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Research article

GC–MS metabolomic differentiation of selected citrus varieties with different sensitivity to citrus huanglongbing

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ABSTRACT

Huanglongbing (HLB) is the most destructive disease of citrus worldwide. The rapid identification of tolerant varieties is considered a critical step towards controlling HLB. GC–MS metabolite profiles were used to differentiate HLB-tolerant citrus varieties '*Poncirus trifoliata*' (TR) and 'Carrizo citrange' (CAR) from HLB-sensitive varieties 'Madam Vinous sweet orange' (MV) and 'Duncan' grapefruit (DG). PCR analyses revealed that MV was the most sensitive variety followed by DG and the tolerant varieties CAR and TR. Metabolomic multivariate analysis allowed classification of the cultivars in apparent agreement with PCR results. Higher levels of the amino acids L-proline, L-serine, and L-aspartic acid, as well as the organic acids butanedioic and tetradecanoic acid, and accumulation of galactose in healthy plants were characteristic of the most sensitive variety MV when compared to all other varieties. Only galactose was significantly higher in DG when compared to the tolerant varieties TR and CAR. The tolerant varieties showed higher levels of L-glycine and mannose when compared to sensitive varieties MV and DG. Profiling of the sensitive varieties MV and DG over a 20-week period after inoculation of those with the HLB-containing material revealed strong responses of metabolites to HLB infection that differed from the response of the tolerant varieties. Significant changes of L-threonine level in the leaves from old mature flushes and L-serine, L-threonine, scyllo-inositol, hexadecanoic acid, and mannose in the leaves from young developing flushes were observed in MV. Significant changes in myo-inositol in old flushes and L-proline, indole, and xylose in new flushes were observed in DG.

1. Introduction

Citrus huanglongbing (HLB), also known as the citrus greening disease, has become one of the greatest challenges for citrus growers across the world. Although Koch postulates have not been confirmed, the disease has been associated with a phloem-limited bacterium, *Candidatus* Liberibacter spp. First detected in China in the early 1900's, HLB has now spread all over the world [1]. Two psyllid species – *Diaphorena citri* (Kuw.), the Asian citrus psyllid; and *Trioza erytreae*, the African citrus psyllid – are responsible for the tree-to-tree transmission of the disease [2]. Currently the disease has no cure. Upon development of HLB infection in a tree, leaves accumulate high amounts of starch and show a pattern of yellow and green blotches [3]; the fruit becomes smaller, lopsided, and color does not fully develop. As HLB progresses, estimated yield reductions from 30 to 100% have made groves unprofitable within 7–10 years of infection [3]. Detection can be done by visual symptoms or by polymerase chain reaction (PCR). However, incidences reported by PCR have been twice as high as those reported by visual examination [4]. Other methods such as starch detection and chlorophyll fluorescence have also been suggested for HLB detection [5].

The development of tolerant and resistant citrus varieties is being emphasized as an alternative to reduce the impact of the disease [6]. Although HLB affects all citrus varieties, certain varieties have been reported to be more susceptible than others. Folimonova et al. [7] classified 30 citrus genotypes ranging from sensitive to tolerant according to their response to HLB in terms of symptom development and bacterium titer determined by PCR. However, the internal factors responsible for HLB susceptibility in citrus are yet to be understood.

Metabolomics is a growing field of analytical chemistry that focuses on the identification of small metabolites. Initially, mainly used in pharmaceutical applications, metabolomics has become

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a powerful tool in agriculture and food science [8,9] and has been used to characterize metabolic changes in plants after biotic and abiotic stresses [10], as well as biotic contamination of foods [11]. Metabolomic techniques have been able to identify changes in the metabolite profile of different citrus varieties [12], including those affected by HLB [13,14], as well as metabolomic changes in both pathogen and host after *Arabidopsis thaliana* infection with *Pseudomonas syringae* [15]. Additionally, GC–MS based metabolic profiling has been used to identify differences between two sunflower genotypes varying in response to *Sclerotinia sclerotiorum* [16]. However, no specific metabolites relative to tolerance were reported.

The objective of this research was to determine GC–MS-based metabolomic differences between two citrus varieties that are sensitive to HLB – 'Madam Vinous' sweet orange (MV) and 'Duncan' grapefruit (DG) – and two tolerant citrus varieties 'Carrizo citrange' (CAR) and '*Poncirus* trifoliata' (TR) [7]. We also monitored metabolomic changes occurring during HLB infection of sensitive varieties as a first step towards understanding the HLB tolerance mechanism of citrus.

2. Results

2.1. PCR and symptom development

Seedlings of both MV and DG varieties demonstrated a strong visible response to the HLB infection, with MV developing more pronounced symptoms earlier than all other varieties. Plants of these varieties developed severe yellowing of young leaves and reduced growth. Symptoms began to appear around 12–14 weeks after graft-inoculation and their severity progressed with time. PCR tests conducted using samples collected from the inoculated plants and HLB-specific primers showed high levels of the HLB bacterium, with the mean values of the threshold cycle (Ct) being 22.1 ± 0.28 for MV and 23.9 ± 0.30 for DG at week 14. At week 20, Ct values for these cultivars measured 23.4 ± 0.19 and 25.1 ± 0.27, respectively. In contrast, at week 14, Ct values for CAR and TR were 30.5 ± 0.45 and 31.6 ± 0.26, respectively. At week 20, CAR and TR had Ct values of 29.3 ± 0.20 and 32.6 ± 0.33, respectively, indicating that these varieties were highly tolerant to HLB. CAR and TR seedlings continued to grow vigorously, similar to control seedlings grafted with PCR-negative twigs for the duration of this experiment.

2.2. Metabolomic differentiation of citrus varieties

Approximately 61 compounds with a signal to noise ratio of at least 3 were detected by GC–MS in each variety. Fig. 1 shows typical chromatograms of each analyzed citrus cultivar. The overall metabolite profile of each variety was used for Principal Components Analysis (PCA, Fig. 2). Sample grouping by PCA correlated with variety as indicated by well-defined cluster regions in Fig. 2A and possibly with susceptibility. Separation of the most sensitive variety MV was mostly characterized by low scores of the first principal component (PC1). A variety less susceptible than MV, DG was separated from the tolerant varieties in the second principal component (PC2). Compounds with highest absolute loading values in PC1 were amino acids such as L-proline, L-serine, L-aspartic acid, L-glycine, and L-threonine; organic acid derivatives such as tetradecanoic acid, butanedioic acid, and hexadecanoic acid trimethyl-ester; and other compounds such as inositol and mannose (Fig. 2). Fig. 3 shows the abundance of the compounds that yielded significant differences ($P < 0.05$) among varieties after ANOVA. The most sensitive variety, MV, showed significantly higher levels of L-proline, L-serine, L-aspartic acid, butanedioic acid, tetradecanoic acid, and galactose oxime. The variety DG only

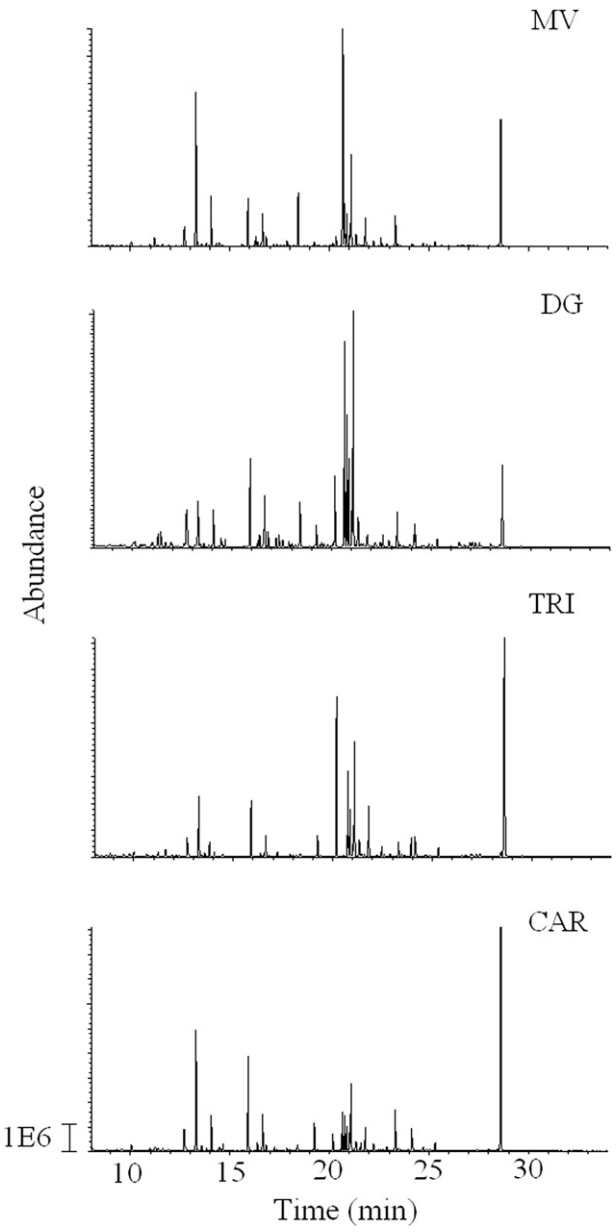

Fig. 1. Typical chromatograms of Madam Vinous' (MV), 'Duncan' grapefruit (DG), 'Trifoliate' (TR), and 'Carrizo' (CAR) orange.

presented significant differences in L-glycine, galactose, and mannose when compared with the tolerant varieties CAR and TR. L-glycine and mannose levels were significantly lower in MV and DG when compared to CAR and TR; whereas, galactose was in a significantly higher concentration in sensitive varieties when compared to the tolerant ones.

2.3. Changes in metabolite profile of sensitive varieties after inoculation with HLB

In this experiment, all varieties were preliminarily analyzed 14 weeks after inoculation. Only the susceptible varieties MV and DG showed significant differences in the metabolite profile and were selected for this part of the study. The complete metabolite profile obtained by GC–MS at 14 and 20 weeks after inoculation was used for PCA. Infected samples of MV were grouped by PCA at week 14 after inoculation (Fig. 4A), showing an improved grouping at week

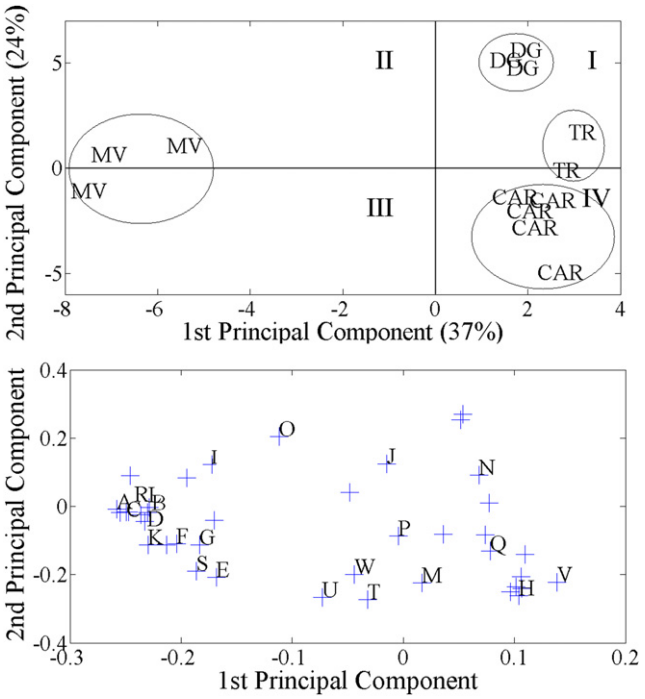

Fig. 2. Score (top figure) and loading values (bottom figure) of principal components analysis comparing varieties 'Madam Vinous' (MV), 'Duncan' grapefruit (DG), 'Trifoliate' (TR), and 'Carrizo' (CAR) orange. Metabolites coded in the loading plot are: (A) silanool trimethyl benzoate, (B) tetronic acid, (C) L-proline TMS, (D) L-serine TMS, (E) L-threonine TMS, (F) butanedioic acid TMS, (G) L-aspartic acid TMS, (H) glycine TMS, (I) pentonic acid TMS, (J) citric acid TMS, (K) ropane TMS, (L) tetradecanoic acid TMS, (M) D-fructose TMS, (N) D-xylose TMS, (O) galactose oxime TMS, (P) D-sorbitol TMS, (Q) inositol TMS, (R) Scyllo-inositol TMS, (S) hexadecanioic acid, trimethyl ester, (T) Myo-inositol TMS, (U) Indole TMS, (V) Mannose TMS, and (W) α-Galactoside TMS.

20 (Fig. 4B). Statistical analyses PCA and ANOVA included healthy and infected samples at each sampled week to account for changes in metabolites occurring as the plants grow. Fig. 5 shows the metabolites that yielded significant differences between healthy and HLB-infected MV at either sampled week. Old flush only yielded differences in L-threonine; whereas, new flush showed significant differences in L-serine, L-threonine, scyllo-inositol, hexadecanoic acid, and mannose.

Although less sensitive than MV, metabolites from DG leaves were also affected by HLB. Fig. 6A shows that 14 weeks after inoculation only new flush developed changes in metabolite profile of DG. However, 20 weeks after inoculation, changes in the metabolite profiles of both old and new flush were detected by PCA (Fig. 6B). Old flush only yielded significant changes in myo-inositol; whereas, new flushes showed significant changes in L-proline, indole, and xylose (Fig. 7).

3. Discussion

3.1. PCR and metabolomic differentiation of citrus varieties

PCR data suggest that MV and DG varieties are sensitive to HLB, whereas CAR and TR are tolerant. Results were in agreement with previous findings, suggesting MV and DG as more HLB-susceptible varieties than TR and CAR [7]. Similarly, PCA grouping of susceptible and tolerant varieties occurred when analyzing the metabolite profile of all varieties (Fig. 2). Separation of the two susceptible varieties in the upper quadrant I and quadrants II and III of the PCA score plot suggested that differences in metabolites may have an effect in the various levels of susceptibility to HLB. However, this observation is not conclusive unless the differences in metabolite profiles are associated to the metabolic response to HLB. The most sensitive variety, MV, showed significantly higher levels of L-proline, L-serine, L-aspartic acid, butanedioic acid, tetradecanoic acid, and galactose oxime. Increased concentration of L-proline has been previously reported in HLB-infected trees [13], and its accumulation has been related to the plant response to several biotic or abiotic stresses [17,18]. However, in this research, the most sensitive variety, MV, showed significantly higher levels of proline prior to inoculation with HLB. Similar results have been reported in rice, where the variety most sensitive to tungro virus disease presented significantly higher levels of L-proline prior to inoculation with the virus followed by significant accumulation of this aminoacid after inoculation [19]. Similarly, lower levels of L-proline were characteristic of rice varieties resistant to the stress caused by *Orseolia oryzae* [20]. High levels of proline have been suggested to prevent the death of pathogen by scavenging most of the bactericidal reactive oxygen species [21], thus enhancing the activity of plant pathogens. Therefore, one can hypothesize that the abundance of L-proline favors *Candidatus* Liberibacter survival and spread *in planta*. The higher levels of L-serine and L-aspartic acid found in MV suggest an important role of these amino acids in the sensitivity of MV to HLB. Endogenous serine was associated to the initiation of senescence in duckweed *Spirodela polyrrhiza* [22]. Conversely, another amino acid, glycine, was significantly higher in tolerant varieties CAR and TR, suggesting a possible involvement in the tolerance mechanism.

Levels of the organic acids butanedioic and tetradecanoic acids were also significantly higher in MV. Similar results have been reported in chrysanthemum resistance to biotic stresses, where significantly higher levels of butanedioic (succinic) acid were found in leaves susceptible to *Frankliniella occidental* [23]. L-glycine was in significantly lower concentrations in MV and DG compared to CAR and TR. High concentrations of L-glycine have been associated to photorespiration which, in turn, has been associated to plant response to stress [24,25]. The variety DG only presented significant differences in L-glycine, galactose, and mannose when compared with the tolerant varieties CAR and TR. The lower amount of differentiating compounds in DG leaves corresponded to the smaller number of PCR-confirmed infections obtained for this variety when compared to MV.

The tolerant varieties CAR and TR showed significantly higher levels of mannose. Mannose has been suggested as an elicitor of defense mechanisms in plants [26]. Therefore, the presence of mannose may increase tolerance of citrus varieties to HLB.

3.2. Changes in metabolite profile of sensitive varieties after inoculation with HLB

Infected samples of MV were grouped by PCA at week 14 after inoculation (Fig. 4A), showing an improved grouping at week 20 (Fig. 4B). Results suggest a direct effect of HLB in the metabolite profile of susceptible varieties. In general, old flush of MV only yielded differences in L-threonine; whereas, new flush showed significant differences in L-serine, L-threonine, scyllo-inositol, hexadecanoic acid, and mannose, indicating that new flush is more responsive to the disease than old flush which can be expected from tissue that is metabolically more active. These results correlate with recent reports showing that anatomical aberrations and higher number of *Candidatus* Liberibacter in HLB-infected plants were mostly found in new flush [27]. Accumulation of amino acids such as L-threonine and L-serine are commonly associated to stress response in plants mostly due to increased photorespiration or overexpression of proteases and peptidases. For instance, a serine carboxypeptidase-like gene has been found to be up-regulated after biotic and oxidative stress in rice [28] and disease resistance

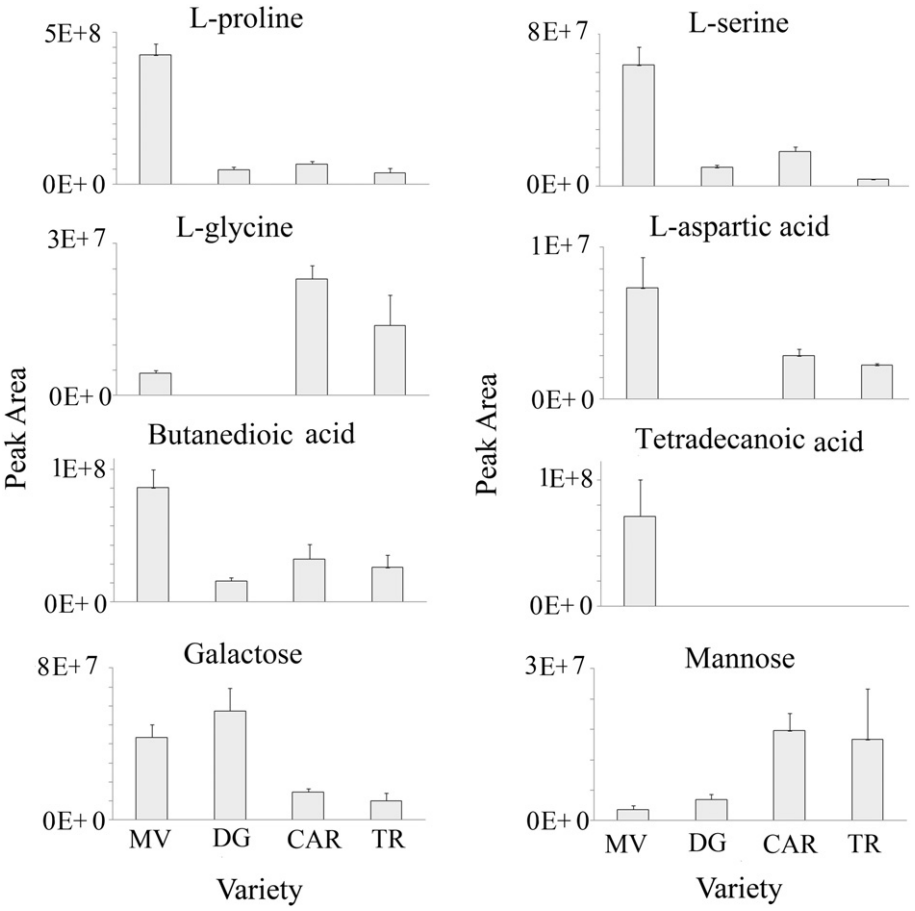

Fig. 3. Metabolites showing significant differences in different varieties. Codes are as in Fig. 1. Error bars represent standard error of all replicates.

in oats [29]. L-serine is also involved in transamination with L-glycine during photorespiration [25]. Additionally, L-threonine and L-serine-rich proteins and peptides have been previously reported as a mechanism of osmotic stress response in plants such as rice, tomatoes, and spinach [30–32]. Accumulation of these amino acids during HLB infection suggests their involvement in plants' general reaction to biotic or abiotic stresses. The role of inositol in citrus diseases is still not well understood. A recent study showed that in plant accumulation of inositol occurring after stress reduced the programmed cell death in *A. thaliana* but also blocked salicylic acid-dependent defense mechanism [33]. Additionally, myo-inositol has been shown to prevent internucleosomal fragmentation normally occurring during stress of *Allium cepa* [34]. In this study, accumulation of scyllo-inositol occurred in MV as a response to HLB. The accumulation of mannose observed in infected leaves of MV may have induced programmed cell death as a result of the

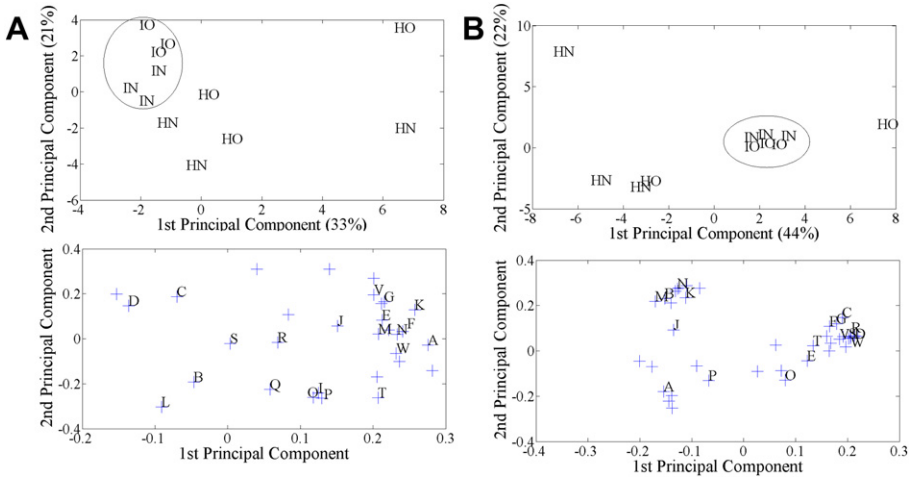

Fig. 4. Score (top) and loading (bottom) plots of healthy-old (HO), healthy-new (HN), infected-old (IO), and infected-new (IN) flushes of MV at 14 (A) and 20 (B) weeks after inoculation with HLB. Metabolite codes in the loading plot are the same as in Fig. 2.

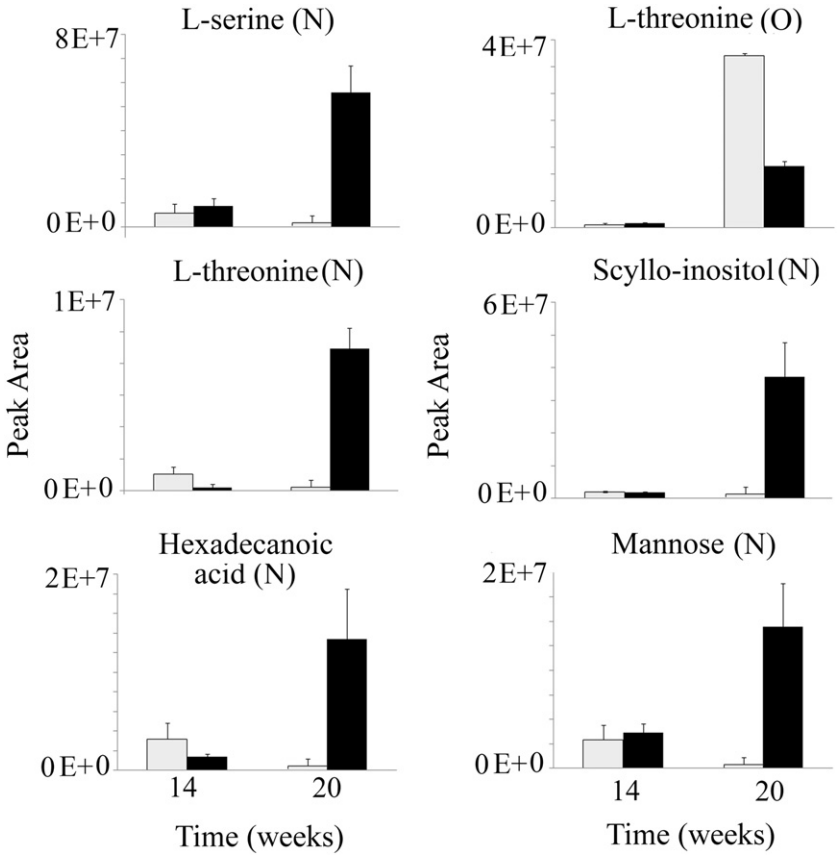

Fig. 5. Metabolites showing significant changes in new (N) and old (O) flushes of healthy (□) and HLB-infected (■) MV at 20 and 14 weeks after inoculation.

pathogenic attack as reported in previous studies [35]. The fatty acid, hexadecanoic acid, also accumulated significantly in HLB-infected MV leaves. Higher levels of hexadecanoic acid have recently been related to the resistance of certain grapevine varieties to downy mildew [36]. The role of fatty acids in plant defense has been suggested as repressors of the type III genes of plant pathogens [37]. However, the lack of type III secretion system in *Candidatus* Liberibacter asiaticus [38] suggests a more general role such as an antioxidant function of fatty acids in the citrus defense mechanism.

In the DG variety, old flush yielded significant changes in myo-inositol only, whereas new flushes showed significant changes in L-proline, indole, and xylose (Fig. 7). As previously discussed for MV, accumulation of myo-inositol in plant disease has mostly been related to prevention of programmed cell death. L-proline accumulation after biotic and abiotic stresses in citrus has been widely reported with several conditions including HLB [13]. Xylose levels were significantly reduced after HLB infection, suggesting a reduction or shift in the metabolism of DG. Indole levels were significantly increased after HLB inoculation, probably as a defense

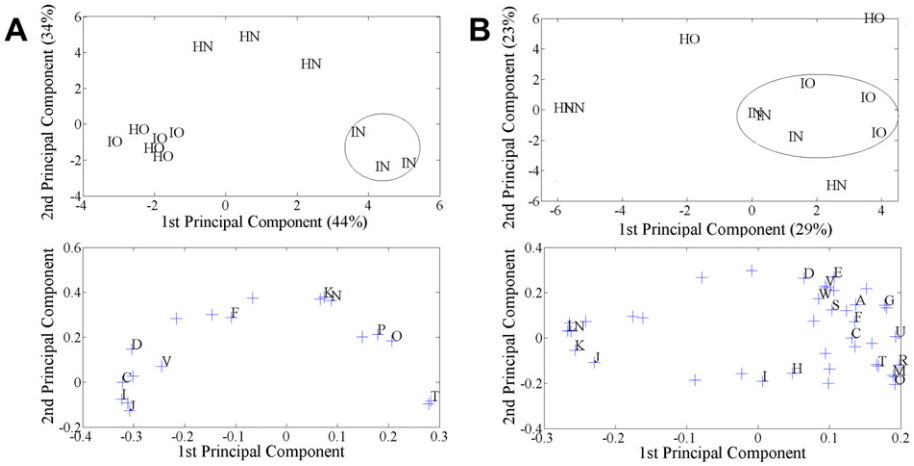

Fig. 6. Score (top) and loading (bottom) plots of healthy-old (HO), healthy-new (HN), infected-old (IO), and infected-new (IN) flushes of DG at 14 (A) and 20 (B) weeks after inoculation with HLB. Metabolite codes in the loading plot are the same as in Fig. 2.

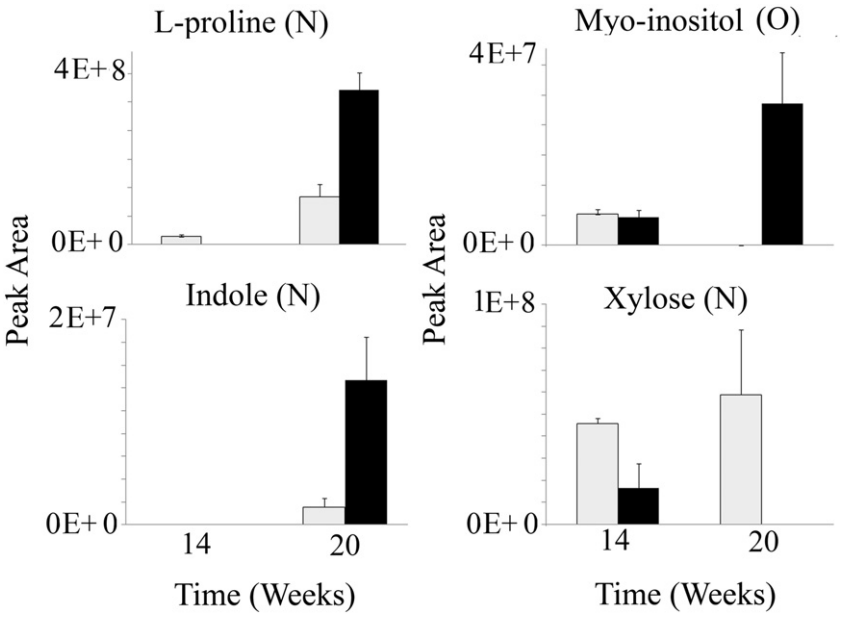

Fig. 7. Metabolites showing significant changes in new (N) and old (O) flushes of healthy (□) and HLB-infected (■) DG at 20 and 14 weeks after inoculation.

mechanism of DG. Accumulation of indole-related compounds after plant pathogenic attack has been widely suggested. For example, indole acetic acid, an indole derivative, was reported to function as an interaction mechanism between pathogens and plants, facilitating bacterial colonization [39].

In conclusion, visual observation of HLB symptoms and PCR results correlated to the metabolomic classification of varieties by PCA, suggesting the suitability of GC-MS-based metabolomics for rapid identification of tolerant varieties. Currently, susceptibility assessments by PCR require inoculation of the plants and PCR monitoring for several months. Even though both MV and DG appear to be sensitive to the HLB infection, the metabolites responding to inoculation were dissimilar in the two varieties, which may suggest that the intrinsic response to HLB infection depends on the citrus cultivar. Both cultivars showed the greatest metabolite changes in newly developed flushes compared to those in the pre-existing flushes. Grouping of metabolite profiles of the studied HLB-sensitive *vs.* HLB-tolerant varieties into two distinct regions allows hypothesizing that metabolomic analyses have the potential for separating HLB-tolerant citrus varieties. Although testing of this hypothesis requires studying of other varieties and their hybrids (currently underway in our laboratories), results presented here are the foundation for continued research on metabolomic profiling for better understanding of the citrus response to HLB infection.

4. Materials and methods

4.1. Plant materials, inoculum sources, and inoculations

Citrus germplasm used in these studies was obtained from DPI (Florida Department of Agriculture and Consumer Services, Division of Plant Industry) or USDA CRC (USDA-Agricultural Research Service, National Clonal Germplasm Repository for Citrus & Dates). Four citrus varieties were used in this work: Madam Vinous sweet orange [*C. sinensis* (L.) Osbeck], Duncan grapefruit (*C. paradisi* Macfadyen), Carrizo citrange (×*Citroncirus webberi* J. Ingram & H. E. Moore) and *Poncirus trifoliata* (L.) Raf. Plants were propagated as seedlings. Six- to eight-month-old seedlings were used for inoculation with HLB-infected budwood obtained from field sources in Florida. All inoculum sources were verified to have HLB via PCR assays with HLB-specific oligonucleotides. Two infected buds were grafted into each treatment seedling. If after 10 days one of the buds dried out, a third bud was grafted to ensure similar density of inoculum in each treatment. Control trees were similarly grafted with healthy buds. Inoculated plants were kept in a USDA-APHIS approved secure greenhouse with the temperature controlled between 28 °C and 32 °C. Photosynthetically active radiation (PAR) was measured using LI-185 Quantum/Radiometer/Photometer (Lambda Instruments Inc., Lincoln, NE). PAR measured above plants in the greenhouse with natural photoperiods ranging between 300 and 570 $\mu Em^{-2}\ sec^{-1}$ during the daytime. Usually, two leaves from each of six inoculated seedlings of each variety, along with two leaves from each of five to six non-inoculated control trees, were sampled immediately after inoculation, 14 weeks after inoculation and 20 weeks after inoculation. One leaf from each seedling was used for PCR analysis and the other for GC–MS. Leaf samples were collected at 4-week intervals for 6 months and kept frozen at −80 °C till extraction for metabolomic analysis. Samples included leaves of trees from all four varieties, both healthy and HLB-infected. When available, both old and new flush were analyzed and compared.

4.2. PCR assays

Midribs from leaf tissue (250 mg) were extracted in 2.5 mL extraction buffer (100 mM Tris-HCL pH 8.0; 50 mM EDTA; 500 mM NaCl; 10 mM dithiothreitol). An aliquot of 1300 μL was transferred to a 1.5-mL Eppendorf tube, 90 μL 20% SDS were added, and incubated at 65 °C for 30 min. To the mixtures, 500 μL of 5 M potassium acetate was added, mixed thoroughly, and incubated on ice for 20 min. The mixture was centrifuged at 13,000 rpm for 10 min, 400 μL of supernatant were recovered, and DNA was precipitated by adding an equal volume of isopropanol. Tubes were kept at −20 °C overnight. The DNA was pelleted, washed, and resuspended in 100 μL water for PCR analysis. Conventional PCR and

real-time quantitative PCR (qPCR) tests were performed as described previously [40], using 0.2 μg of DNA per assay.

4.3. Extraction and sample preparation

All preparation, extraction, and GC–MS analysis conditions were similar to those reported as optimum in previous studies [13]. Briefly, each fresh leaf was weighed and ground to a fine power under liquid nitrogen. Methanol, water, and chloroform (MWC) were added at a 7.2:0.9:0.9 ratio to make a final leaf concentration of 10%. Extraction was done by stirring the suspension overnight at 0 °C in a Fisher Scientific Isotemp 3016 D (Saint Louis, MN) water bath. The suspension was then passed through a 0.45-μm Fisher brand filter (Pittsburg, PA) attached to a Popper & Sons Micro Mate glass syringe (New Hyde Park, New York). The resulting extracts were then stored at −20 °C until derivatization. One hundred and eighty microliters of extracts were then dried under a flow of nitrogen. Thirty microliters of methoxyamine (MOX) (Thermo Scientific, Rockford, IL) were then added to the dried extract and left to react for at least 17 h at room temperature. To the mixture, 80 μL of N-methyl-N-trifluoroacetamide (MSTFA) (Thermo Scientific, Rockford, IL) were then added to the resulting solution and let react for exactly 2 h.

4.4. Gas chromatography analysis

The gas chromatograph (GC) used in this experiment was model HP5890 coupled to an HP5971 series mass spectrometer (MS) from Hewlett Packard, (Santa Clara, CA). Chromatogram analysis was completed using HP ChemStation software. A volume of 0.3 μL of the derivatized extract was injected into the GC–MS using a Hamilton Microliter (Reno, NV) syringe. The syringe was cleaned 10 times before each use with hexane. The GC method settings were: injector temperature of 250 °C, initial oven temperature of 70 °C with a 10 °C min^{-1} ramp to 310 °C and a 5-min hold at 310 °C. Ultrapure hydrogen was used as the carrier gas at 1 mL min^{-1}. The MS was tuned to maximum sensitivity in electron impact mode, positive polarity, and the total ion current was recorded for a mass range of 50–650 amu. The GC–MS interface was set to 318 °C. The scan was recorded after a solvent delay of 8 min with a scan frequency of 4 s^{-1}.

4.5. Compound identification

Compound identification was done by library matching of mass spectra of each compound (3:1 signal to noise ratio) using the Wiley 138 K mass spectral library (Hoboken, NJ) and our internal databases that include several amino acids, organic acids, and sugar standards. Compound identity was obtained and reported only when the matching value of the mass spectra comparison was 70 or higher, and an increase in the size of the peak was observed when spiking the sample with the corresponding pure standard. Mass spectra of all peaks were analyzed at the beginning, middle, and end width of each peak to detect coelution. No coelution was found in any of the detected peaks.

4.6. Statistical analysis

Data from the chromatogram was aligned to correct differences in retention time using an in-house program written in C++. Principal components analysis (PCA) was carried out to compare the overall metabolite profile of each sample group, and analysis of variance (ANOVA) was run to determine significant difference of individual compounds. Statistical analyses were performed in MATLAB R2008a from The MathWorks (Natick, MA), and significant differences were reported at 95% confidence level.

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