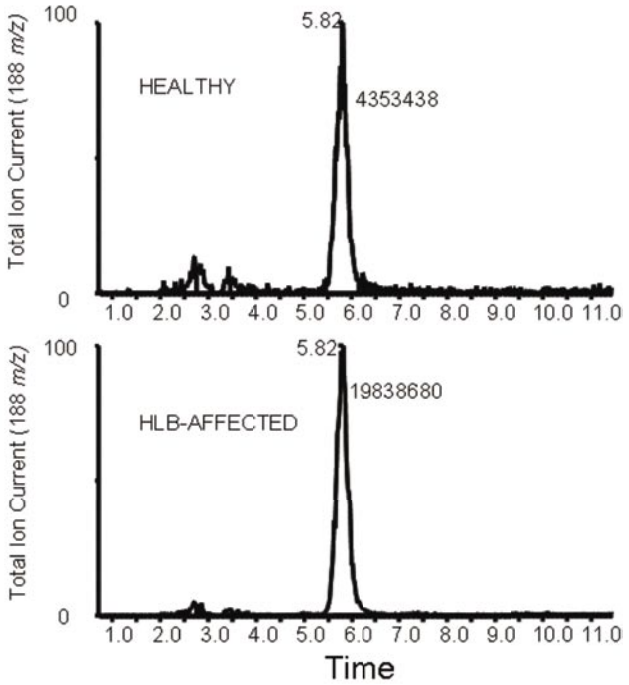

Fig. 4. Mass extracted (188 amu) total ion chromatograms of 'Valencia' HLB-symptomatic and healthy leaves. Values listed are peak integrations for identical injection volumes and leaf extract concentrations.

was useful as a means of detecting HLB-affected trees (Schwarz, 1965, 1968). This fluorescent compound was later identified as gentisoyl glucoside (Feldman and Hanks, 1969), and the levels of this compound were subsequently shown to be significantly correlated with the severity of HLB symptoms (Hooker, 1993).

These earlier reported changes in gentisoyl glucoside accumulation in HLB-affected citrus tissues are further mirrored by the changes in numerous other phenolic secondary metabolites, as illustrated in this present study. Of particular interest were the different effects of HLB on the profiles of the different classes of flavonoids and the hydroxycinnamates. The decreases in the apigenin glycosides, both as *O*-glycosides and *C*-glycosides in the HLB symptomatic leaves, are in sharp contrast to the nearly unchanged levels of the diosmetin glycoside, diosmin, and of the main flavanone glycoside, hesperidin. Also striking were the steep increases in the hydroxycinnamates in the HLB-affected leaves.

In light of the dramatic internal anatomical and physiological changes in HLB-infected leaves, particularly pertaining to the vascular tissue, the effects on the biosynthesis of limonin glucoside are also of interest. Limonin biosynthesis occurs in leaf stem phloem (Ou et al., 1988), and the higher limonin glucoside concentrations detected in the HLB-symptomatic leaves suggest a possible influence of the additional phloem formed in these leaves on the levels of biosynthesis of this compound. What role this actually plays on the limonoid glucoside biosynthesis is still unknown.

Finally, the discovery of the increased levels of an Ehrlich reagent positive compound in the HLB-affected leaves provides yet additional evidence of changes in the secondary metabolite profiles. Little is known of this compound, although the odd-numbered molecular weight of this compound, 188 amu, indicates that this compound contains an odd number of N atoms, possibly indicative of an alkaloid. This is supported by the formation of a violet-colored Ehrlich reagent spot for this compound on TLC plates, a property frequently observed with N-containing alkaloid compounds (Steelink, 1959). Work is in progress to complete the isolation and structural determination of this compound. The identification of this compound may provide additional information about biochemical changes occurring in HLB-affected citrus tissues.

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