



Research article

All roads lead to Rome: Towards understanding different avenues of tolerance to huanglongbing in citrus cultivars

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ABSTRACT

Citrus tolerance to huanglongbing could result from tolerance to the pathogen *Candidatus Liberibacter asiaticus* (CLas) and/or to its vector *Diaphorina citri*. Field observations and greenhouse-controlled studies showed that some citrus cultivars were more tolerant than others. However, the mechanism(s) behind the tolerance has not been determined yet. Using GC-MS, we investigated the volatile organic compounds (VOCs) and the non-volatile metabolite profiles of two tolerant citrus cultivars- Australian finger lime, 'LB8-9' Sugar Belle[®] mandarin hybrid, and a recently released mandarin hybrid 'Bingo'. The three were grafted onto the rootstock, *Carrizo citrange*. Our findings showed that the metabolomic profiles of Australian finger lime were different from that of 'LB8-9'. Finger lime was high in many amino acids and tricarboxylic acid intermediates, whereas 'LB8-9' was high in several amino acids, sugars, and sugar alcohols. 'LB8-9' was high in thymol, which is known for its strong antimicrobial activity against a panel of pathogenic bacteria. The metabolomic profiles of 'Bingo' were intensely different from the other mandarin hybrid, 'LB8-9', including a reduced thymol biosynthetic pathway and low amounts of most of the amino acids and sugar alcohols. Remarkably, 1,8-cineole (eucalyptol) was only detected in 'Bingo', indicating that eucalyptol could have feeding and ovipositional repellency against *D. citri*. The metabolite profiles generated for HLB-tolerant citrus species will improve the ability of citrus breeders and will allow them to take more informed decisions. Metabolomic profiling of HLB-tolerant citrus species could identify tolerance specific markers that can be introduced to other commercial citrus cultivars to improve their tolerance to HLB disease.

1. Introduction

Citrus huanglongbing disease (HLB), also called citrus greening, has decimated many of the world's citrus industries in recent years. In Florida, the disease is caused by *Candidatus Liberibacter asiaticus*, a fastidious gram-negative phloem-limited bacterium transmitted by a tiny Hemipteran insect, the Asian citrus psyllid, *Diaphorina citri* (Garnier et al., 2000; Jagoueix et al., 1996). The bacterium resides and multiplies in the gut and salivary glands of the psyllid vector (Ammar et al., 2011). Psyllids inject the bacteria into host plants through their mouthparts during feeding. Most often, *D. citri* acquires the bacterium as nymphs while feeding on the phloem sap of infected plants (Ammar et al., 2016). After reaching maturity, adults can fly up to 2 km in search of new host plants for feeding and reproduction (Lewis-Rosenblum et al., 2015). Once inoculated into the host tree, the bacteria accumulate in the sieve elements (Achor et al., 2010), consume the

supply of nutrients present in the phloem sap (Killiny, 2017), and initiate a cascade of host plant responses which lead to phloem plugging, starch accumulation, and leaf chlorosis (Achor et al., 2010), root loss (Johnson et al., 2014), and reduced yield due to small and lopsided fruit unfit for processing (Gottwald, 2010). Most of citrus cultivars are susceptible to this bacterium and once infected trees show a slow decline in production and often eventually die within 5–10 years (Grafton-Cardwell et al., 2013; Halbert and Manjunath, 2004).

Because the citrus industry has relied heavily on the use of chemical insecticides to combat the insect vector, increasing reports of insecticidal resistance within the Florida psyllid population have appeared (Tiwari et al., 2011). Consequently, citrus growers are in desperate need of alternative strategies such as new, more HLB-tolerant citrus varieties. Because transgenic approaches are not well accepted by consumers, breeding programs remain the best option for the current economic situation. Increasing the genetic diversity through citrus

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breeding programs has the potential to introduce favorable traits such as disease resistance and reduce the risks associated with monoculture.

Although there are currently no commercial citrus cultivars with strong tolerance to CLAs, greenhouse studies and field observations showed that some citrus species are more tolerant to CLAs bacteria than others. Citrus accessions which show some tolerance to HLB have been reported (Albrecht and Bowman, 2012; Cevallos-Cevallos et al., 2012; Folimonova et al., 2009). In a broad study of potentially tolerant hybrids, a 'LB8-9/Sour orange' scion/rootstock combination maintained a healthy appearance and good yield over the five-year study (Stover et al., 2016). Recently, we studied in detail the volatile and non-volatile leaf metabolite profiles of 'LB8-9' Sugar Belle[®] mandarin hybrid and its closest relatives, 'Dancy' tangerine, 'Minneola' tangelo, 'Duncan' grapefruit, and 'Clementine' mandarin in order to identify compounds related to HLB tolerance (Killiny et al., 2017). In that study, VOCs such as thymol, *p*-cymene, and γ -terpinene were identified as compounds specific to 'LB8-9' which might confer antibacterial properties. In fact, previous studies showed that thymol has a strong antimicrobial activity against a panel of pathogenic bacteria (Du et al., 2015). Phenolic compounds present included benzoic acid, ferulic acid, caffeic acid, and synephrine, and many of these have been implicated in plant response to biotic stresses (Blodgett and Stanosz, 1998; Wallis and Chen, 2012; Wilhelm et al., 2011).

In this follow-up study, we examined the volatile and non-volatile metabolite profiles of 'LB8-9', 'Bingo', and 'Australian Finger Lime' grafted onto 'Carrizo' citrange rootstock. 'Carrizo' citrange is a hybrid of 'Washington' navel orange \times trifoliolate orange (Castle et al., 2016). 'Carrizo' has been in use in Florida since the 1930s (Castle et al., 2016). Its use as a rootstock in Florida is preferred in areas with poorly draining soil because of its tolerance to *Phytophthora* root rot, but it does not perform well under high pH or high salinity conditions (Castle et al., 2016). New evidence suggests that a close citrus relative, 'Australian finger lime' *Microcitrus australasica* (F. Muell.) Swingle showed potential HLB tolerance as CLAs titers were low for the six years of the study and it was a poor host for *D. citri* (Ramadugu et al., 2016). 'Bingo' mandarin hybrid is a relatively new hybrid consisting of 'Clementine' mandarin \times 'Valencia' sweet orange as female parent and 'Seedless Kishu' as male parent (Gmitter, 2015). The original seedling 'Bingo' tree was symptomless and continued to test negative for CLAs by polymerase chain reaction (PCR) assays through the first 9 years in the field. However, in the 10th year symptoms were observed in leaves and fruit, so the cultivar is apparently not resistant to CLAs. Its long term tolerance and ability to continue good performance under infection remains to be determined. (Gmitter, 2015). Recently, 'Bingo' has become widely available to growers, and several thousand trees have been planted in Florida.

Plants produce a wide variety of complex organic molecules as defenses against bacterial and fungal pathogens (Giamperi et al., 2002; Marchese et al., 2016), animal and insect herbivory (Bennett and Wallsgrove, 1994; Hijaz et al., 2013), and abiotic stresses such as drought or high salinity, and sometimes through interaction of both biotic and abiotic events (Nguyen et al., 2016). Some chemical defense compounds are formed as saps, toxic alkaloids, and phytohormones through their organs (glands, trichomes, leaves, stems, fruit, flowers, etc.), while others may be released into the air to attractant beneficial insects or other pollinators (Bennett and Wallsgrove, 1994). Volatile organic compounds (VOCs) such as terpenes can be easily extracted through use of organic solvents (i.e. hexane, chloroform) while water-soluble metabolites (organic acids, sugars) can be extracted using solvents blended with water. Analysis of these molecules, which are often metabolic pathway intermediates, can help identify subtle changes in complex metabolic systems and can be used comparatively. These studies can be achieved through a variety of analytical tools including liquid and gas chromatography (GC), as well as by using different chemical derivatization techniques. Each method has its own advantages and disadvantages, and may be optimal for some compounds and not

others. Using multiple techniques on the same tissue is advantageous in that a more complete picture of the underlying biochemistry can be obtained. Therefore, in this study we chose to extract both the volatile (using hexane extraction) and non-volatile metabolites (using a mixed organic solvent and derivatization) from two aliquots of the same leaf tissue samples. Gas chromatography-mass spectrometry (GC-MS) analysis provided both quantification and identification of compounds from among the four studied cultivars.

In this study, we investigated the volatile and nonvolatile metabolites of finger lime, 'Bingo', 'Sugar Belle' mandarin, and their rootstock 'Carrizo' in order to test if there is a relationship between these metabolites and citrus tolerance to CLAs and *D. citri*. In our previous study of 'LB8-9' Sugar Belle[®] mandarin hybrid, tolerance was associated with an overall higher level of extracted VOC compounds than the other cultivars examined (Killiny et al., 2017) as well as the presence of specific antimicrobial compounds. We also expected that the chemical profile of 'Bingo' mandarin hybrid to be closer to 'LB8-9' than finger lime due to its mandarin heritage. Furthermore, by comparing 'Carrizo' citrange rootstock VOC and metabolite profiles to that of the scion/rootstock combinations, specific compounds passed from rootstock to scion may be revealed which may or may not be involved in conferring tolerance to the scion.

2. Material and methods

2.1. Plant material

Healthy one-year old trees of 'Carrizo' citrange trifoliolate hybrid [*Citrus sinensis* (L.) Osb. 'Washington' \times *Poncirus trifoliata* (L.) Raf.], *Microcitrus australasica* (F. Muell.) Swingle 'Australian finger lime' trees and, two mandarin hybrids, 'LB8-9' Sugar Belle[®] ['Clementine' mandarin (*Citrus reticulata*) \times 'Minneola' tangelo] and 'Bingo' [*Citrus reticulata* hybrid \times 'Seedless Kishu' mandarin (*Citrus kinokuni* 'Mukakukishu')] previously grafted onto 'Carrizo' citrange six months earlier were used this study. All trees were maintained together in a climate controlled growth chamber (16 h:8 h light:dark photoperiod, with $27 \pm 2^\circ\text{C}$ and $70 \pm 5\%$ RH) prior to sampling. Trees were randomly placed in the greenhouse. Trees were watered three times per week and fertilized monthly with 20:10:20 NPK water soluble fertilizer. Five trees of each type were sampled. Five leaves were harvested from each cultivar on the same day and were frozen at -80°C overnight before analyses. VOC and metabolites content were extracted from the same biological samples. Samples were analyzed in duplicate [5 replicates \times 2 injections] for each type of analysis. Foliar morphology and HLB-tolerance of the cultivars used in this study are presented in Fig. 1 and Table 1. Note the small leaf surface area and the presence of large thorns in finger lime compared to the mandarins, 'LB8-9' and 'Bingo'.

2.2. VOC and metabolite extractions

Leaves were frozen in liquid nitrogen and homogenized using a Tissuelyzer II (Qiagen). Two aliquots of ~ 0.1 g leaf tissue were taken from each sample for separate analyses. Leaf volatile organic compounds (VOCs) were extracted from the first tissue aliquot using *n*-hexane exactly as reported in Killiny et al. (2017). Similarly, leaf polar metabolites were extracted from the second aliquot of 0.1 g leaf tissue according to Killiny et al. (2017) except that the extraction solvent was methanol:chloroform:water (8:1:1 v/v/v) and took place at 6°C overnight on a lab rotator. It should be noted that due to the small leaf size for finger lime and 'Carrizo' trifoliolate leaves, whole leaves including the petioles were used for both VOC and metabolite extractions, whereas for larger leaves of 'LB8-9' and 'Bingo' only the middle section of the leaf blade was used for extraction (avoiding the tip and the petiole). All sample sizes were $\sim 0.1 \pm 0.05$ g fresh weight.

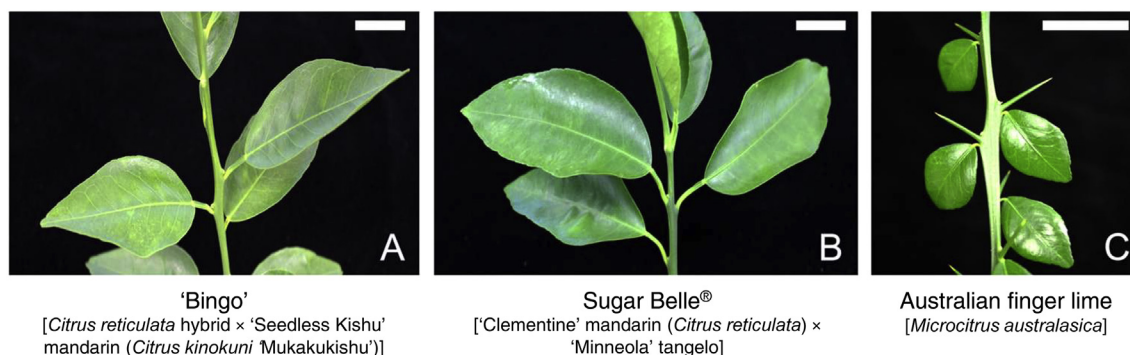


Fig. 1. Foliar morphology of 'Bingo' mandarin hybrid (A), 'LB8-9' Sugar Belle[®] mandarin hybrid (B) and *Microcitrus australasica* (Australian finger lime) (C). The white bar represents 1 cm in length for scale.

Table 1

Citrus cultivars used in this study, their tolerance to Huanglongbing disease (HLB), and their preference by *Diaphorina citri*.

Cultivar	Genotype	Phenotype	HLB susceptibility ^a	<i>D. citri</i> preference	Reference
'LB8-9' Sugar Belle [®] Mandarin	'Clementine' mandarin (<i>Citrus reticulata</i>) × 'Minneola' tangelo	Wide leaf blade and no thorns	Tolerant	Good host	Stover et al., 2016 Killiny et al., 2017 Patent U.S. PP21,356
'Bingo' Mandarin	<i>Citrus reticulata</i> hybrid × <i>Citrus kinokuni</i> 'Mukakukishu'	Wide leaf blade and no thorns	Not confirmed at this time	Not confirmed at this time	Gmitter, 2015 Patent US PP27,778
Finger lime	<i>Microcitrus australasica</i>	Very narrow leaf blade and long thorns	Tolerant	Poor host	Ramadugu et al., 2016 Westbrook et al., 2011
'Carrizo' Rootstock	<i>Citrus sinensis</i> (L.) Osb. × <i>Poncirus trifoliata</i> (L.) Raf.	Trifoliolate narrow leaves and moderate thorns	Tolerant	Poor host, but not tolerant compared to trifoliolate	Hall et al., 2017 Folimonova et al., 2009

^a HLB evaluation depended on *D. citri* inoculations not grafting.

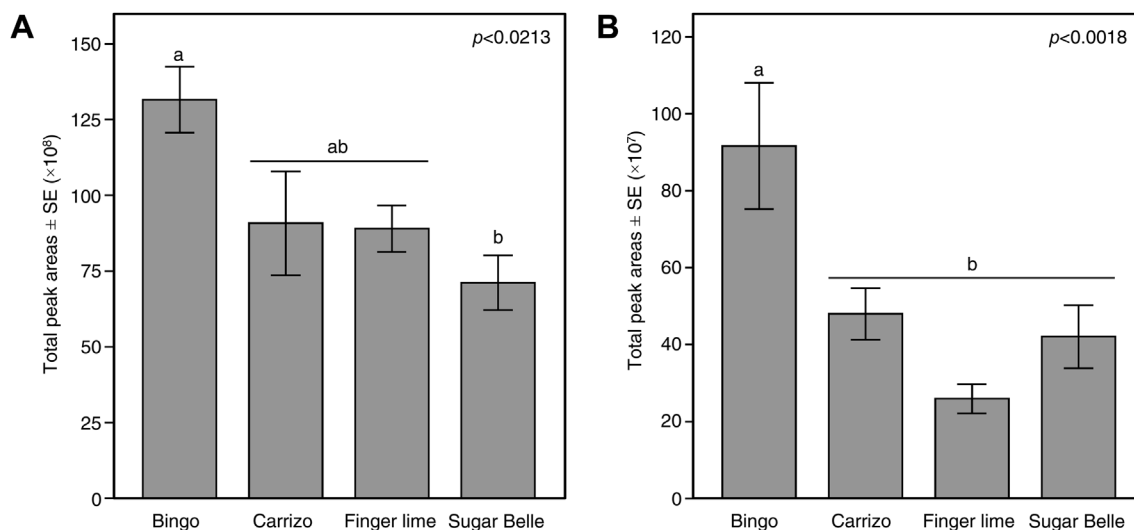


Fig. 2. Abundance of total peak areas of non-volatile polar metabolites (A) and volatile organic compounds (B) detected in citrus leaf extracts using GC-MS ($n = 10$). Leaf polar metabolites were extracted using an extraction solvent of methanol:chloroform:water (8:1:1 v/v/v) and derivatized with TMS, while leaf volatile organic compounds (VOCs) were extracted using *n*-hexane and injected directly into the GC-MS. Different letters indicate statistically significant differences among the studied varieties ($p < 0.05$), while "ns" or the same letter signify no significant differences among them using Tukey-Kramer honestly significant different test (Tukey HSD).

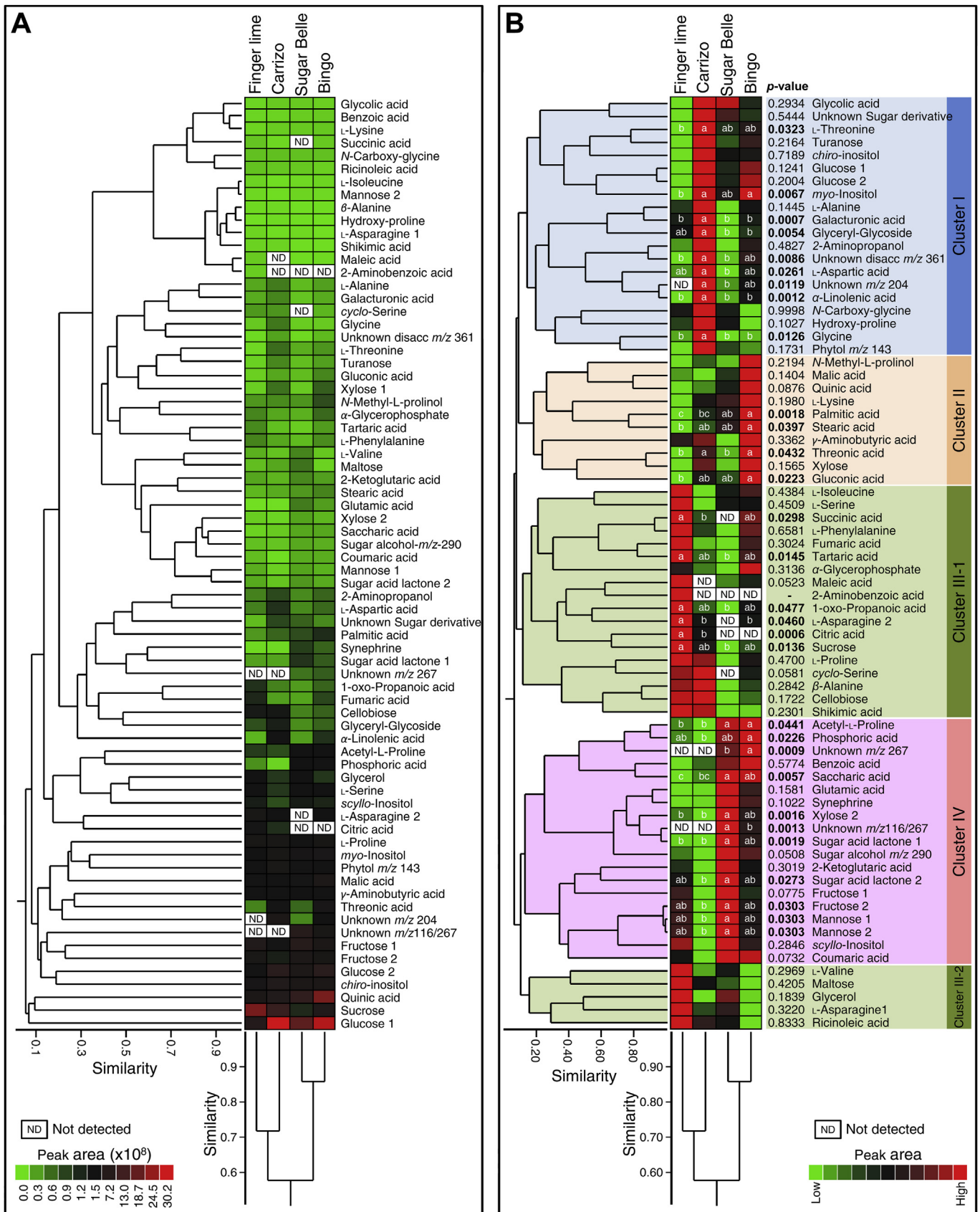
2.3. GC-MS analysis

For leaf VOC analysis by GC-MS, 0.5 μ L of each hexane extract was injected splitlessly into the GC. For leaf metabolites, dried leaf extracts were derivatized using our trimethylsilylation (TMS) protocol prior to injection. All procedures and conditions including GC-MS column and oven conditions, compound identification, and peak area normalization

were carried out as previously reported in Killiny et al. (2017).

2.4. Statistical analyses

For both leaf metabolites and extracted volatiles analysis, five biological and two technical replicates per treatment were analyzed. Data were normally distributed. Two way-hierarchical cluster analysis (HCA)



(caption on next page)

Fig. 3. Two-way hierarchical cluster analysis (HCA) using the non-standardized (A) and standardized (B) means of individual non-volatile polar metabolites detected in citrus leaf extracts using GC-MS ($n = 10$). The differences in the metabolites' abundance between the three cultivars and their rootstock are visualized in the heat map diagram. Rows represent the individual metabolites while columns represent the varieties. Lower peak areas are colored green and higher peak areas are colored red (see the scale at the corner of the bottom of the heat map). Metabolites and varieties were organized using hierarchical cluster analysis (HCA) based on similarities in auto-scaled values and correlations, respectively, with 95% confidence between groups from the discriminant function analysis (DFA) to construct the similarity dendrograms. In panel (B), different letters indicate statistically significant differences among the studied varieties ($p < 0.05$), while cells without letters or with the same letter signify no significant differences among them using Tukey-Kramer honestly significant different test (Tukey HSD). P -values are listed to the right-side of the heat map in panel (B) and bolded if less than 0.05.

was performed with the standardized and non-standardized means of the matrices for the four studied treatments. Distance and linkage were done using the Bray-Curtis similarity measure method (Michie, 1982) with 95% confidence between groups from the discriminant function analysis (DFA) to construct the similarity dendrograms. Multivariate compound similarities were presented as a heat map, combined with two way-HCA as described above. All compound (as peak areas) were statistically analyzed according to the analysis of variance technique (ANOVA). Post hoc pairwise comparisons between the four studied treatments were performed with the Tukey-Kramer honestly significant different test (Tukey HSD). Statistical significance was established as $p < 0.05$. Principal component analysis (PCA) was performed using normalized data of individual metabolites and volatile compounds. Additionally, the loading-plots were generated associated with the PCA by using the singular value decomposition (SVD).

3. Results

Herein, we studied the differential chemical composition of leaf extracts of three citrus cultivars and one common rootstock. We focused on non-volatile metabolites after derivatization with TMS and volatile organic compounds. TMS derivatization resulted in highly reproducible chromatograms (data non-shown). Overall, 'Bingo' was significantly higher in total peak area of detectable metabolites ($p < 0.0213$; Fig. 2A) and VOCs ($p < 0.0018$; Fig. 2B) compared to other cultivars, whereas 'LB8-9' and M finger lime' had the lowest total peak area of detectable metabolites and VOCs, respectively (Fig. 2).

3.1. Citrus leaf metabolites as a tolerance marker against huanglongbing

After TMS derivatization, 72 metabolites compounds were detected among the three citrus cultivars and rootstock (Fig. 3), which included carboxylic acids, amino acids, amino alcohols, organic acids, sugars, sugar alcohols, sugar acids and phosphoric acid. The non-standardized two way-hierarchical cluster analysis (HCA) showed that the peak areas of detected metabolites ranged from 0.01×10^8 to 30.2×10^8 . Most of the detectable metabolites were sugars that were the most abundant metabolites in the leaf extracts. Glucose and fructose were each detected as two peaks (named as glucose 1, glucose 2, fructose 1 and fructose 2, respectively). Glucose 1 was the most abundant metabolite in all studied cultivars except finger lime where sucrose was the most abundant metabolite (Fig. 3A). On the other hand, many metabolites (about 45 compounds) had a small peak area less than 1×10^8 . Glycolic acid, benzoic acid, L -lysine, succinic acid, N -carboxy-glycine, and ricinoleic acid had the lowest peak areas among all detected metabolites, which were clustered together in the top of the cluster dendrogram (Fig. 3A). 2-Aminobenzoic acid was only detected in finger lime, but not in the other studied cultivars.

The standardized-HCA combined with ANOVA analysis and Tukey HSD test using the means of peak areas of individual metabolites was used to differentiate the individual metabolites among the cultivars (Fig. 3B). The differences in the metabolites are also visualized in the heat map presented. The total HCA dendrogram among varieties (presented in the bottom of Fig. 3B) showed that the metabolites profile of citrus leaves from 'Bingo' was closer to the profile of 'LB8-9' (more than 85% similarity), whereas finger lime was closer to 'Carrizo' (about 72%

similarity). In addition, the HCA dendrogram among metabolites showed that all detected compounds separated into four clusters. Cluster I includes 20 metabolites which were higher in 'Carrizo' and nine compounds were significantly different compared to other varieties. Cluster II includes 10 compounds, which were higher in 'Bingo', four of which were significantly different compared to other cultivars (palmitic acid, stearic acid, threonic acid, and gluconic acid). Cluster III includes 23 metabolites which were higher in finger lime and split into two separate clusters (cluster III-1 and cluster III-2). Six out of 23 metabolites, all from cluster III-1, were significantly different compared to other varieties (succinic acid, tartaric acid, citric acid, 1-oxo-propanoic acid, L -asparagine 2, and sucrose) (Fig. 3B). Interestingly, five compounds of this cluster were not detected in 'LB8-9' including succinic acid, 2-aminobenzoic acid, L -asparagine 2, citric acid, and *cyclo*-serine. Cluster IV includes 19 metabolites, which were higher in 'LB8-9'. Out of these 19 compounds, 11 compounds were significantly different compared to the other cultivars (Fig. 3B), most of them sugars or sugar derivatives (saccharic acid, xylose 2, sugar acid lactone 1, sugar acid lactone 2, fructose 2, mannose 1, and mannose 2) in addition to acetyl- L -proline and phosphoric acid.

3.2. Principal component analysis reveals the differences in metabolites between citrus cultivars

The principal component analysis (PCA) performed using the peak area of individual metabolites its associated loading-plot are shown in Fig. 4A and B. The scatter plot obtained from the PCA showed a clear separation among all studied cultivars which clustered at the top of the scatter plot except finger lime (Fig. 4A). Principal component 1 and 2 explained about 81.43% of the variation. Furthermore, the loading plot (Fig. 4B) showed that about 30 compounds (out of 72 detected metabolites) correlated positively with finger lime. These compounds included amino acids (L -valine, L -isoleucine, L -serine, L -asparagine 1, L -asparagine 2, L -phenylalanine, L -proline, and β -alanine), organic acids (fumaric acid, maleic acid, tartaric acid, citric acid, ricinoleic acid, succinic acid, and shikimic acid), sugars (mannose 1, mannose 2, fructose 1, fructose 2, sucrose, and cellobiose), and sugar alcohols (*scyllo*-inositol and glycerol). In addition, 2-aminobenzoic acid had the highest absolute score value correlated with finger lime (Fig. 4B).

3.3. Citrus leaf volatiles as a bio-marker for HLB-tolerant cultivars

Forty-eight different compounds were detected and identified in the n -hexane extracts of citrus leaves. The abundances of these compounds after normalizing to the internal standard are visualized in the heat map and presented in Fig. 5. Most of the detected compounds belonged to three main groups (monoterpenes, sesquiterpenes, and aliphatic aldehydes). The non-standardized-HCA showed that the peak areas of detected volatiles ranged from 0.004×10^7 to 27.471×10^7 . Among the 48 detected VOCs, citronellal, *d*-limonene, and linalool were the most abundant compounds. Finger lime had the highest levels of citronellal, while 'Carrizo' had the highest levels of *d*-limonene. On the other hand, linalool was the predominant compound in both 'Bingo' and 'Sugar Belle' mandarin hybrids (Fig. 5A). Many VOCs (about 18 compounds) had a peak area less than 0.5×10 , which were clustered together in the top of the cluster dendrogram (Fig. 5A). α -Thujene, α -humulene,

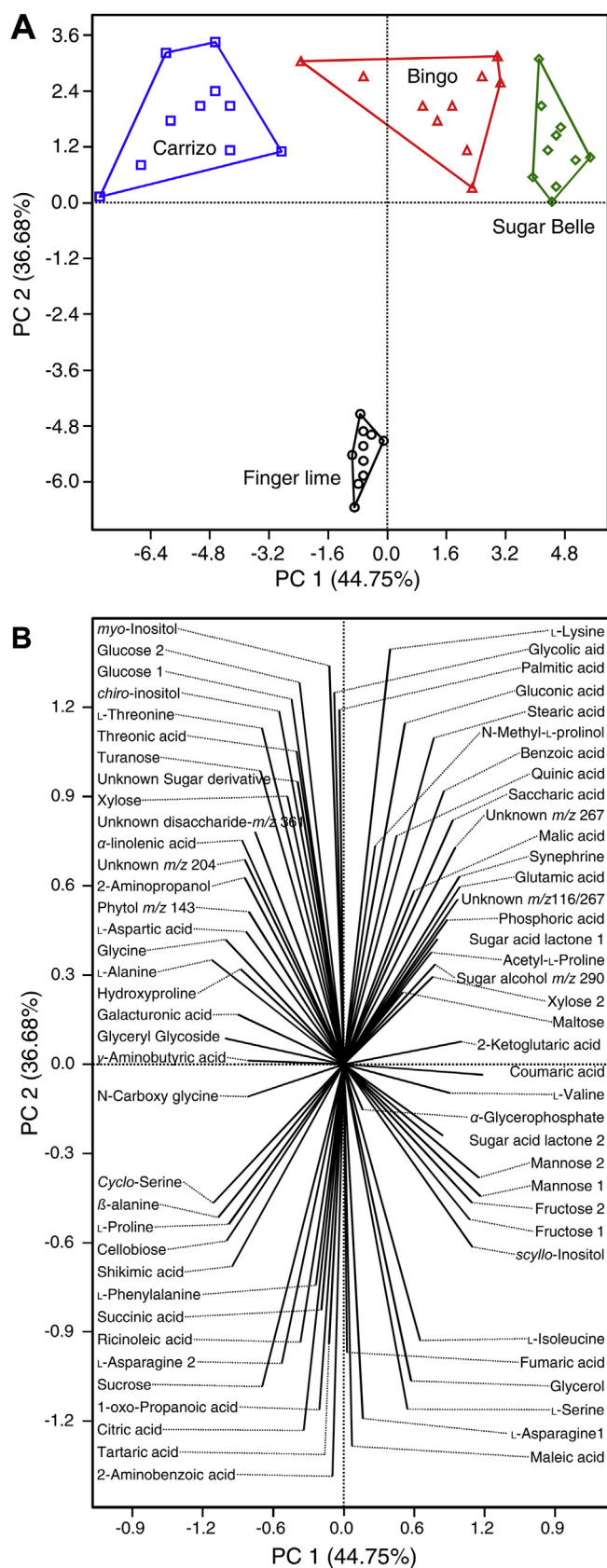


Fig. 4. Principal component analysis (PCA) of different non-volatile polar metabolites detected in citrus leaf extracts using GC-MS ($n = 10$). (A) PCA-scatter-plot; (B) PCA-loading-plot.

phytol, β -phellandrene, citronellol, unknown terpene alcohol-1, α -farnesol, β -farnesol, 1,8-cineole, sabinene hydrate, neryl acetate, sesquithujene, unknown terpene alcohol-2, *allo*-ocimene, nonanol, terpinen-4-ol, an unknown ketone, and decanal had the lowest peak areas among all detected volatiles. Interestingly, the volatile profiles of the three citrus cultivars were different from each other.

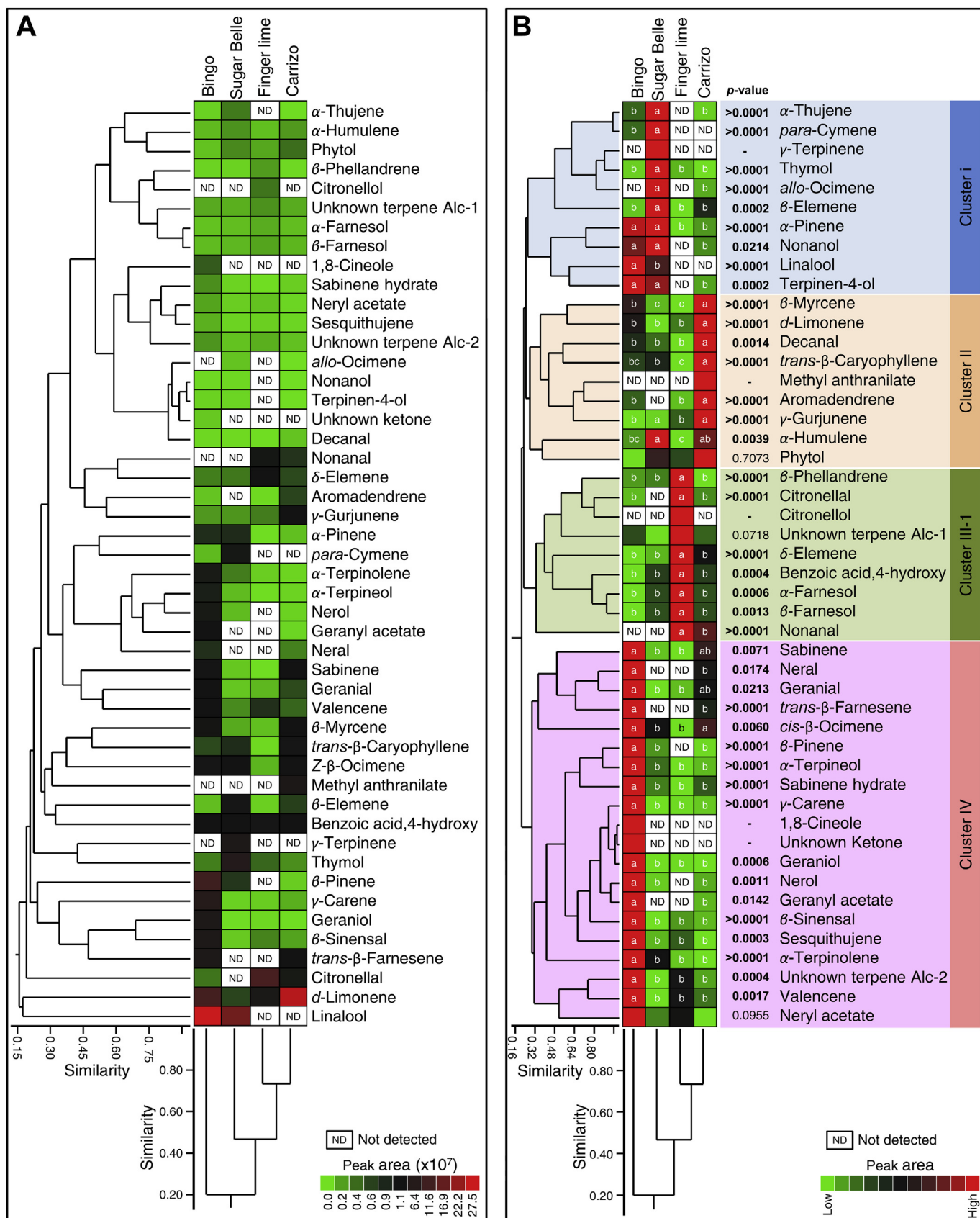
The standardized-HCA combined with ANOVA analysis and Tukey HSD test using the means of normalized-peak areas of individual VOCs was used to differentiate between the studied cultivars and is visualized in the heat map (Fig. 5B). The total HCA dendrogram among cultivars (presented in the bottom of Fig. 5B) showed that the VOC profile of finger lime was closer to that of 'Carrizo' (about 75% similarity), followed by 'LB8-9' (less than 50% similarity), whereas 'Bingo' was separated from other cultivars (about 20% similarity). In addition, the HCA dendrogram among individual volatiles showed that all detected compounds separated into four clusters. Cluster I includes 10 volatile compounds that were significantly higher in 'LB8-9' than the other cultivars. Interestingly, no significant differences were observed between both 'Bingo' and 'LB8-9' in α -pinene, nonanol, and terpinen-4-ol. Moreover, γ -terpinene was detected only in 'LB8-9', but not detected in the other cultivars (Fig. 5B). Both cluster II and cluster III include nine compounds each, which were higher in 'Carrizo' and finger lime, respectively. Interestingly, methyl anthranilate was detected only in 'Carrizo', while citronellol was found only in finger lime. Furthermore, there are no significant differences in both phytol and unknown terpene alcohol 1 levels from both clusters (cluster II and cluster III, respectively). Cluster IV includes the majority of detected volatiles (20 compounds), which were higher in 'Bingo'. Out of these 20 compounds, only neryl acetate was not significantly different among all studied cultivars. Remarkably, both 1,8-cineole (eucalyptol) and unknown ketone were only detected in 'Bingo' leaves (Fig. 5B).

3.4. Principal component analysis reveals the differences in leaf volatiles between citrus cultivars

The PCA using the normalized-peak areas of individual volatiles is shown in Fig. 6A and the associated loading-plot is shown in Fig. 6B. The scatter plot obtained from the PCA showed a clear separation among all studied cultivars. All studied cultivars clustered in the left side of the scatter plot except 'Bingo' (Fig. 6A), with respect to PC1 and PC2 (total variance up to 49.76% for PC1 and 27.46% for PC2). Furthermore, the loading plot (Fig. 6B) showed that most detected volatiles were grouped in three sets. Unknown terpene alcohol-1, citronellal, citronellol, β -phellandrene, *d*-elemene, nonanal, β -farnesol, α -farnesol, and 4-hydroxybenzoic acid, were positively correlated with finger lime. Additionally, thymol, γ -terpinene, *allo*-ocimene, *para*-cymene, α -thujene, β -elemene, and α -humulene had the highest absolute score value correlated with 'LB8-9'. The rest of the detected volatiles were positively correlated with 'Bingo', which clustered together to the right side of the loading-plot (Fig. 6).

4. Discussion

Plant tolerance to vector-borne diseases could result from their tolerance to the plant pathogen and/or to its vector. In fact, some plant physical characteristics such as thorns, trichomes, leaf surface wax, and thick cell walls may deter or form physical barriers against herbivores (War et al., 2012), consequently protecting plants from insect-vector pathogens. Additionally, many plants possess specific chemical defense mechanisms against both insects and pathogens. Plants with the ability to synthesize both allelochemicals and antimicrobial compounds would seemingly be more disease tolerant than plants without these attributes. Thus, development of citrus cultivars with these defenses would increase their disease tolerance, reduce the costs of citrus production and minimize insecticide use. In this study, we investigated the volatile and the non-volatile leaf metabolites of two HLB-tolerant cultivars and one



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Fig. 5. Two-way hierarchical cluster analysis (HCA) using the non-standardized (A) and standardized (B) means of individual volatile organic compounds (VOCs) detected in citrus leaf extracts using GC-MS ($n = 10$). The differences in the VOCs' abundance between the three cultivars and their rootstock are visualized in the heat map diagram. Rows represent the individual volatiles while columns represent the varieties. Lower peak areas are colored green and higher peak areas are colored red (see the scale at the corner of the bottom of the heat map). VOCs and varieties were organized using hierarchical cluster analysis (HCA) based on similarities in auto-scaled values and correlations, respectively, with 95% confidence between groups from the discriminant function analysis (DFA) to construct the similarity dendrograms. In panel (B), different letters indicate statistically significant differences among the studied varieties ($p < 0.05$), while cells without letters or with the same letter signify no significant differences among them using Tukey-Kramer honestly significant different test (Tukey HSD). P -values are listed to the right-side of the heat map in panel (B) and bolded if less than 0.05.

new mandarin hybrid propagated on 'Carrizo' rootstock in order to understand why some citrus cultivars are more tolerant to HLB than others. Understanding biochemical profiles of the disease-tolerant cultivars may lead to identifying specific chemical biomarkers which can help predict tolerance or susceptibility of new citrus cultivars.

The Asian citrus psyllid is a pest of nearly all *Citrus* and related Rutaceae species. However, finger lime was found to be a poor host for *D. citri* (Ramadugu et al., 2016), and this criterion could prevent *D. citri* from transmitting the vector-borne pathogen, CLAs. The tolerance of finger lime to *D. citri* could result from the presence of physical barriers or chemical toxins. The presence of anthocyanins in the young leaves of finger lime could play a key role in its tolerance to *D. citri*. In fact, previous studies showed that anthocyanins can protect plants against biotic stresses such as insect and pathogen attacks (Gould et al., 2000; Xu et al., 2017). In addition, anthocyanins can make plants less discernible to insects and decrease their feeding activities as a result of the high phenolic concentrations (Karageorgou and Manetas, 2006). The presence of these red pigments in finger lime could impair the visual signals to *D. citri*, discourage their feeding, and consequently prevent the transmission of CLAs.

Compared to other cultivars, finger lime was the highest in citronellal, nonanal, citronellol, β -phellandrene, δ -elemene, α -farnesol, β -farnesol, and an unknown terpene alcohol. In our previous study, we found that the levels of some volatiles such as undecanal, citronellal, β -phellandrene and *d*-limonene increased in CLAs-infected Valencia leaves, indicating that they could play a role in citrus response to CLAs (Hijaz et al., 2013). Interestingly, we also found that CLAs-tolerant cultivars were high in aldehyde compounds such as undecanal and citronellal, which are known for their antibacterial activities (Hijaz et al., 2016). Aldehydes such as neral and citronellal showed moderate antibacterial activity against several pathogens (Dorman and Deans, 2000; Yamasaki et al., 2007). Citronellol, the alcohol form of citronellal, also showed an antibacterial activity against *Staphylococcus aureus* (Griffin et al., 1999). In addition, accumulation of β -citronellol and nerol in peel of citrus fruits improved their resistance to pathogens and insect attack (Rodriguez et al., 2011). Farnesol was also active against several microorganisms and showed synergetic effects with several antibacterial agents (Derengowski et al., 2009). The high levels of these volatiles in finger lime indicated that they could act as antimicrobial agents against CLAs. In addition, these VOCs can act as deterrents or antifeedants against *D. citri*.

Our results also showed that finger lime was rich in several amino acids (*L*-isoleucine, *L*-serine, *L*-phenylalanine, *L*-asparagine, and *L*-proline, 2-aminobenzoic acid, *cyclo*-serine, and β -alanine), organic acids (succinic acid, fumaric acid, tartaric acid, maleic acid, citric acid, 1-oxo-propanoic, shikimic acid, and ricinoleic acid), and sugars (sucrose, maltose and *scyllo*-inositol). The role of amino acids in plant response to pathogens' attack has been extensively studied (Bennett and Wallsgrove, 1994). Previous studies showed that many amino acids including phenylalanine, tyrosine, and tryptophan are associated with plant response to pathogens (Killiny and Hijaz, 2016). Interestingly, the level of shikimic acid, the precursor of the aromatic amino acids, was also high in finger lime. The elevated levels of these amino acids, which are known to be implicated in plant defense, can enhance finger lime tolerance to CLAs.

The elevated levels of many of the TCA cycle intermediates (succinic

acid, fumaric acid, maleic acid, and citric acid) in finger lime suggested that it could have a high respiration rate. Resistance response is metabolically expensive, and it relies on many metabolic pathways including the TCA cycle to support the required energy (Yang et al., 2017). Under biotic and abiotic stresses, γ -aminobutyric acid (GABA) is converted to succinic acid and fed into the TCA cycle to enhance the production of energy (Yang et al., 2017). The high levels of the TCA cycle intermediates makes it tolerate biotic and abiotic stresses better than other cultivars. The level of *scyllo*-inositol was also high in finger lime, indicating that it could be implicated in its tolerance. Sugar alcohols can provide protection against osmotic and photo-oxidative stress, and some sugar alcohols such as mannitol play important roles in plant pathogen interactions (Williamson et al., 2002).

Stover et al. (2016) reported 'LB8-9' to be tolerant to CLAs. Our previous work showed that the main volatiles in "LB8-9" were linalool, thymol, γ -terpinene, β -elemene, z - β -ocimene, and α -pinene (Killiny et al., 2017). Compared with other cultivars, 'LB8-9' was high in α -thujene, *para*-cymene, γ -terpinene, thymol, *allo*-ocimene, β -elemene, α -pinene, and nonanol. The possible role of these compounds in 'LB8-9' tolerance to CLAs was extensively discussed in our previous work (Killiny et al., 2017). In agreement with our previous study, we also found that 'LB8-9' mandarin on 'Carrizo' rootstock was high in thymol and its precursors (γ -terpinene and *p*-cymene). Interestingly, pure thymol showed strong antibacterial activity against pathogenic *Escherichia coli* and *Clostridium perfringens* (Du et al., 2015). The high level of thymol in 'LB8-9' indicated that it could be involved in its tolerance to CLAs.

Compared with other cultivars, 'LB8-9' was high in several amino acids (acetyl-*L*-proline, *L*-valine, *L*-glutamic acid, synephrine), and was the highest in non-protein amino acids such as GABA and synephrine. High levels of non-protein amino acids can be toxic to herbivorous insects, thereby functioning as defense compounds (Bennett and Wallsgrove, 1994). Sugar acid lactone, fructose, mannose, maltose, saccharic acid, *scyllo*-inositol, and unknown sugar alcohol, phosphoric acid, benzoic acid, 2-ketoglutaric acid, and coumaric acid were also high in LB8-9. Any of these could be tolerance biomarkers worthy of further study. We found similar results when we compared this cultivar with its parents, 'Duncan' grapefruit, 'Dancy' tangerine, 'Clementine' mandarin, and 'Minneola' tangelo (Killiny et al., 2017). The possible roles of these metabolites, especially sugar alcohols, in 'LB8-9' tolerance to CLAs were also discussed (Killiny et al., 2017).

According to its patent, 'Bingo' mandarin did not show any HLB symptoms in its first 10 years in the field. However, since this cultivar has not been tested under greenhouse-controlled studies, these preliminary observations do not guarantee it is tolerant to HLB disease. More recently, the original tree in the field, now more than 10 years old, has shown some decline and is now testing positive for Liberibacter. Moreover, its VOC and polar metabolite profiles were dramatically different from the tolerant mandarin, 'LB8-9'. Although 'Bingo' was relatively high in twenty-four volatile compounds, only four of these volatiles (α -pinene, nonanol, linalool, terpinen-4-ol) were found at high levels in finger lime and LB8-9. On the other hand, 'Bingo' was low in most volatiles that were high in finger lime and LB8-9, indicating lower activity in most of the biosynthesis pathways related to production of defense compounds thought to be required for CLAs tolerance.

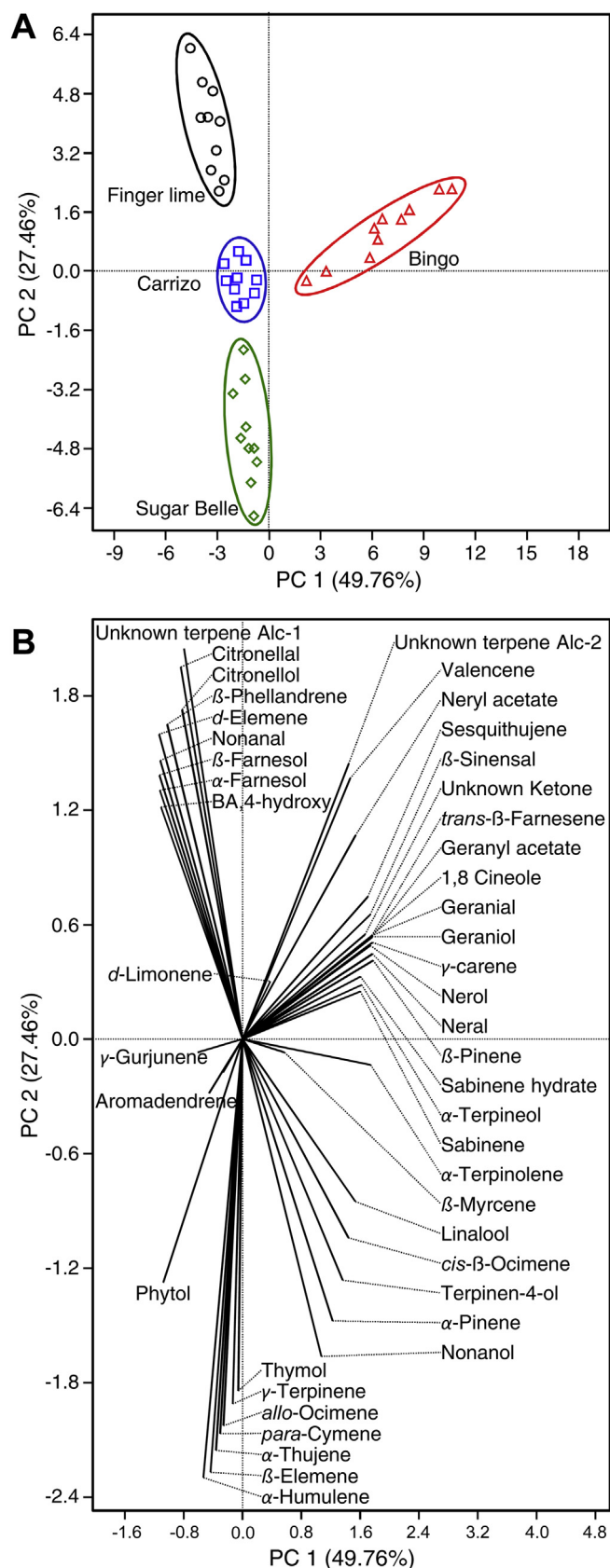


Fig. 6. Principal component analysis (PCA) of different volatile organic compounds detected in leaf extract of four citrus varieties using GC-MS ($n = 10$). (A) PCA-scatter-plot; (B) PCA-loading-plot.

Low levels of thymol was detected in 'Bingo' and its precursors (γ -terpinene and *p*-cymene) were not even detected, indicating that the thymol biosynthetic pathway was less active than in 'LB8-9'. 'Bingo' and 'LB8-9' share a common parent in 'Clementine' mandarin, which is susceptible to HLB. Our previous work showed that 'Minneola' tangelo was the source of the antibacterial compound thymol in 'LB8-9', as it was nearly absent in 'Clementine' (Killiny et al., 2017). The other 'Bingo' grandparent, 'Valencia' sweet orange, also shows little tolerance to HLB. Remarkably, eucalyptol was only detected in 'Bingo'. In fact, previous studies showed that eucalyptol possess a strong feeding and ovipositional repellency against *Aedes aegypti* (yellow fever mosquito) (Klocke et al., 1987). Eucalyptol was reported in citrus and its biosynthetic enzyme (1,8-cineole synthases) was also characterized in *Citrus unshiu* Marc (Shimada et al., 2005). Eucalyptol could act as a repellent for *D. citri*, however this suggestion need to be tested. In the same manner, the level of total polar metabolites in 'Bingo' was relatively higher than the other cultivars. However, as single metabolites, only eight metabolites (palmitic acid, stearic acid, threonic acid, gluconic acid, acetyl-L-proline, phosphoric acid, *myo*-inositol, and an unknown compound) were significantly higher in 'Bingo' compared with the other cultivars.

'Carrizo' citrange is very common rootstock and it was found to be relatively tolerant to CLAs (Folimonova et al., 2009) and to *D. citri*, but not like its parent, *P. trifoliata* (Hall et al., 2017). Compared with other selected cultivars, 'Carrizo' contained the highest level of β -myrcene, *d*-limonene, decanal, *trans*- β -caryophyllene, methyl anthranilate, aromadendrene, γ -gurjunene, and phytol. The central location of 'Carrizo' in the PCA plot (Fig. 6A) demonstrated that it had low effects on the VOCs of selected scions. In our previous study, we found that 'Carrizo' was the highest, among fourteen cultivars in *d*-limonene, *trans*- β -caryophyllene, γ -elemene, β -elemene, and germacrene D (Hijaz et al., 2016) and suggested that these volatiles could play a role in its tolerance to CLAs.

Our current results also showed that 'Carrizo' was high in some amino acids (L-threonine, L-alanine, L-aspartic acid, L-proline, glycine, 2-aminopropanol, β -alanine, N-carboxy-glycine, *cyclo*-serine, hydroxyproline), several sugars and sugar alcohols (turanose, glucose, *chiro*-inositol, and *myo*-inositol), and α -linolenic acid and shikimic acid. In a similar study, we also found that 'Carrizo' was relatively high in quinic acid and glycine compared to other cultivars (Killiny and Hijaz, 2016). This result also indicated that the rootstock 'Carrizo' had low effects on the non-volatile metabolite profile of the selected scions. The effect of rootstock on citrus leaf metabolites is controversial. Some studies showed that rootstock could affect citrus leaf metabolites (volatiles, phytohormones, flavonoids), whereas others did not show any effects (Martinez-Cuenca et al., 2017; Santos dos et al., 2017; Scora et al., 1981). The previous results together suggested that rootstock effects depend on the scion/rootstock combinations.

Although field observations and controlled greenhouse studies showed that some citrus were more tolerant to CLAs pathogen and its vector than others, only few studies were performed in order to explain these observations. Ammar et al. (2014) found that thick fibrous ring (sclerenchyma) could prevent the feeding and/or probing of *D. citri* adult into the vascular bundle (phloem and/or xylem) and consequently halt or slow the transmission of the CLAs bacterium by *D. citri*. In our effort to investigate the reasons behind the tolerance of some citrus species to CLAs and *D. citri*, we conducted a several metabolic studies on a large number of HLB-tolerant and sensitive citrus species (Hijaz et al., 2016; Killiny and Hijaz, 2016; Killiny, 2017; Killiny et al., 2017). We believe that metabolic profiling of CLAs- and *D. citri*-tolerant citrus species will reveal insights about the active metabolites that could be implicated in their tolerance to HLB disease. Information generated from these metabolic studies will be a great tool for the citrus breeding programs. Consequently, we think that future studies should take advantageous of other strong analytical tools such as liquid chromatography-mass spectrometry (LC-MS) in order to identify other secondary

metabolites that cannot be detected using GC-MS.

Contribution

Nabil Killiny conceived the study. All authors contributed in conducted the experiments and analyzing of data. Writing of the manuscript was by all authors. Nabil Killiny obtained the funding for the work.

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