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Research article

Metabolic variations in different citrus rootstock cultivars associated with different responses to Huanglongbing

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ABSTRACT

Huanglongbing (HLB) is one of the most destructive bacterial diseases of citrus. No resistant cultivars have been identified, although tolerance has been observed in the genus *Poncirus* and some of its hybrids with *Citrus* that are commonly used as rootstocks. In this study we exploited this tolerance by comparing five different tolerant hybrids with a cultivar that shows pronounced HLB sensitivity to discern potential contributing metabolic factors. Whole leaves of infected and non-infected greenhouse-grown seedlings were extracted and subjected to untargeted GC-TOF MS based metabolomics. After BinBase data filtering, 342 (experiment 1) and 650 (experiment 2) unique metabolites were quantified, of which 122 and 195, respectively, were assigned by chemical structures. The number of metabolites found to be differently regulated in the infected state compared with the non-infected state varied between the cultivars and was largest (166) in the susceptible cultivar Cleopatra mandarin (*Citrus reticulata*) and lowest (3) in the tolerant cultivars US-897 (*C. reticulata* 'Cleopatra' × *Poncirus trifoliata*) and US-942 (*C. reticulata* 'Sunki' × *P. trifoliata*) from experiment 2. Tolerance to HLB did not appear to be associated with accumulation of higher amounts of protective metabolites in response to infection. Many metabolites were found in higher concentrations in the tolerant cultivars compared with susceptible Cleopatra mandarin and may play important roles in conferring tolerance to HLB. Lower availability of specific sugars necessary for survival of the pathogen may also be a contributing factor in the decreased disease severity observed for these cultivars.

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1. Introduction

Huanglongbing (HLB) is one of the most destructive and economically important diseases of citrus. In Florida and in most citrus producing countries, HLB is associated with *Candidatus* Liberibacter asiaticus (Las), a non-culturable phloem-limited and gram-negative bacterium which is transmitted by the Asian citrus psyllid, *Diaphorina citri* Kuwayama. HLB is expressed by the appearance of foliar disease symptoms, such as irregular blotchy mottling and severe chlorosis often resembling zinc- or other nutritional deficiencies (McClean and Schwarz, 1970), followed by tree decline and major reduction in fruit quality and yield. Management practices for trees with HLB include enhanced nutritional programs to remediate symptoms which often result in improved tree appearance (Stansly et al., 2014). However, despite such targeted management practices, Florida citrus production has continued to decline because of HLB damage (NASS, 2015), and the overall economic impact of HLB was estimated to be $4.5 billion.

Las infects all known *Citrus* species and *Citrus* relatives, and most commercial cultivars exhibit strong disease symptoms following infection (McClean and Schwarz, 1970). Different responses to HLB were found between different citrus cultivars and tolerance was described for some hybrids between *Citrus* and *Poncirus trifoliata* commonly used as rootstocks (Albrecht and Bowman, 2011, 2012a; Folimonova et al., 2009). The rootstock is

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an important component of commercially grown citrus trees and can determine success or failure of a citrus operation (Castle, 2010). In addition to the desired effect on scion vigor, fruit size, fruit quality, and yield, rootstock selection is based on tolerance to different environmental conditions and resistance to pests and diseases. Greenhouse and field studies have shown that hybrids of citrus with *P. trifoliata*, show higher tolerance to HLB (Albrecht and Bowman, 2011, 2012a). In addition, recent studies have shown that in Las-infected sweet orange trees fruit production is 200–300 percent greater for trees on some new rootstock cultivars compared to standard commercial rootstocks (Bowman and McCollum, 2015; Bowman et al., 2016). It is evident that rootstock plays a major role in determining a tree's ability to tolerate Las, and that methods are needed that assist in the selection of superior rootstock candidates prior to long-term evaluation in the field.

Genomics methodologies, such as high-throughput DNA sequencing and gene expression/microarray analysis allow plant breeders to directly study the correlation between genotype and phenotype and are therefore valuable tools to improve and accelerate breeding efforts (Pérez-de-Castro et al., 2012). Using such tools, much progress has been made understanding the transcriptional and physiological effects of HLB in citrus (Albrecht and Bowman, 2008, 2012b; Fan et al., 2011; Kim et al., 2009; Martinelli et al., 2012; Xu et al., 2015; Zhong et al., 2015). Proteomic studies aimed at understanding citrus responses to HLB include the studies by Fan et al. (2011), Nwugo et al. (2013a, b), and Zhong et al. (2015). Whereas most of these studies focused on susceptible sweet orange (*Citrus sinensis*) cultivars, Albrecht and Bowman (2012b) and Nwugo et al. (2013b) included cultivars with tolerance or reduced sensitivity to HLB in their investigations.

In addition to the study of the transcriptome and the proteome, study of the metabolome has considerably gained in popularity within the plant sciences. Metabolites are the end products of cellular regulatory processes, and their levels can be regarded as the ultimate response of a biological system to genetic or environmental changes (Fiehn, 2002). In contrast, changes in mRNA or protein levels do not provide direct information about how these changes are linked to a change in biological function (Fiehn et al., 2000). Since the metabolite composition not only depends on the type and strength of the stress, but also on the cultivar and the plant species, metabolomics present an ideal tool for plant breeders (Fernie and Schauer, 2008; Krasensky and Jonak, 2012). Although our transcriptomic studies have revealed many expressed sequences that appear to be playing an important role in the tolerance to HLB (Albrecht and Bowman, 2008, 2012b), the majority of these sequences require further molecular genetic approaches in order to be applicable for transcriptomic profiling. One major advantage of metabolomics studies is that they do not rely on available genomic information. In addition, costs for metabolic profiling have decreased in recent years to a level that is much more cost-efficient compared with transcriptomic profiling.

Many studies on metabolites are targeted analyses which involve targeting compounds from a preselected and well-defined class of compounds, contrary to untargeted analyses which allow for the analysis of all detectable metabolites in a sample, including chemical unknowns (Cajka and Fiehn, 2016). Recent metabolite analyses on citrus compared leaf, root, or fruit metabolic profiles of non-infected and Las-infected sweet orange cultivars (Chin et al., 2014; Freitas et al., 2015) and other citrus cultivars with different sensitivity to HLB (Cevallos-Cevallos et al., 2009, 2011, 2012; Nwugo et al., 2013b). These studies focused on metabolites with known chemical structure. In this study, we conducted an untargeted metabolite analysis using gas chromatography-time-of-flight mass-spectrometry (GC-TOF MS) methodology that focused not only on known metabolites, but also on the large group of chemical unknowns. Our objectives were to: 1) identify metabolites associated with response to Las infection, and 2) define metabolic variations of different citrus rootstock cultivars with different levels of tolerance or susceptibility to HLB. Stress tolerant plants are generally found to have higher levels of stress-related metabolites under normal growth conditions and/or are able to accumulate larger amounts of protective metabolites under unfavorable conditions (Krasensky and Jonak, 2012). We hypothesized that different citrus cultivars will exhibit distinctive metabolic profiles that not only depend on their genotype but that are also associated with their differential response to Las infection. The identification of metabolite profiles that correlate with HLB disease tolerance will improve our ability to select the most promising citrus cultivars and reduce time and costs associated with breeding programs that are targeting this trait.

2. Materials and methods

2.1. Study design

2.1.1. Experiment 1

Twenty-one greenhouse-grown 15 month-old Cleopatra mandarin (*Citrus reticulata*) seedlings and 21 greenhouse-grown 15 month-old US-897 (*C. reticulata* 'Cleopatra' × *Poncirus trifoliata* 'Flying Dragon') seedlings were inoculated by grafting two bark and two leaf pieces onto each plant. To produce infected plants, bark and leaf pieces were obtained from infected greenhouse-grown 'Valencia' scions, PCR-positive for Las and symptomatic for HLB. To produce non-infected plants, bark and leaf pieces were obtained from healthy greenhouse-grown Valencia scions. Six plants were inoculated with disease-free tissue pieces and 15 plants were inoculated with infected tissue. Studies in our laboratory have shown US-897 to be tolerant to HLB with almost no visible effects, while Cleopatra mandarin was found to have strong leaf symptoms and pronounced stunting (Albrecht and Bowman, 2011).

2.1.2. Experiment 2

Thirty-six greenhouse-grown 16 month-old seedlings of the genotypes Carrizo citrange (*Citrus sinensis* × *P. trifoliata*), US-802 (*C. grandis* 'Siamese pummelo' × *P. trifoliata* 'Gotha Road'), US-812 (*C. reticulata* 'Sunki' × *P. trifoliata* 'Benecke'), US-897, and US-942 (*C. reticulata* 'Sunki' × *P. trifoliata* 'Flying Dragon') and 29 greenhouse-grown 16 month-old 'Cleopatra' mandarin seedlings were inoculated as described above, but using three bark- or bud pieces per plant. Previous studies in our laboratory (Albrecht and Bowman, 2012a) have categorized these rootstock cultivars as tolerant (Carrizo, US-897, US-942), moderately tolerant (US-802, US-812), or susceptible (Cleopatra) to HLB. Nine plants of each genotype were mock-inoculated with disease-free tissue pieces and 27 plants were inoculated with infected tissue. For Cleopatra, six plants were mock-inoculated and 23 plants were inoculated with infected tissue.

All inoculations were performed in groups containing one plant per genotype to ensure that different genotypes received tissue pieces from the same source. Plants were arranged randomly on the greenhouse benches and kept under natural light conditions at a temperature of 21–28 °C. Plants were irrigated and treated with insecticides as needed and were fertilized every three weeks using a water-soluble fertilizer mix, 20N-10P-20K (Peters Professional, The Scotts Company, Marysville, OH). Plants were pruned immediately after graft-inoculation and at 6 months after inoculation to promote new leaf growth and enhance HLB disease symptom development. Plants were evaluated every two (experiment 1) or three (experiment 2) months for foliar disease symptoms (chlorosis, blotchy mottle, leaf size) and growth reductions.

2.1.3. PCR detection of Las

To monitor disease progression in experimental plants, four to six fully expanded leaves were collected from each plant every two (experiment 1) or every three months (experiment 2). Petioles were severed and ground in liquid nitrogen using mortar and pestle. One hundred milligrams of ground tissue per sample were used for DNA extraction using the Plant DNeasy kit (Qiagen) according to the manufacturer's instructions. PCR analyses were performed by quantitative real-time polymerase chain reaction (PCR) as described in Albrecht and Bowman (2012a). For analysis of samples collected for metabolite studies (see next paragraph), PCR detection of Las was conducted on whole leaves using the same described procedures.

2.2. Metabolite analysis

2.2.1. Tissue collection

Six infected plants from each genotype were selected based on uniformity of disease symptom development and PCR results. Leaves were immediately frozen in liquid nitrogen, and stored at −80 °C until used for Las detection and metabolite extraction. Experiment 1: Depending on leaf size, 6–8 leaves from six non-infected and six infected plants from each genotype were collected at 8 and at 10 months after inoculation (mai). Leaves from US-897 seedlings were dark green and did not differ in appearance from non-infected plants. Leaves from infected Cleopatra seedlings were of reduced size and severely chlorotic. Average cycle threshold values for Las (CtLas) after PCR analysis ranged from 22.6 for leaves from Cleopatra to 27.8 for leaves from US-897 seedlings (Table 1). Experiment 2: Four to six leaves from six non-infected and six infected plants from each genotype were collected at 12 mai. Leaves from infected plants were blotchy mottled, except for leaves from US-897 and US-942, which were without any discernable disease symptom (Table 1). The average CtLas values ranged from 21.0 for leaves from US-802 to 24.0 for leaves from US-897.

2.2.2. Metabolite extraction

Leaves were ground in liquid nitrogen using mortar and pestle. Twenty milligrams of ground tissue per sample were extracted twice in 1 ml of a mixture of methanol, chloroform and water (5:2:2) for 20 min at 4 °C under constant agitation. After centrifugation at 14,000 g for 3 min, supernatants were pooled, evaporated to dryness under vacuum in a speedvac concentrator (Savant, Thermo Scientific, Hudson, NH), and stored at −80 °C until GC-TOF MS analysis.

Table 1
Disease symptom type and average CtLas values of leaves from plants from experiments 1 and 2 collected at 8–12 months after inoculation (mai).

Genotype	Average CtLas value			Disease symptom type
	8 mai	10 mai	12 mai	
Experiment 1				
Cleopatra mandarin	22.6	24.7	–	Small chlorotic leaves
US-897	27.3	27.8	–	None
Experiment 2				
Cleopatra mandarin	–	–	21.6	Blotchy mottled leaves
Carrizo citrange	–	–	22.3	Blotchy mottled leaves
US-802	–	–	21.0	Blotchy mottled leaves
US-812	–	–	22.4	Blotchy mottled leaves
US-897	–	–	24.0	None
US-942	–	–	23.1	None

Ct (cycle threshold) values were obtained through quantitative real-time polymerase chain reaction of whole leaves.

2.2.3. Metabolite profiling

Samples were derivatized by methoximation and trimethylsilyation according to Fiehn et al. (2008). Samples were injected into a Gerstel (Gerstel, Muehlheim, Germany) automatic liner exchange system using a Gerstel CIS cold injection system. Gas chromatography and mass spectrometry were performed on an Agilent 6890 gas chromatograph (Agilent, Santa Clara, CA) and a Leco Pegasus IV time of flight mass spectrometer, respectively, both controlled by the Leco ChromaTOF software v.2.32 (Leco, St. Joseph, MI). Data were processed using the algorithms implemented in the open-source BinBase metabolome database as described by Fiehn et al. (2005).

2.3. Statistical analysis

Metabolite data were normalized by dividing each sample peak by the sum of peaks of all metabolites for the sample and multiplying with the average sum of peaks of all biological replicates within a treatment group. Univariate and multivariate statistical analysis of normalized data was performed using Statistica software version 10 (Dell, formerly StatSoft) to assess leaf metabolite profiles of the different cultivars in the non-infected and in the Las-infected state. Principal component analysis (PCA) and partial least squares analysis (PLS) were performed using Statistica's nonlinear iterative partial least squares (NIPALS) algorithm.

3. Results

3.1. Overview of metabolic profiles

To reduce the highly dimensional data sets to a lower dimension and to reveal the underlying structure of the dataset, principal component analysis (PCA) was carried out. PCA is a non-parametric, unsupervised multivariate method that aims to find the maximum variation within the data without referring to any class labels.

3.1.1. Experiment 1

In the first experiment we compared leaf metabolic profiles of HLB tolerant US-897 seedlings and HLB susceptible Cleopatra mandarin seedlings at two different times (8 and 10 months) after inoculation with Las. Untargeted GC-TOF MS analysis identified a total of 342 unique metabolites of which 122 were assigned by chemical structure. PCA was carried out using all 342 identified compounds and a total of 7 principal components (PCs) were extracted which accounted for 77.1% of the total variance. The total variation explained by the first three principal components was 60.9%, with PC1 contributing 30.6%, PC2 contributing 17.2% and PC3 contributing 13.1% of variance. The score plot (Fig. 1A) shows separation of samples from US-897 and from Cleopatra along PC3. As can be seen in the loading plot (Fig. 1B), this separation was primarily due to the high levels of palatinose and several chemically unknown compounds, such as 228,833, 202,663, 208,792, and 309,580 in US-897 and a high abundance of synephrine, ornithine, citrulline, and proline, and several unknown compounds, such as 338,771, 208,662, 208,668, and 208,688 in Cleopatra in the infected state. Whereas samples from Cleopatra clearly separated depending on infection with Las, no clear separation of non-infected and infected samples was observed for US-897. Mass spectra for unidentified compounds are presented in Suppl. Table S1.

3.1.2. Experiment 2

In our second study we extended our investigations to include six rootstock cultivars which have been characterized in our laboratory as responding differently to Las (Albrecht and Bowman, 2012a). Using the same methodologies as in the first study, we

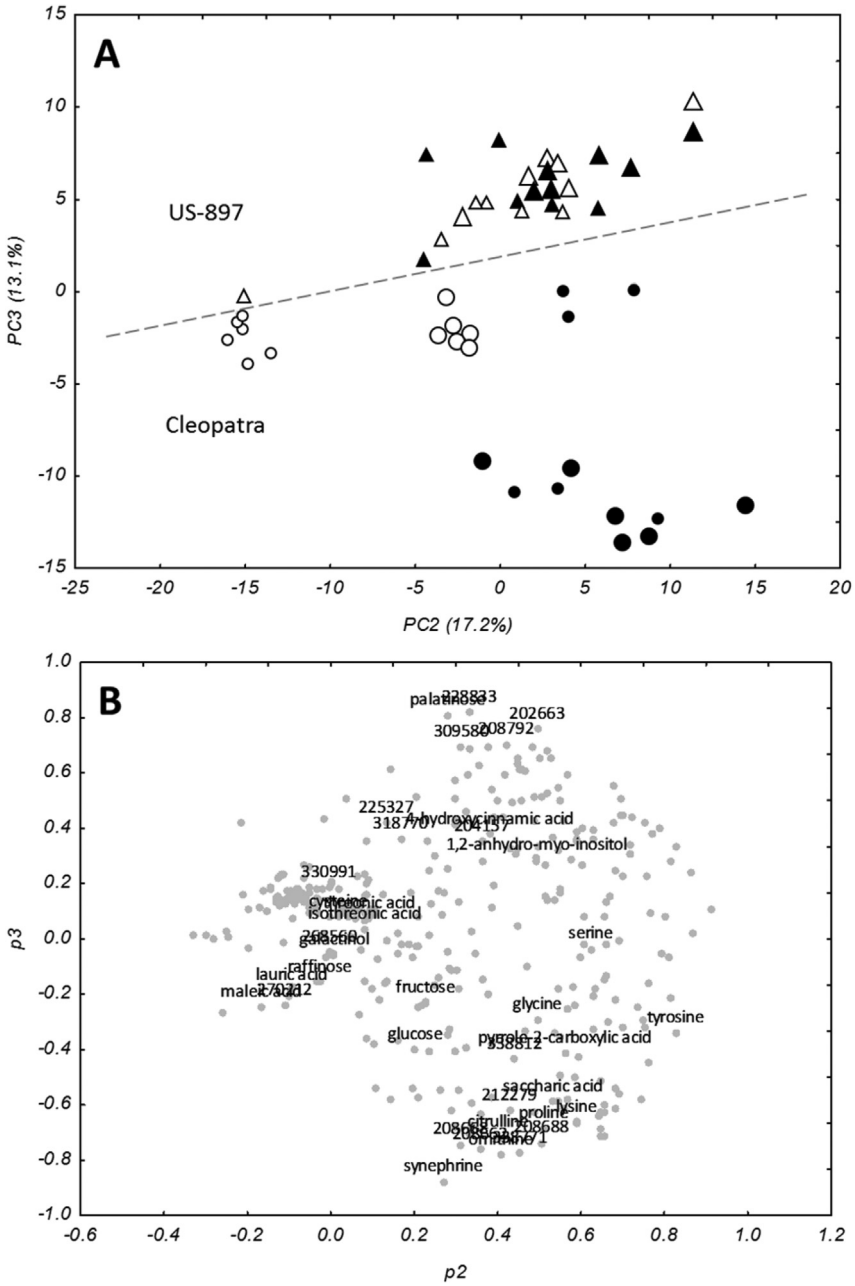

Fig. 1. Principal component analysis (PCA) of leaf GC-TOF MS profiles of US-897 and Cleopatra mandarin seedlings. A) Score plot of metabolite profiles of *Cleopatra mandarin* (circles) and US-897 (triangles) 8 months (small circles and small triangles) and 10 months (large circles and large triangles) after mock-inoculation (no fill) or inoculation (black fill) with *Ca.* L. asiaticus. B) Loading plot depicting selected metabolites mentioned in the text.

identified 650 unique metabolites of which 195 were identified by chemical structure. PCA using all 650 identified compounds extracted a total of 5 PCs which accounted for 56.1% of the total variance. The total variation explained by the first three principal components was 47.0%, with PC1 contributing 22.5%, PC2 contributing 16.7%, and PC3 contributing 7.8% of variance. The PCA score plot (Fig. 2A) shows separation of rootstock cultivars into different groups, with Cleopatra mandarin samples forming one group, the mandarin × trifoliate hybrids US-812, US-897, and US-942 forming a second group, and Carrizo citrange and US-802 forming a third group. Additional separation of samples was observed within the cultivars Cleopatra, Carrizo, and US-802, depending on whether plants were non-infected or infected with Las. No clear separation based on the state of infection was found for US-812, US-897, and US-942 samples. The loading plot (Fig. 2B) highlights some of the metabolites responsible for the observed pattern of separation and includes compounds also observed in the first experiment.

3.2. Metabolic response of plants to infection with Las

3.2.1. Experiment 1

In Cleopatra, 67% of all metabolites detected were found to be differentially regulated in response to infection with Las. Metabolic profiles of US-897 were found almost unaltered and only 7% of metabolites were found to be significantly ($P < 0.05$) affected in response to infection. PCA analysis (Fig. 1) showed a clear

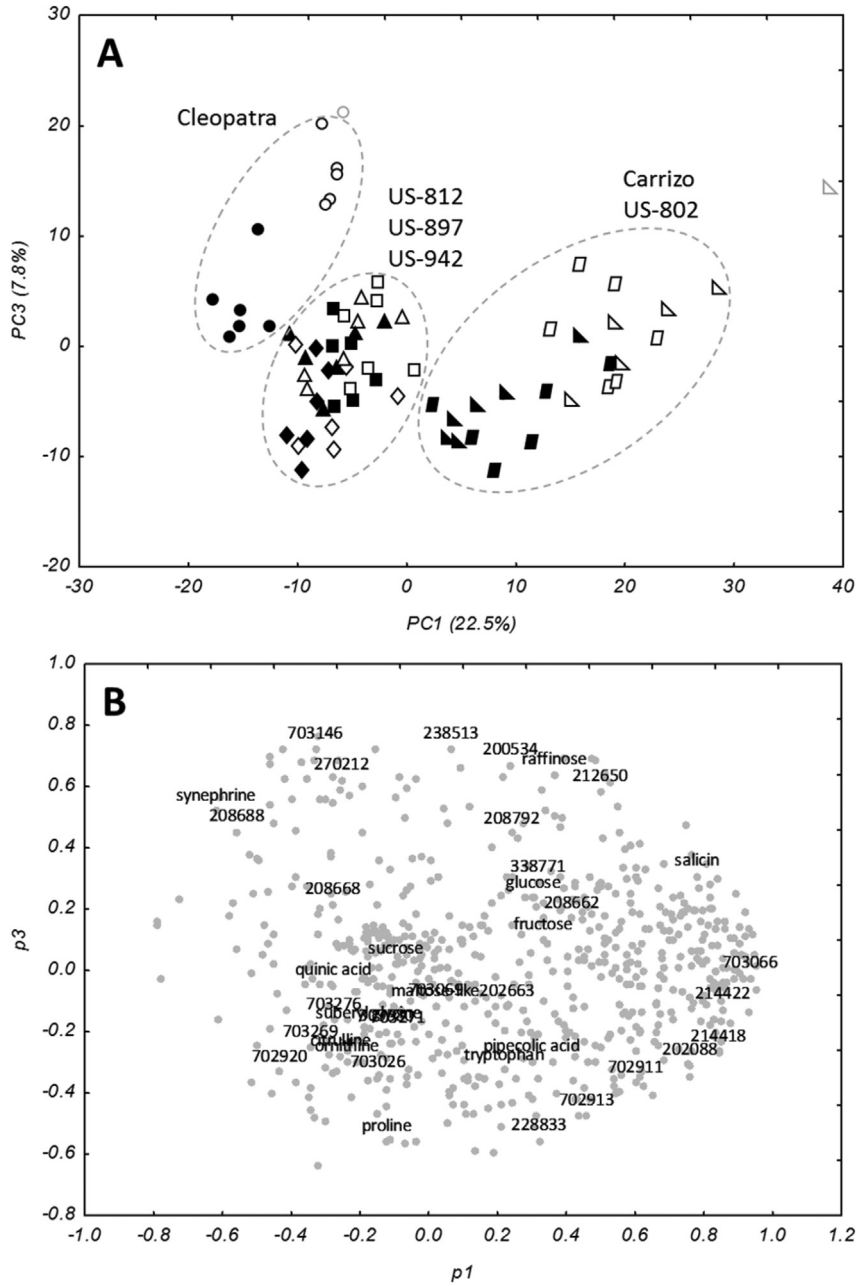

Fig. 2. Principal component analysis (PCA) score plot of leaf GC-TOF MS profiles of six citrus cultivars. A) Score plot of metabolite profiles 12 months after mock-inoculation (no fill) or inoculation with *Ca.* L. asiaticus (black fill). Outliers are marked in gray. *Cleopatra mandarin* (circles), US-812 (squares), US-897 (triangles), US-942 (diamonds), *Carrizo citrange* (right triangles), and US-802 (parallelograms). B) Loading plot depicting selected metabolites mentioned in the text.

separation of non-infected and infected Cleopatra seedlings at 8 and 10 months after inoculation (mai). However, infected Cleopatra samples from the earlier time point separated into two groups. No clear separation of non-infected and infected US-897 plants was observed at either time point. Among the compounds most highly (4–233-fold) induced in Cleopatra at both time points in response to infection with Las were the arginine pathway metabolites ornithine, citrulline, and proline as well as several organic acids (saccharic acid, pyrrole-2-carboxylic acid, maleic acid), amino acids (lysine, tyrosine, glycine) and metabolites of unknown structure (Table 2). Leaf metabolites 4–7-fold reduced in Cleopatra seedlings in response to infection at 10 mai were threonic acid, cysteine, galactinol, raffinose, isothreonic acid, and the unknown compounds 270,212, 268,560, and 225,327. Interestingly, the carbohydrates glucose, fructose, and 1,2-anhydro-myo-inositol were significantly induced by 8–14-fold at 8 mai, but reduced by 3–4-fold at 10 mai. A complete list of metabolites differentially regulated in Cleopatra is presented in Suppl. Table S2. Metabolites in higher abundance (2–3-fold) in US-897 leaves in response to infection with Las at one or both time points after inoculation were the carbohydrates glucose, fructose, and raffinose, as well as 4-hydroxycinnamic acid and the unknown compounds 204,157, 338,812 and 212,279. Compound 318,770 was considerably more abundant (42 fold) in response to infection in US-897 at 10 mai. Only two metabolites (lauric acid and 330,991) were found to be significantly reduced in infected US-897 compared with non-

Table 2
Leaf metabolites significantly ($P < 0.05$) induced at 8 months after inoculation (mai) and at 10 mai in Cleopatra mandarin (Cleo) seedlings in response to infection with *Ca.* L. asiaticus. Only compounds with 4 or more fold abundance are shown. Underlined compounds are significantly more abundant in US-897 seedlings independent of infection with *Ca.* L. asiaticus. Ctrl, mock-inoculated control plants, Las, Las-inoculated infected plants.

	Cleo Ctrl (8 mai)	Cleo Las (8 mai)	Fold difference	Cleo Ctrl (10 mai)	Cleo Las (10 mai)	Fold difference
Known compounds						
ornithine	6967	359,869	51.7	3463	807,023	233.0
citrulline	3102	61,569	19.9	1226	135,047	110.2
biuret	369	3019	8.2	262	6084	23.2
proline	189,732	2835955	14.9	205,407	3120156	15.2
saccharic acid	37,382	334,713	9.0	50,610	762,070	15.1
suberyl glycine	2142	17,974	8.4	2175	27,386	12.6
pyrrole-2-carboxylic acid	1941	22,809	11.8	1514	16,732	11.1
lysine	3181	43,881	13.8	7491	60,925	8.1
3-phosphoglycerate	648	2919	4.5	480	2897	6.0
hexuronic acid	1016	5825	5.7	2484	11,978	4.8
serine	111,208	510,469	4.6	140,808	637,844	4.5
maleic acid	40,187	333,651	8.3	96,617	437,203	4.5
glycine	10,086	65,309	6.5	18,253	72,250	4.0
Unknown compounds						
212,279	269	3312	12.3	157	3523	22.5
289,052	1043	16,367	15.7	852	13,316	15.6
208,850	6722	88,110	13.1	6863	98,959	14.4
280,564	412	4119	10.0	362	4369	12.1
338,818	160	814	5.1	84	916	10.9
289,101	393	3521	9.0	405	3980	9.8
208,874	8665	56,280	6.5	8599	80,861	9.4
299,159	1755	12,326	7.0	1118	9987	8.9
338,812	431	1504	3.5	389	3316	8.5
202,088	710	5630	7.9	799	6315	7.9
211,900	348	1554	4.5	228	1733	7.6
214,418	757	6125	8.1	856	6269	7.3
338,481	584	3381	5.8	535	3656	6.8
214,414	456	3411	7.5	710	4488	6.3
208,661	21,844	122,951	5.6	40,789	226,320	5.5
208,682	1414	7063	5.0	1951	9192	4.7
337,157	14,483	59,514	4.1	36,911	165,164	4.5
310,987	3967	18,953	4.8	7585	31,601	4.2
208,660	20,447	84,062	4.1	45,228	181,427	4.0

infected US-897.

3.2.2. Experiment 2

The number of metabolites that were differentially regulated in response to infection with Las varied considerably between cultivars and was highest in the susceptible cultivar (Cleopatra), followed by Carrizo, US-802, and US-812 (Table 3). Only three metabolites each were differentially regulated in the tolerant cultivars US-897 and US-942. The total number of metabolites that were significantly down-regulated in the six citrus cultivars in response to infection with Las was nearly 4-fold higher than the number of metabolites that were upregulated. Metabolites most highly (3–6-fold) induced in the infected susceptible cultivar Cleopatra were inulobiose, trans-4-hydroxyproline, and proline and several unknown compounds. Among the metabolites most reduced (3–8-fold) in infected Cleopatra leaves were threitol, raffinose, isothreonic acid, salicin, α-ketoglutaric acid, galactinol, glucose, and fructose as well as many compounds with unknown chemical structure. The only metabolite that was found to be in higher abundance in four (Cleopatra, Carrizo, US-802, US-812) of the six cultivars in response to infection, was proline (Table 4). Down-regulated in the same four cultivars were 2-hydroxyglutaric acid, alpha ketoglutaric acid, salicin, and 7 metabolites of unknown chemical structure. A list of all metabolites with different abundance in the non-infected and infected state in all six cultivars is presented in Suppl. Table S3.

3.3. Metabolic variations in different rootstock cultivars with different responses to HLB

3.3.1. Experiment 1

Thirty-six percent of the 342 detected leaf metabolites differed significantly between the two cultivars in the non-infected state at one or both time points. Of the compounds most important for the

Table 3
Number of known and unknown metabolites significantly ($P < 0.05$) up- and down-regulated by 2.0 or more fold in six citrus cultivars 12 months after inoculation with *Ca.* L. asiaticus.

	Up-regulated compounds			Down-regulated compounds			Total regulated compounds
	Known	Unknown	Total	Known	Unknown	Total	
Cleopatra mandarin	5	24	29	39	98	137	166
Carrizo citrange	3	8	11	14	69	83	94
US-812	11	9	20	14	26	40	60
US-802	4	9	13	13	31	44	48
US-897	1	2	3	0	0	0	3
US-942	1	1	2	0	1	1	3

Table 4
Metabolites significantly ($P < 0.05$) up- and down-regulated by 2.0 or more fold and common in three or more citrus cultivars 12 months after inoculation with *Ca.* L. asiaticus (Las).

Differentially regulated compounds common in three or more cultivars
Up-regulated
Cleopatra mandarin, *Carrizo citrange*, *U-812*, *and US-802*:
proline
Cleopatra mandarin, *Carrizo citrange and US-802*:
338,481, 503,549
Carrizo citrange, *US-802 and US-812*:
maltose-like, tryptophan
Down-regulated
Cleopatra mandarin, *Carrizo citrange*, *US-802*, *and US-812*:
2-hydroxyglutaric acid, alpha ketoglutaric acid, salicin, 200,908, 202,737, 296,071, 428,788, 607,446, 703,113, 703,134
Carrizo citrange, *US-802*, *and US-812*:
199,275, 487,274
Cleopatra mandarin, *Carrizo citrange*, *and US-812*:
1,2-anhydro-myo-inositol NIST, raffinose, 200961
Cleopatra mandarin, *Carrizo citrange*, *and US-802*:
Succinic acid, 199,216, 200,420, 200,534, 214,680, 299,828, 703,040, 703,114
Cleopatra mandarin, *US-802 and US-812*:
537,761

separation of the two cultivars, palatinose, citric acid, and the unknown compounds 309,580, 309,738, 338,667, 338,465, and 228,833 were 5–278-fold more abundant in tolerant US-897 compared with susceptible Cleopatra at both time points

Table 5
Leaf metabolites with significantly ($P < 0.05$) higher abundance in US-897 seedlings compared with Cleopatra (Cleo) seedlings. Underlined compounds are significantly induced in Cleopatra seedlings in response to infection with *Ca.* L. asiaticus. Ctrl, mock-inoculated control plants. Only compounds with two- or more fold differences between the cultivars at 8 and 10 months after inoculation (mai) are shown.

	Cleo Ctrl (8 mai)	US-897 Ctrl (8 mai)	Fold diffe rence	Cleo Ctrl (10 mai)	US-897 Ctrl (10 mai)	Fold diffe rence
Known compounds						
palatinose	490	9000	18.4	567	12,834	22.6
citric acid	155,190	1102387	7.1	134,815	773,534	5.7
3-phosphoglycerate	648	2527	3.9	480	1951	4.1
serine	111,208	548,913	4.9	140,808	497,951	3.5
inositol allo-	63,266	603,146	9.5	303,192	1017399	3.4
saccharic acid	37,382	247,549	6.6	50,610	145,664	2.9
pyrrole-2-carboxylic acid	1941	5358	2.8	1514	4225	2.8
rhamnose	2359	6767	2.9	3852	9136	2.4
nicotianamine	1609	6088	3.8	3027	6975	2.3
galactonic acid	11,146	31,250	2.8	23,569	52,624	2.2
fumaric acid	2445	5588	2.3	5568	11,910	2.1
2-ketoglucose dimethylacetal NIST	1081	3660	3.4	3669	7374	2.0
isorhamnose	344	693	2.0	409	818	2.0
Unknown compounds						
309,580	614	44,548	72.5	401	111,371	278.0
309,738	322	12,842	39.9	151	31,143	206.5
338,667	614	7626	12.4	2179	17,243	7.9
338,465	4608	62,415	13.5	12,963	98,195	7.6
228,833	291	1479	5.1	248	1800	7.3
309,866	197	637	3.2	107	761	7.1
208,682	1414	12,534	8.9	1951	10,090	5.2
208,702	1784	4244	2.4	1307	6661	5.1
202,088	710	5082	7.2	799	3692	4.6
214,418	757	5329	7.0	856	3720	4.3
208,850	6722	38,077	5.7	6863	29,676	4.3
208,874	8665	25,925	3.0	8599	29,816	3.5
202,071	1402	10,910	7.8	2053	6674	3.3
338,467	2711	12,930	4.8	15,134	41,958	2.8
215,066	424	2048	4.8	631	1715	2.7
337,159	10,516	45,721	4.3	22,754	61,208	2.7
208,871	2162	8031	3.7	2914	7674	2.6
202,663	420	1880	4.5	1224	2835	2.3
202,832	3390	16,837	5.0	9401	21,008	2.2
202,885	692	2488	3.6	1696	3777	2.2
201,009	8749	38,781	4.4	20,103	44,279	2.2
199,205	831	3673	4.4	2907	6339	2.2
211,896	12,058	36,414	3.0	34,354	74,576	2.2
268,610	1511	16,874	11.2	8767	18,672	2.1
231,576	862	5092	5.9	3923	8232	2.1
289,101	393	858	2.2	405	823	2.0
208,841	8310	33,107	4.0	23,752	47,407	2.0

(Table 5). Interestingly, many of the metabolites found in higher concentrations in US-897 were also significantly upregulated in Cleopatra in response to infection. Results showed a 2–21-fold lower abundance in US-897 compared with Cleopatra at both time points for synephrine, galactinol, and raffinose, and the unknown compounds 338,607, 338,492, 210,468, 337,151, 337,197, 338,773, and 213,301. Among the metabolites with at least 5-fold lower abundance in US-897 at only one time point (10 mai), were glucose, fructose, and the unknown compounds 338,512, 270,212, and 335,365.

3.3.2. Experiment 2

Partial least squares (PLS) analysis comprising all 650 detected metabolites was used to identify the variables responsible for distinguishing the six different citrus rootstock cultivars from experiment 2 and their levels of tolerance or susceptibility to HLB. Contrary to PCA, PLS analysis is a supervised dimension reduction methodology that takes into account the correlation between the dependent variable and the independent variable. PLS analysis according to cultivars in the non-infected state resulted in 36.1% of dependent variation (cultivars) explained by 37.7% of the predictors (metabolites). Analysis of samples in the infected state resulted in 34.1% of cultivar variation explained by 35.0% of metabolite variation, however, only the first component chosen by cross-validation was significant. The PLS score plots (Fig. 3A and C) shows a clear separation of cultivars into three groups: one group with Cleopatra samples, a second group containing US-812, US-897, and US-942 samples, and a third group containing the samples from US-802 and Carrizo. The top 30 metabolites extracted based on variable importance in projection (VIP) values are depicted in the loading plots (Fig. 3B and D). Only three compounds of unknown chemical structure (208649, 411,030, and 702,926) were common in the non-infected and in the infected state.

PLS analysis according to response (susceptible, moderately tolerant, or tolerant) to HLB resulted in 72.4% of dependent variation explained by 29.7% of metabolite variation under mock-inoculated conditions and in 76.0% of dependent variation explained by 30.6% of metabolite variation under infected conditions. The PLS score plots (Fig. 4A and C) show a clear grouping of samples from the susceptible cultivar Cleopatra, whereas grouping is not as well defined between the moderately tolerant cultivars (US-802 and US-812) and the tolerant cultivars (US-897, US-942, and Carrizo). The top 30 metabolites extracted based on VIP values are depicted in the loading plots (Fig. 4B and D). Only

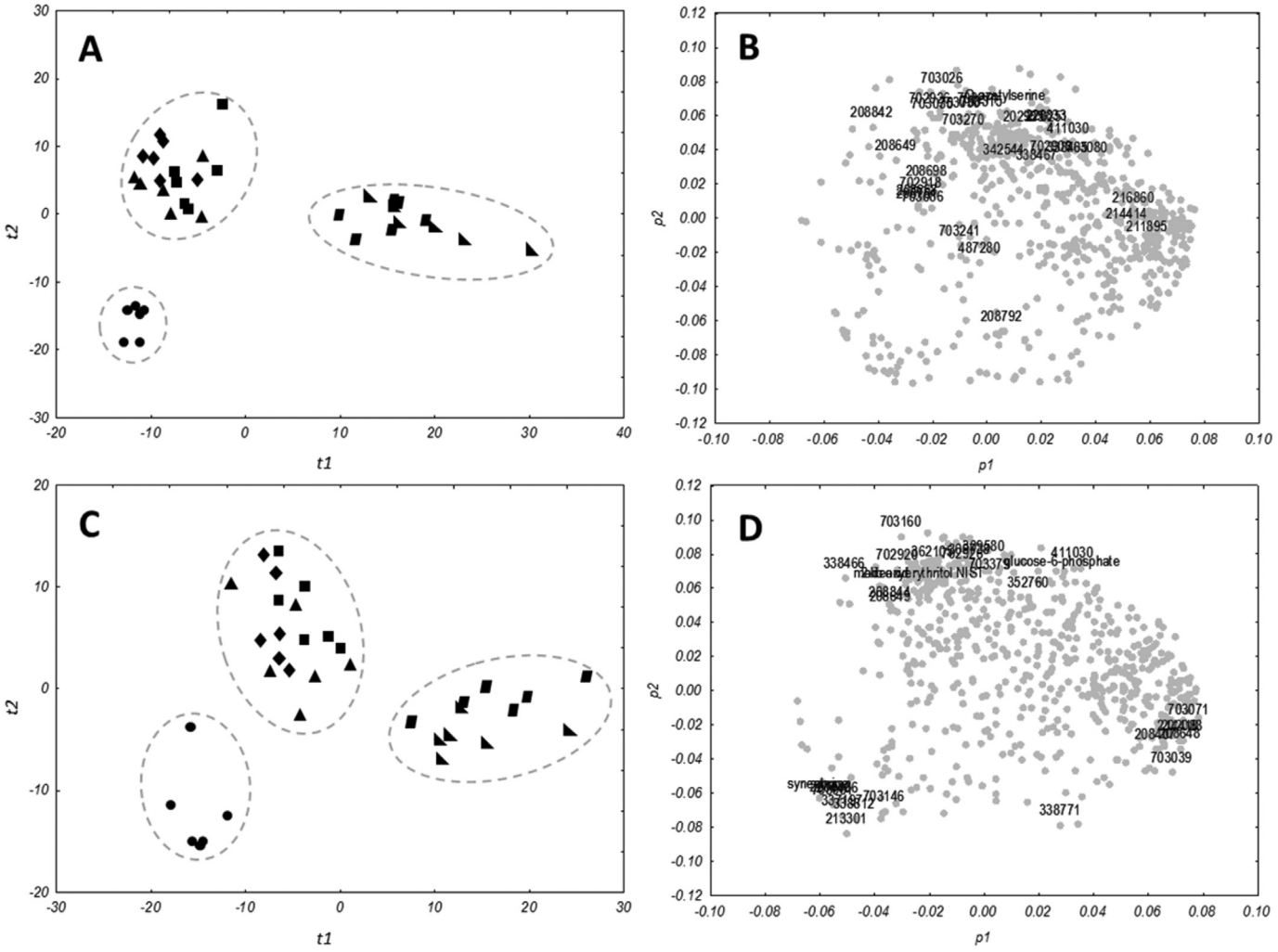

Fig. 3. Partial least squares analysis (PLS) of leaf GC-MS profiles of citrus seedlings according to cultivar. (**A**) Score plot and (**B**) loading plot of leaf GC-MS profiles 12 months after mock-inoculation. (**C**) Score plot and (**D**) loading plot of leaf GC-MS profiles 12 months after inoculation with *Ca.* L. asiaticus. The top 30 metabolites responsible for separation of cultivars are marked in the loading plots. Cleopatra mandarin (circles), US-812 (squares), US-897 (triangles), US-942 (diamonds), and Carrizo citrange (right triangles), US-802 (parallelograms).

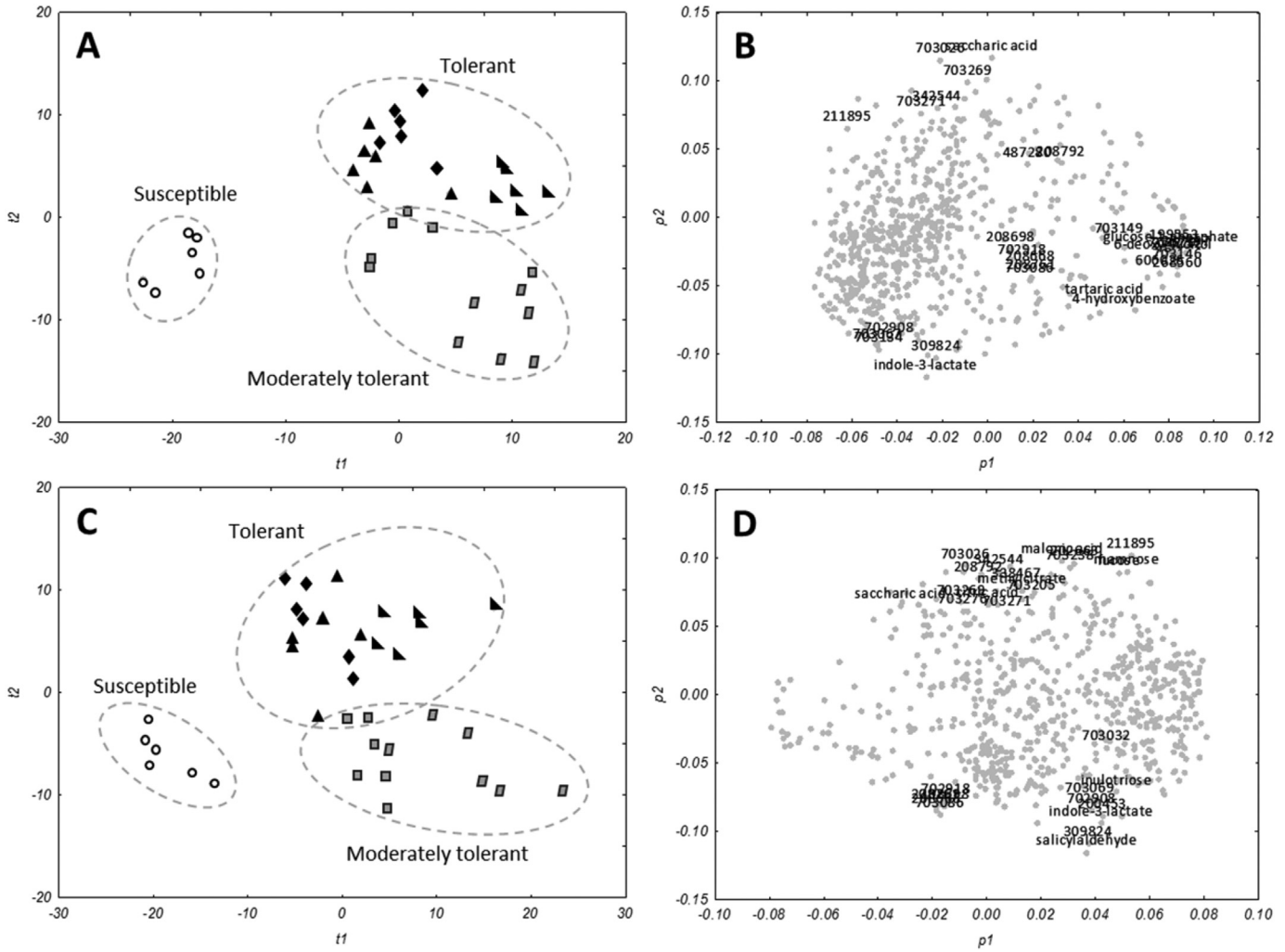

Fig. 4. Partial least squares analysis (PLS) of leaf GC-MS profiles of citrus seedlings according to response to HLB. (**A**) Score plot and (**B**) loading plot of leaf GC-MS profiles 12 months after mock-inoculation. (**C**) Score plot and (**D**) loading plot of leaf GC-MS profiles 12 months after inoculation with *Ca.* L. asiaticus. The top 30 metabolites responsible for separation of susceptible, moderately tolerant, and tolerant plants are marked in the loading plots. Susceptible (no fill): Cleopatra mandarin (circles), moderately tolerant (gray fill): US-812 (squares) and US-802 (parallelograms), and tolerant (black fill): US-897 (triangles), US-942 (diamonds), and Carrizo citrange (right triangles).

compounds of unknown chemical structure (208,668, 208,698, 208,761, 208,792, 211,895, 309,824, 342,544, 702,908, 702,918, 703,026, 703,086, 703,269, and 703,271) were common in the non-infected and in the infected state. A list of all metabolites detected in the six rootstock cultivars including signal intensities and VIP values is presented in Suppl. Table S4. Many of the metabolites with unknown chemical structure were considerably higher in abundance in the tolerant cultivars compared with Cleopatra, such as the compounds 309,738, 342,544, 703,026, 702,907, and 309,580. Other metabolites were found in much lower concentrations in the tolerant cultivars and include the unknowns 702,965, 703,146, 268,560, and several sugars, such as raffinose, fructose, inulotriose, glucose-1-phosphate, and glucose. A complete list of fold differences can be found in Suppl. Table S5.

4. Discussion

Use of rootstocks with disease resistance or tolerance to HLB would be a valuable tool to sustain citrus production in areas affected by this destructive disease. In previous studies, several rootstock varieties were identified that respond differently in the presence of the pathogen associated with HLB (Albrecht and Bowman, 2011 and 2012a; Folimonova et al., 2009). This study sought to identify metabolic profiles that are not only associated with disease response, but that are also associated with disease tolerance.

4.1. Metabolic response to infection with Las

Using untargeted GC-TOF MS analysis, we identified 342 unique leaf metabolites in our first experiment involving a susceptible (Cleopatra) and a tolerant (US-897) rootstock cultivar. Among the 36% of metabolites identified by chemical structure were the arginine pathway metabolites ornithine, citrulline and proline, which were found in much higher abundance in the susceptible cultivar in response to infection and which contributed much of the variation observed by PCA analysis. These compounds are often reported to be associated with the response of plants to different types of abiotic and biotic stress (Alcázar et al., 2010; Mollayi et al., 2015). As components of arginine metabolism, it is also very likely that they are involved in the nitric oxide signaling pathway (Winter et al., 2015). In addition to its role as osmoprotectant and antioxidant, increasing evidence points to proline as an important signaling molecule and regulator of plant development, and it has been

suggested that engineering of proline metabolism may improve plant tolerance to environmental stresses (Szabados and Savouré, 2010). Studies on HLB-affected sweet orange and grapefruit plants (Cevallos-Cevallos et al., 2012) and on witches' broom disease-affected Mexican lime plants (Mollayi et al., 2015) also revealed significant increases in proline concentrations in response to infection with Las or *Ca*. Phytoplasma aurantifolia, respectively. No significant variation in proline content was observed in symptomatic Las-infected sweet orange leaves in a study by Freitas et al. (2015) and proline betaine content was decreased in response to infection. The reason for the differing results of this study in comparison with other studies on HLB is unclear.

Besides proline, other amino acids and several organic acids were found in higher concentrations in infected Cleopatra leaves compared with healthy leaves. Variable roles have been attributed to amino acids and organic acids in plant response to stress, which range from osmotic stress protection to regulation of ion transport and detoxification of heavy metals (Rai, 2002). Organic acids specifically have been implicated in the enhanced uptake of soil nutrients by plants (Ryan et al., 2001). It is well established that HLB-affected citrus plants suffer from nutritional depletion, and nutritional applications are among the management strategies used to sustain citrus production in affected areas (Stansly et al., 2014). The increase in amino acid and organic acid concentrations observed in the susceptible citrus cultivar under Las infection may be associated with an attempt to enhance nutrient uptake in response to disease. Further studies involving root metabolic profiles of citrus are currently under way in our laboratory. It has also been suggested that specific amino acids are involved in plant defense (Rojas et al., 2014), since metabolite profiling showed that, dependent on the host-pathogen system, some amino acids increase in concentration whilst others decrease in the diseased state (Buhtz et al., 2015).

Several carbohydrates, including glucose and fructose, were found in higher concentrations in infected plants compared with non-infected plants in Cleopatra as well as in US-897, although concentrations were reduced at the later stage of infection in the former. Cevallos-Cevallos et al. (2011) found significantly higher concentrations of fructose in HLB-affected sweet orange leaves, but did not detect significant differences in glucose content. The effects of HLB on carbohydrate metabolism are well described (Albrecht and Bowman, 2008; Fan et al., 2010; Kim et al., 2009) and are in accordance with observations in other plant-pathosystems involving phloem-limited microorganisms such as phytoplasmas and spiroplasmas (André et al., 2005; Renaudin, 2006). Since sugar accumulation was shown to activate pathogenesis-related (PR) proteins and their genes, it was proposed that sugars act as amplifiers for plant defense responses during the interaction with a pathogen and are important components of plant immunity (Rojas et al., 2014; Trouvelot et al., 2014).

In our second study, which compared metabolic profiles of six citrus cultivars with different sensitivity to HLB, we detected 650 metabolites, of which 30% were identified by chemical structure. Compared with the first study, this second study revealed a much larger proportion of leaf metabolites that decreased in abundance in the infected state and which included the carbohydrates glucose, fructose, and raffinose, as well as several organic acids. The only compound that was found to be increased in infected plants of all cultivars, except US-897 and US-942, was proline. The difference in metabolic response to infection between the two studies is likely the result of the different stage of disease development in the two studies along with different environmental conditions or plant developmental stage. We suggest that source-sink relationships of leaves at the time of infection with Las are of great importance in determining their metabolic response and the type of disease symptom (chlorosis or blotchy mottle) they display. Differences in metabolic profiles depending on the developmental stage of the leaf were also found by Cevallos-Cevallos et al. (2012) in HLB-sensitive sweet orange and grapefruit plants. Among the compounds significantly induced in these cultivars in response to infection were proline, serine, threonine, hexadecanoic acid, scyllo-inositol, and mannose. In an earlier study, Cevallos-Cevallos et al. (2011) detected a combination of biomarkers able to distinguish HLB affected leaves from leaves with zinc-deficiency symptoms. One of the identified potential biomarkers for HLB in that study was proline. Using HPLC-MS, Hijaz and Killini (2012) compared secondary metabolites in leaves from non-infected and Las-infected Valencia and Hamlin plants. Although a number of metabolites were significantly affected by HLB, responses were not consistent and the authors suggested the uneven spread of Las and disease symptom development as a possible cause. Studies on HLB-affected citrus fruit also revealed significant differences in the concentrations of sugars, amino acids and other classes of metabolites (Slisz et al., 2012). Contrary to our observations on leaves, proline concentrations were significantly reduced in infected fruit compared with healthy fruit. In addition, Chin et al. (2014) found decreased concentrations of the amino acids phenylalanine, histidine, and asparagine, and other metabolites in infected symptomatic sweet orange fruit compared with non-symptomatic and healthy fruit. The reverse effect of Las in leaves and fruit has also been observed in studies on citrus involving transcriptomics and other methodologies (Martinelli et al., 2012; Rosales and Burns, 2011) and is likely associated with the disruption of phloem transport due to HLB (Achor et al., 2010; Kim et al., 2009) in combination with differences in source and sink status of these different organs.

Few metabolites were found to be differentially regulated in response to Las infection in the tolerant rootstock cultivars US-897 and US-942, which is in accordance with the absence of disease symptoms observed for these cultivars. Similarly, Cevallos-Cevallos et al. (2012) did not find any significant differences of metabolic profiles for infected and non-infected HLB-tolerant *P. trifoliata*. However, contrary to our results, the study also found no differences between the metabolic profiles of infected and non-infected Carrizo citrange. It appears that tolerance to HLB is not primarily associated with the accumulation of higher amounts of protective metabolites in response to infection in the cultivars used for this study.

4.2. Metabolic variations in different rootstock cultivars with different levels of tolerance or susceptibility to HLB

Many studies have demonstrated the suitability of metabolic profiling for identifying different plant cultivars and include rice (Hu et al., 2013), soybean (Lin et al., 2014), and citrus (Chin et al., 2014). In the present study PCA and PLS analysis showed a clear separation of the six citrus cultivars into three groups which appears to be largely associated with the genetic background of the cultivars. One group included all samples from US-812, US-897, and US-942, rootstock cultivars which originated from crosses of mandarin and trifoliate orange. Samples from the mandarin cultivar Cleopatra used in this study form a separate group. Interestingly, samples from US-802 and Carrizo were found to form a third group, which separated from the other hybrids of trifoliate orange. The grouping of US-802, a pummelo × trifoliate hybrid, together with Carrizo citrange, a sweet orange × trifoliate hybrid, may be associated with the genetic influence of pummelo and the common assumption of sweet orange having arisen from introgression of pummelo genes into a mandarin genotype (Barrett and Rhodes, 1976). It is apparent that the genetic background plays a large role in the metabolic variation observed in this study.

Fewer studies investigated whether cultivars can be

discriminated based on tolerance or susceptibility to diseases. Ali et al. (2009) was able to distinguish grapevine cultivars based on their resistance to downy mildew infection and identified quercetin-3-O-glucoside and a trans-feruloyl derivative as the metabolites associated with this trait. Similarly, leaf metabolic profiling clearly differentiated between mango cultivars with different responses to *Fusarium* infection(Augustyn et al., 2014). To our knowledge, only one study investigated metabolite profiles on different citrus cultivars and assessed their correlation with HLB resistance (Cevallos-Cevallos et al., 2012). Using GC-MS, this study analyzed four citrus varieties (sweet orange, grapefruit, trifoliate orange, and Carrizo citrange) and revealed leaf metabolic profiles that appeared to be associated with different degrees of tolerance to HLB. Whereas the most sensitive variety Madam Vinous sweet orange was characterized by high levels of proline, serine, aspartic acid, butanedioic acid, tetradecanoic acid, and galactose, higher levels of glycine and mannose were suggested to be possible components of HLB tolerance.

Previous studies in our laboratory (Albrecht and Bowman, 2012b) identified the cultivars US-897, US-942, and Carrizo as the most tolerant of the six cultivars used in the present study. Similarly, Folimonova et al. (2009) characterized Carrizo citrange as tolerant to HLB but found inconsistent results for *P. trifoliata*. PLS analysis based on tolerance revealed a clear grouping of samples from the susceptible cultivar Cleopatra, whereas the moderately tolerant and tolerant cultivars did not clearly separate from each other. T-tests showed many unidentified metabolites to be in considerable higher concentrations in some of the tolerant cultivars, especially US-897 and US-942, compared with Cleopatra in the non-infected healthy state. These compounds may play important roles in conferring tolerance to HLB and will be very valuable for selection of superior rootstock candidates in breeding programs.

Duan et al. (2009), who unraveled the complete genome structure of Las, found that the pathogen does not produces toxins, enzymes or specialized secretion systems, and suggested that Las is parasitic rather than pathogenic, with disease symptoms arising primarily as a result of host metabolic imbalances caused by nutrient depletion or interference of transportation. Interestingly, our study found lower concentrations of raffinose, fructose, and glucose in the HLB-tolerant cultivars independent of infection. These carbohydrates were also reduced by infection in those cultivars that expressed foliar disease symptoms. Fan et al. (2010) found higher accumulations of glucose and sucrose, but not of fructose in HLB-symptomatic sweet orange plants, in addition to much reduced levels of mannose. Results from research on the pathogenic mechanisms of a different group of phloem-limited pathogens, the cultivable spiroplasmas, suggest that they cause disease symptoms by depleting the phloem of specific sugar molecules (Firrao et al., 2007). Fructose depletion was identified as the cause of yellow symptoms in periwinkle infected by *Spiroplasma citri* (Gaurivaud et al., 2000). It may be speculated that nutrient depletion in citrus as a result of infection with Las may be associated with the utilization of specific carbohydrates, thus altering sugar balances in the plants. This would also explain the reduced sugar concentrations found for Cleopatra at the later stage of disease in experiment 1. Since sugar molecules play an important role in long-distance signaling and regulation of gene expression (Liu et al., 2009), such imbalances are likely to cause physiological disorders and disease symptoms in affected plants. Based on the presence of genes for the key enzymes 6-phosphofructokinase and phosphoglucomutase in the Las genome, glycolysis appears to be the major pathway for the catabolism of monosaccharides in Las (Duan et al., 2009). Thus, it is possible that tolerance to HLB is at least partially associated with a lesser availability of specific carbohydrates in the host.

5. Conclusions

Metabolic profiling of leaves identified many metabolites that responded to infection of citrus with Las, although responses were low in the tolerant cultivars US-897 and US-942, which were visually not affected by disease. Metabolic profiles allowed the separation of citrus cultivars based on their level of tolerance to HLB, and were largely influenced by their genetic background. Tolerance to HLB did not appear to be associated with the accumulation of higher amounts of protective metabolites in response to infection, but rather with different concentrations of specific metabolites independent of infection. The large proportion of chemically unknown metabolites detected in this study not only provides ample opportunity for the discovery of new compounds associated with the citrus-HLB complex, but will also be valuable for the selection of new rootstock candidates prior to the long term evaluation in the field.

Author's contribution

KDB provided resources and obtained grant funding to support the research. UA and KDB designed and conducted the experiments and performed Las detections and metabolite extractions. OF conducted the metabolite analysis and data processing. UA analyzed the data and wrote the manuscript. All authors read and approved the final manuscript for its publication in Plant Physiology and Biochemistry Journal.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.05.030

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