**Metabolically speaking: Possible reasons behind the tolerance of ‘Sugar Belle’ mandarin
hybrid to huanglongbing**

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Abstract

Huanglongbing (HLB) is currently considered the most destructive disease of citrus. Since its spread to the Americas, HLB has killed millions of trees and caused a sharp decline in production in many citrus growing regions. With the continuous spread of HLB disease in Florida and worldwide, there is an urgent need for the development of commercial citrus cultivars with a strong tolerance to HLB. Interestingly, field observations showed that some of the recently released mandarin hybrids such as ‘Sugar Belle’ were tolerant to HLB. In this study, we investigated the volatile and non-volatile metabolites of greenhouse-grown ‘Sugar Belle’ mandarin and four of its ancestors in order to understand why ‘Sugar Belle’ mandarin is relatively tolerant to HLB. Leaf volatiles were directly extracted with hexane and analyzed using gas chromatography-mass spectrometry (GC-MS). Leaf polar metabolites were extracted with a mixture of methanol:water (1:1, v/v), derivatized to their trimethylsilyl ethers, and analyzed using GC-MS. Forty-seven volatile compounds and forty-two polar metabolites were detected in ‘Sugar Belle’ mandarin leaves and its ancestors. ‘Sugar Belle’ was high in several volatiles such as α -thujene, *para*-cymene, γ -terpinene, thymol, β -elemene, and (*E*)- β -caryophyllene. Some of these volatiles, especially thymol, β -elemene, and (*E*)- β -caryophyllene are known for their anti-microbial activity. In addition, ‘Sugar Belle’ mandarin was the highest in synephrine, benzoic acid, ferulic acid, caffeic acid, *chiro*-inositol, fructose, glucose, threonic acid, saccharic acid, and galactaric acid, and the second in threonine, malic acid, and *myo*-inositol compared to the ancestors. Phenolic compounds such as benzoic, ferulic, and caffeic acids may act as antibacterial agents, whereas others like sugar alcohols may protect ‘Sugar Belle’ mandarin from stress during pathogen attack. The tolerance of ‘Sugar Belle’ and other newly released mandarin hybrids should be further evaluated using greenhouse controlled studies. If tolerance of these

hybrids is confirmed, they could be used to replace the traditionally susceptible cultivars.

1. Introduction

Huanglongbing (HLB), also called citrus greening, was first discovered in southern China in 1919 (Bové, 2006). North America was free of HLB until it was reported in Florida, USA in 2004 (Bové, 2006). Since its discovery in Americas, HLB has spread in many citrus growing regions in Brazil and U.S.A (Teixeira et al., 2008). HLB has been responsible for the death of millions of citrus trees and it resulted in a sharp decline in citrus production worldwide (Hodges et al., 2014). Due to the spread of HLB disease in Florida, Florida's orange production during 2015-2016 season was the lowest in the past fifty years (NASS, 2017).

Candidatus Liberibacter, the actual agent for citrus greening, is a phloem-limited, Gram-negative bacterium. Three species of yet-uncultured *Candidatus Liberibacter* were associated with citrus greening: *Candidatus Liberibacter asiaticus* (CLas), *Candidatus Liberibacter americanus* (CLam), and *Candidatus Liberibacter africanus* (CLaf). The CLas has been found in Asia, North America, and Brazil, while CLam and CLaf are endemic to Brazil and Africa, respectively. CLas and CLam are transmitted by the Asian citrus psyllid, *Diaphorina citri* Kuwayama, whereas CLaf is transmitted by the African citrus psyllid, *Trioza erytreae* (Tatineni et al., 2008).

Although *Candidatus Liberibacter* can be spread by grafting, psyllids are the main transmission vector of the CLas pathogen in the field. Psyllids acquire the citrus greening pathogen while feeding on citrus phloem sap of the infected trees then transmit it by moving to healthy trees. Field observation showed that *D. citri* prefers some host plants more than others, for example *Poncirus trifoliata* was an occasional host, while sweet orange and *Murraya*

paniculata (L.) Jack (orange jasmine) were the most preferred hosts for *D. citri* (Halbert and Manjunath, 2004). In addition, comparative laboratory studies showed that some host plants were more preferred by *D. citri* than others. Tsai and Liu tested the preference of *D. citri* to several host plants and found that grapefruit was the most preferable host, followed by rough lemon, orange jasmine, and sour orange (Tsai and Liu, 2000). Richardson and Hall also showed that hybrids of *Poncirus trifoliata* x *Citroncirus* spp. were more tolerant to *D. citri* than *Citrus macrophylla* Wester (Alemow). Although, they did not investigate the mechanism behind the tolerance of *P. trifoliata* to *D. citri*, it is believed that *P. trifoliata* may have antixenotic resistance to *D. citri* (Richardson and Hall, 2014).

Although there are no commercial citrus cultivars with strong tolerance to CLAs, which makes it remarkably difficult to apply effective control measures (Fan et al., 2013), field observations showed that some citrus cultivars are more tolerant to CLAs bacteria than others (Halbert and Manjunath, 2004). In addition, greenhouse controlled studies also showed that some citrus cultivars are more tolerant to CLAs pathogen (Folimonova et al., 2009). Sensitive cultivars showed severe leaf chlorosis, decreased growth and death, while the tolerant cultivars showed fewer symptoms (Folimonova et al., 2009). In another study, it was also found that some Malaysian citrus cultivars were also more tolerant to CLAs pathogens than others (Shokrollah et al., 2009).

In our previous work, we investigated the phloem sap composition of fourteen different citrus cultivars with different degrees of tolerance to CLAs pathogen. We reported that the levels of most of the amino acids, especially those implicated in plant defense to pathogen attack such as phenylalanine, tryptophan, and tyrosine were higher in CLAs-tolerant cultivars (Killiny and Hijaz, 2016). In another study, we investigated the volatile composition of the same citrus

cultivars and indicated that citrus leaf volatiles could play a role in citrustolerance against the CLas pathogen. Tolerant cultivars contained higher amounts of volatiles especially those with strong antimicrobial activities such as aldehydes than susceptible cultivars(Hijaz et al., 2016).

To date, there is no effective tool to stop the spread of the CLas pathogen or manage HLB disease. Management mainly depends on the control of *D. citri* vector with insecticides, geographical isolation, use of pathogen-free nursery trees, and removal of infected trees to reduce inoculum (Gottwald et al., 2012; Halbert and Manjunath, 2004). Although removal of infected plants was proposed for eradication of HLB, this strategy is problematic and expensive (Halbert and Manjunath, 2004). Since the previous management strategies were unable to stop the fastspread of HLB, commercial citrus producers have tried various means to mitigate the disease (Gottwald et al., 2012). Recently, enhanced nutritional programs (ENPs) were proposed to minimize the deleterious effects of HLB on infected trees and maintain their productivity. However, well-designed field studies showed that the ENP was not able to maintain long-term tree health, productivity, or fruit quality (Gottwald et al., 2012). On the other hand, it is believed that the use of ENP strategies may increase the spread of the CLas pathogen since it prolongs tree life but does not affect the CLas titer (Gottwald et al., 2012).

Field observations showed that some of the recently released mandarin hybrid cultivars were tolerant to the HLB disease (Stover et al., 2016). If the tolerance of these mandarin hybrids is confirmed, they could be used to replace the traditional susceptible cultivars such as sweet orange and grapefruit (Stover et al., 2016). In 2009, the University of Florida IFAS/CREC cultivar improvement team released many mandarin hybrids including the ‘Sugar Belle’ previously known as LB8-9 [‘Clementine’ mandarin (*Citrus reticulata*) x ‘Minneola’ tangelo (*Citrus x Tangelo*), ‘Duncan’ grapefruit (*Citrus paradisi*) x ‘Dancy’ tangerine (*Citrus*

reticulata)]. ‘Sugar Belle’ mandarin is a mid-season mandarin that matures between late November and early January under Florida conditions. An earlier evaluation of ‘Sugar Belle’ mandarin cultivar and ‘Sunburst’ tangerine and ‘Minneola’ tangelo revealed that ‘Sugar Belle’ mandarin decay was lower compared to ‘Sunburst’ tangerine and its peel, juice color, and vitamin C content were higher than ‘Minneola’ tangelo (Dou and Gmitter, 2007).

Recently published data from Stover et al., (2016) on the field tolerance of several cultivars to HLB, suggested that growth and yield were not suppressed in ‘Sugar Belle’ mandarin trees even in the presence of HLB symptoms, which indicated that ‘Sugar Belle’ mandarin could be tolerant to the HLB disease. In addition, field observation indicated that ‘Sugar Belle’ mandarin is tolerant to HLB regardless of the rootstock (Dr. Tripti Vashisth, CREC-IFAS-UF, personal communication). Surprisingly, field observation showed that ‘Sugar Belle’ mandarin was a good host for *D. citri* (observation of Dr. Fred G. Gmitter, Jr.), suggesting that ‘Sugar Belle’ mandarin may have an innate antimicrobial resistance.

With the current epidemic spread of HLB in the United States as well as in many citrus producing countries, there is an urgent need to provide growers with commercial citrus cultivars that are tolerant to HLB. Our objective was to evaluate the volatile and non-volatile metabolites of ‘Sugar Belle’ mandarin which shows tolerance to HLB (Stover et al., 2016) and its parents which were evaluated as susceptible cultivars (Folimonova et al., 2009; McCollum et al., 2016) in order to identify which compounds may be involved in tolerance to CLAs. Correlating plant metabolites to HLB tolerance, by identifying specific compounds with anti-microbial activity in naturally tolerant cultivars, may provide important information about plant–pathogen

interactions. In addition, results from these studies may open new avenues to improving the disease tolerance/resistance for the highly susceptible cultivars via biotechnology applications.

2. Material and Methods

2.1. Plant Material

The budwood for ‘Sugar Belle’ mandarin, previously known as LB8-9 and its parents, [(‘Clementine’ mandarin (*Citrus reticulata*) × ‘Minneola’ tangelo, ‘Duncan’ grapefruit (*C. paradisi*) × ‘Dancy’ tangerine (*C. reticulata*)] and its parents (Figure 1) were obtained from Chiefland Budwood Foundation at Chiefland, Florida. The degree of tolerance of selected cultivars to CLas pathogen is shown in Table 1. The budwoods were side-grafted onto one-year-old UF-15 rootstocks [Hirado Buntan pummelo (*Citrus maxima*) x ‘Cleopatra’ mandarin (*Citrus reshni* hort.)]. Six months later, several leaves were collected from each plant from three different locations (top, middle, and bottom). Seven plants were sampled from each cultivar. Collected leaves were placed inside zip bags and kept on ice until analysis (less than one hour).

2.2. Analysis of Leaf Volatiles

Leaf volatiles were extracted using hexane as described by (Hijaz et al., 2016) with modifications. Briefly, fresh leaf samples were collected from the five cultivars and placed in zip bags on ice. Three leaves from each tree were pooled together for analysis. Seven different biological samples (each sample from a different tree) were collected and each sample was analyzed twice (two technical replicates) and averaged. Leaf samples of ~ 0.1 g were diced from leaf blades using a razor blade and placed into 2 mL screw cap tubes with 5 mm stainless steel balls for maceration. Sample tubes were placed into liquid nitrogen for at least 5 min and then

macerated using a TissueLyser II (Qiagen, Valencia, CA). For extraction of volatiles from the leaf tissue, 0.5 mL of hexane was added to each tube of leaf powder; the tubes were placed on ice and sonicated for 5 min, then placed on a tube rotator for 1 h at 8 °C. Following extraction, the samples were centrifuged for 3 min at 10,000 rpm and 200 µL of supernatant was transferred to an amber vial with 350 µL fused inserts (C4000-LV2, National Scientific, Rockwood, TN). Trans-2-nonenal was added at a final concentration of 1 µLmL⁻¹ as an internal standard. Hexane extracts (0.5 µL) were injected splitlessly into the injector heated to 250 °C. The GC-MS system consisted of a Clarus 680 GC fitted with Elite-5MS column (30 m x 0.25 mm I.D. x 0.25 µm film thickness) for separation of analytes (Perkin Elmer, Waltham, PA). The carrier gas was ultrapure helium at a flowrate of 1 mL·min⁻¹. The oven program was 40 °C for 2 min, then increased to 200 °C at a rate of 7°C·min⁻¹ for a total run time of 25 min. The mass spectrometer (Clarus SQ8) was operated in electron impact mode, 70 eV, 180 °C ion source temperature, and scanning from 40-400 *m/z*.

2.3. Analysis of polar Metabolites

Citrus leaf polar metabolites (organic acids, amino acids, and sugars) were determined according to the method of (Cevallos-Cevallos et al., 2011) with slight modifications. Briefly, three leaves from each plant were pooled together and ground to a fine powder in liquid nitrogen using a mortar and pestle. Seven plants (seven biological replicates) from each cultivar were sampled and each plant was analyzed twice (two technical replicates) and averaged. About 0.1 g of the leaf powder was mixed with 1 mL methanol:water (1:1, *v/v*) and was extracted by stirring the sample overnight at 8 °C in a Fisher Scientific Isotemp 3016 D (Saint Louis, MN) water bath. Samples were centrifuged at 5 °C for 5 min at 5000 rpm to precipitate the leaf debris. A 180-µL aliquot of the leaf extract was transferred to a 200 µL silanized vial insert, ribitol (10 µL of 1

mgmL⁻¹) was added as the internal standard, vortexed, and the sample was dried under a stream of nitrogen gas. Dried residues were derivatized using the trimethylsilylation (TMS) procedure and GC-MS conditions as described by (Killiny and Hijaz, 2016) except the injection volume was increased from 0.2 µL to 0.5 µL.

2.4. Compound identifications

For all detected volatiles and non-volatiles metabolites, analyte peaks were integrated using TurboMass software v. 5.4.2 (Perkin Elmer, Waltham, MA) and mass spectra were compared to those of authentic standards and/or identified by at least two of three mass spectral libraries (Wiley Flavor and Fragrances of Synthetic and Natural Compounds, Wiley 9th ed. Mass Spectral Database, and NIST 2011 MSD) with a matching score of at least 700. Unidentified metabolites were reported by *m/z* and retention time or linear retention index. Reference standard compounds were purchased from Sigma-Aldrich (St. Louis, MO) at the highest available purity.

2.5. Quantification of compounds

Compound peak areas were normalized to the internal standards used for each type of compound (volatile or polar metabolite) and then to 1 g of fresh leaf weight. Quantification of leaf volatiles was based on the peak areas obtained from a series of reference standards diluted in hexane and injected under the same chromatographic conditions as samples. Calibration curves were constructed from the linear regressions obtained by plotting the concentration vs. peak area for each standard at each concentration. Similarly, reference standards from the major classes of metabolites were derivatized and used for the construction of calibration curves.

2.6. Statistical analyses

Data was manually aligned using retention time and mass values. For each treatment, seven biological replicates were analyzed and each replicate was analyzed twice (two technical replicates per biological replicate). Statistical analyses were performed using JMP version 9.0 (SAS Institute Inc.). Data were normally distributed. Analysis of variance (ANOVA), followed by Post hoc pairwise comparisons with the Tukey-Kramer honestly significant different test (Tukey HSD) was used to compare the level of each metabolite in the different cultivars. For further characterization of the detected metabolites, scatter and loading plot for volatile and non-volatile compounds were generated using principal component analysis (PCA). Additionally, two way-hierarchical cluster analysis (HCA) was performed using the means of the data matrices. Distance and linkage were done using the Bray-Curtis similarity measure method (Michie, 1982). The multivariate compounds similarities were presented as a heat-map, combined with two way-HCA as described above.

3. Results

3.1. Leaf volatiles

Forty-seven volatile compounds were detected in the hexane extracts of ‘Sugar Belle’ mandarin leaves and its ancestors (Table 2). Linalool, γ -terpinene, α -pinene, β -elemen, Z- β -ocimene, β -pinene, and thymol were the major volatiles in ‘Sugar Belle’ mandarin and they made up more than 84% of the total volatile compounds (Table 2). Linalool, sabinene, γ -carene, β -pinene, *d*-limonene, geraniol, and geranial were the most abundant volatiles in ‘Clementine’ mandarin and they made up more than two-third of the total volatiles (Table 2). Linalool, *d*-limonene, γ -terpinene, Z- β -ocimene, β -pinene, and α -pinene were the most abundant volatiles in ‘Minneola’ tangelo and the constituted about 70 % of the total volatiles (Table 2).

224 Low levels of volatiles were detected in ‘Duncan’ grapefruit. Sabinene, linalool, β -elemene, 4-
225 ethoxybenzoic acid, and δ -elemene were the major volatiles in ‘Duncan’ grapefruit and they
226 accounted for about 55% of the total volatiles (Table 2). Linalool, δ -elemene, α -pinene, Z- β -
227 ocimene, β -pinene, γ -terpinene, and γ -elemene were the main volatiles in ‘Dancy’ tangerine
228 (Table 2) and they made about two-third of the total volatiles.

229 The Tukey’s test results showed that ‘Sugar Belle’ mandarin was the highest in α -
230 thujene, para-cymene, γ -terpinene, thymol, β -elemene, trans- β -caryophyllene and the second
231 highest in linalool and α -humulene (**Table 2**). ‘Clementine’ mandarin was the highest in
232 sabinene, β -myrcene, γ -carene, sabinene hydrate, geraniol, geranial, and geranyl acetate (Table 2).
233 ‘Dancy’ tangerine was the highest in most of the compounds including linalool, δ -elemene, α -
234 pinene, Z- β -ocimene, β -pinene, γ -terpinene, and γ -elemene (Table 2). On the other hand,
235 ‘Minneola’ tangelo and ‘Duncan’ grapefruit were low in most of these volatiles (Table 2).

236 The principle component analysis (PCA) using the concentrations of the detected volatiles
237 (Table 2) is shown in figure (2). PC1 and PC2 accounted about 80% of the variation (Figure 2A).
238 No clear clustering was observed among the selected varieties except for ‘Minneola’ tangelo,
239 which clustered alone in the left side of the scatter plot (Figure 2A). As shown in the loading plot
240 (Figure 2B), ‘Dancy’ tangerine was high in many of the volatiles such as linalool, δ -elemene, α -
241 pinene, Z- β -ocimene, β -pinene, γ -terpinene, and γ -elemene (top of the right loading plot; Figure
242 2B).

243 For further characterization of the detected volatiles, the 2-way hierarchical cluster
244 analysis (HCA), followed by heat-map using the concentrations in Table 2 were performed
245 (Figure 2C). The cluster analysis showed that the volatile profile of ‘Sugar Belle’ mandarin was

closer to ‘Minneola’ tangelo (0.8 similarity) from the rest of other cultivars (Figure 2C). ‘Duncan’ grapefruit was the second closest (0.65 similarity) variety to ‘Sugar Belle’ mandarin followed by ‘Clementine’ mandarin (0.45 similarity) (Figure 2C).

As shown in the heat-map (Figure 2C), most of the detected volatiles were high (black-red) in ‘Dancy’ tangerine. On the other hand, only few volatiles were high (black-red) in ‘Duncan’ grapefruit (Figure 2C). Several volatiles mainly from C1 & C2 group (α -thujene, linalool, thymol, para-cymene, γ -terpinene, chavicol, 4-ethoxybenzoic acid EE, β -elemene, and (*E*)- β -caryophyllene were high (black-red) in ‘Sugar Belle’ mandarin (Figure 2C). All of the volatiles from C5 group (sabinene, trans- β -farnesene, γ -carene, nerol, geraniol, sabinene hydrate, neral, geranial, geranyl acetate, β -myrcene, α -terpineol, α -terpinolene and few from C2 group (chavicol, 4-ethoxybenzoic acid EE, (*E*)- β -caryophyllene) and C3 group (*d*-limonene) were high (black-red) in ‘Clementine’ mandarin (Figure 2C). Volatiles in C3 group (*d*-limonene, citronellal, nonanol, anthranilate, *cis*-carveol, δ -cadinene) were high ‘Minneola’ tangerine (Figure 2C).

Thymol and its precursors were high in ‘Sugar Belle’ mandarin and ‘Dancy’ tangerine (Figure 3A-D). The thymol methyl ether was only detected in ‘Dancy’ tangerine (Figure 3D). The mass spectra of thymol, its precursors, and its methyl ether derivative are shown in Figure 3E-H. The proposed biosynthetic pathway for thymol in citrus is shown in Figure 3I.

‘Dancy’ tangerine was the highest in total volatiles (216 ± 96 mmolg⁻¹) followed by ‘Sugar Belle’ mandarin (117 ± 54 mmolg⁻¹), ‘Clementine’ mandarin (105 ± 40 mmolg⁻¹), ‘Minneola’ tangelo (68 ± 61 mmolg⁻¹) and ‘Duncan’ grapefruit (20 ± 10 mmolg⁻¹) (Figure 4A). The HCA using the area of the main volatile groups is shown in Figure 4B. The cluster analysis using the

volatile groups also showed that the volatile profile of ‘Sugar Belle’ mandarin was close to ‘Duncan’ grapefruit (0.85 similarity) and ‘Minneola’ tangelo (0.7 similarity) (Figure 4B). As shown in the heat map (Figure 4B), ‘Dancy’ tangerine was high in most of the volatile groups, ‘Clementine’ mandarin was high in monoterpene hydrocarbons and monoterpene esters, ‘Minneola’ tangelo was high in esters and monoterpene aldehydes. ‘Sugar Belle’ mandarin was moderate in most of the volatile groups, whereas ‘Duncan’ grapefruit was low in most of them (Figure 4B).

3.2. Leaf non-volatile metabolites

Forty-two non-volatile metabolites were detected (Table 3) in the leaves of ‘Sugar Belle’ mandarin and its ancestors. These metabolites belong to four main groups (amino acids, organic acids, sugars, and fatty acids). Proline was the most abundant amino acid in all of the selected cultivars and its concentration ranged from 740-1589 $\mu\text{g g}^{-1}$. The non-proteinogenic amino acid γ -aminobutyric acid (623-876 $\mu\text{g g}^{-1}$) was detected in all cultivars. Synephrine was detected in all cultivars, except in ‘Duncan’ grapefruit. Citric, malic, and quinic were the main organic acids in all cultivars. Inositol isomers (*chiro*-inositol, *scyllo*-inositol, and *myo*-inositol) were found in all cultivars and their concentrations ranged from 68 to 517 $\mu\text{g g}^{-1}$ (Table 3). Sucrose (1663-5000 $\mu\text{g g}^{-1}$), glucose (527-1678 $\mu\text{g g}^{-1}$), and fructose (498-1009 $\mu\text{g g}^{-1}$) were the most abundant sugars. Several sugar acids and two fatty acids (palmitic and stearic acid) were detected in the leaves of the selected cultivars.

Compared with other cultivars ‘Sugar Belle’ mandarin was the highest in synephrine, benzoic acid, ferulic acid, caffeic acid, *chiro*-inositol, fructose, glucose, threonic acid, saccharic acid, galactaric acid, and arabino-hexaric acid (Table 3) and the second in threonine, malic acid,

and *myo*-inositol. ‘Clementine’ mandarin was the highest in succinic, 1-oxopropanoic acid, and *myo*-inositol and was the second in proline, serine, synephrine, and *chiro*-inositol. ‘Minneola’ tangelo was the highest in all of the proteinogenic amino acids, maleic acid, malic acid, citric acid, xylose, sucrose, palmitic acid, and stearic acid (Table 3). ‘Dancy’ tangerine was the second highest cultivar in valine, synephrine (Table 3). ‘Duncan’ grapefruit was the highest in xylitol, ribonic acid, and gluconic acid (Table 3).

The PCA and the HCA along with the heat-map were also generated for the polar metabolites (Figure 5). As shown in the scatter plot, ‘Sugar Belle’ mandarin and ‘Minneola’ tangelo clustered away from each other and from the rest of the cultivars (Figure 5A). ‘Clementine’ mandarin, ‘Dancy’ tangerine, and ‘Duncan’ grapefruit clustered close to each other at the center of the scatter plot (Figure 5A). The loading plot and the heat-map show the relative amount of each compound in each variety (Figure 5B-C). As shown in the loading plot (Figure 5B), ‘Minneola’ tangelo was high in many compounds, especially those in the right of the loading plot. Compounds on the left side of the loading plot were high ‘Sugar Belle’ mandarin (Figure 5B). The rest of the compounds in the middle of the loading plot were high in the other cultivars (CM, DG, and DT) (Figure 5B). The cluster analysis also showed that the polar metabolites profile of ‘Sugar Belle’ mandarin was different from ‘Minneola’ tangelo (0.25 similarity) and from the rest of other cultivars (0.45 similarity) (Figure 5C). The heat-map (Figure 5C) also shows that ‘Minneola’ tangelo was high (red) in many compounds (mainly from C1 group). Furthermore, the heat-map shows that all the compounds from C3 group were high (red) in the ‘Sugar Belle’ mandarin (Figure 5C). Compounds in C2 group were high in grapefruit and those in C4 were high in ‘Clementine’ mandarin (Figure 5C).

The total level of polar metabolites was not significantly different among the selected cultivars (Figure 6A). The PCA and the heat map were also generated using the main groups of polar metabolites (Figure 6B). In agreement with the PCA results (Figure 5A), the cluster analysis showed that the ‘Sugar Belle’ mandarin polar metabolite profile was closer to ‘Clementine’ mandarin (0.8 similarity) and ‘Duncan’ grapefruit (0.65 similarity) than the rest of other cultivars (Figure 6B).

The heat-map (Figure 6B) shows that ‘Minneola’ tangelo was high in most of these groups, except sugar acids and sugar alcohols. ‘Dancy’ tangerine was the highest in non-proteinogenic amino acid. ‘Sugar Belle’ mandarin was the highest in sugar acids and sugar alcohols and it contained moderate to high amounts of organic and inorganic acid, sugars, and non-protein amino acids. ‘Duncan’ grapefruit contained moderate amounts of organic acids, sugar acids, and sugar alcohols. ‘Clementine’ mandarin was the highest in sugars and contained moderate amounts of inorganic acids, non-proteinogenic amino acids, amino acids, sugar acids, and sugar alcohols.

4. Discussion

Although field and greenhouse studies showed that some citrus cultivars are more tolerant to CLas pathogen than others, the mechanism underpinning this tolerance has not been determined yet (Folimonova et al., 2009). Understanding of the mechanisms behind citrus tolerance to CLas could help in developing commercially tolerant citrus cultivars. In this study, we investigated the leaf volatile and non-volatile metabolites of ‘Sugar Belle’ mandarin and its ancestors in order to identify metabolites that could be implicated in its tolerance to CLas.

Our results showed that ‘Dancy’ tangerine was the highest in total volatiles, followed by ‘Sugar Belle’ mandarin. On the other hand, ‘Duncan’ grapefruit was the lowest in total volatiles. In agreement with our current results, ‘Duncan’ grapefruit was the lowest in total volatiles among a study of fourteen different cultivars(Hijaz et al., 2016). On the other hand, we found that tolerant cultivars such as *Carrizo citrange*, *Citrus latipes*, and *Severiniabuxifolia* contained the highest amount of volatile compounds (Hijaz et al., 2016). Our current and previous results together suggest that citrus leaf volatiles could be implicated in citrus tolerance to CLas.

Previous research showed that infection with plant pathogens increase the production of plant volatiles that possess a strong antimicrobial activity to inhibit the movement of the pathogens from the site of infection to other tissues(Maffei, 2010). In agreement with the previous results, we also found that the levels of undecanal, citronellal, β -phellandrene and *d*-limonene were induced in ‘Valencia’ orange leaves upon infection with CLas (Hijaz et al., 2013).Huang et al., (2012) also showed that (*E*)- β -caryophyllene protects *Arabidopsis thaliana*flowers against *Pseudomonas syringae* pv. *tomato* DC3000 infection. Since the citrus oil glands are found in the midribs and veins of the citrus leaves as well as in the phloem tissues, direct effect of volatile compounds on the CLas pathogen is possible. Besides the direct effect of volatiles on plant pathogens, volatiles can trigger the resistance to pathogens by the induction of resistance genes in the emitting plant itself as well as in neighboring plants (Quintana-Rodriguez et al., 2015).

In addition, the current results showed that ‘Sugar Belle’ mandarin was high in some monoterpenes (α -thujene, *para*-cymene, γ -terpinene, and linalool). In fact previous reports showed that monoterpenes possess an antibiotic activity against many pathogens and it is believed that monoterpenes kill microorganisms by disrupting the lipid layer of their cell

membrane (Trombetta et al., 2005). The disruption of the lipid layer of cell membrane increases cell permeability and resulting in leakage of intracellular components and consequently leads to cell death (Trombetta et al., 2005).

The current results showed that ‘Sugar Belle’ mandarin was high in some sesquiterpenes such as β -elemene, trans- β -caryophyllene, and α -humulene. Previous results also showed that some sesquiterpenes including (*E*)- β -caryophyllene, γ -elemene and β -elemene, and α -humulene were also high in some of the CLas-tolerant cultivars (Hijaz et al., 2016). These findings indicated that these volatiles could possess an antimicrobial activity against CLas. Previous reports also showed that some sesquiterpenes such as (*E*)- β -caryophyllene and geranyl acetate were active against many microorganisms (Dorman and Deans, 2000). Pure (*E*)- β -caryophyllene and the essential oil of *Stachys cretica* subsp. *smyrnaea* (δ -elemene: 2.1%; δ -cadinene: 2.1%; α -humulene: 3.1%; germacrene-D: 32.8%; (*E*)- β -caryophyllene: 51.0%) showed a strong antimicrobial activity against *Pseudomonas aeruginosa* and *Bacillus subtilis* (Oztürk et al., 2009). β -elemene showed a promising anti-cancer activity against a broad spectrum of tumors (Liu et al., 2011).

‘Sugar Belle’ mandarin was also high in thymol and its precursors (γ -terpinene and *p*-cymene). Interestingly, pure thymol, carvacrol, and a commercial essential oil (25 % thymol and 25 % carvacrol) showed strong antibacterial activity against pathogenic *Escherichia coli* and *Clostridium perfringens* (Du et al., 2015). In addition, previous studies showed that essential oil components with a phenolic structure, such as thymol, eugenol, and carvacrol have the highest antibacterial activities, followed by aldehydes, ketones, alcohols, esters and hydrocarbons (Du et al., 2015). The presence of monoterpene alcohols, diterpene alcohols, thymol and its precursors at high levels in ‘Sugar Belle’ mandarin may contribute to its tolerance to CLas pathogen.

Although the level of total polar metabolites was not significantly different among the studied cultivars, the average level of total polar metabolites in ‘Sugar Belle’ mandarin was relatively higher than that in the rest of selected cultivars. Previous study showed that some of the tolerant citrus cultivars such as *Citrus latipes* and *Severinia buxifolia* were high in total metabolites (Killiny and Hijaz, 2016). Furthermore, amino acids implicated in plant defense such as phenylalanine, tyrosine, and tryptophan, and their total were also higher in CLas-tolerant cultivars (Killiny and Hijaz, 2016). Unfortunately, since the derivatization method used in this study was not specific for amino acids, none of these amino acids were detected. However, the high levels of phenolic compounds such as caffeic, ferulic, and benzoic acids in ‘Sugar Belle’ mandarin indicated that it could be high in these three amino acids. Amino acids like phenylalanine, tyrosine and tryptophan are precursors for many secondary metabolites including phenolic compounds which are implicated in plant response to biotic stress (Bennett and Wallsgrove, 1994). Accumulation of phenolic compounds was also observed in citrus plants upon *D. citri* infestation (non-published data).

In addition, ‘Sugar Belle’ mandarin was relatively high in phosphoric acids and some organic acids. Interestingly, phosphoric acid was also found to increase in citrus plants upon *D. citri* infestation (non-published data), which suggests that it is implicated in plant response to biotic stress. Phosphate deficiency reduces plant growth and increases the jasmonic acid production and plant resistance to herbivores (Khan et al., 2016). The previous results suggested that phosphates are implicated in plant response to biotic stresses. The level of some organic acids such as malic and threonic acid were high in ‘Sugar Belle’ mandarin. An increase in many organic acids including citric acid, maleic acid, and fumaric acid was observed in ‘Cleopatra’ mandarin (*Citrus reticulata*) upon infection with CLas pathogen (Albrecht et al., 2016). The

increase in organic acid was explained as a strategy to enhance nutrient uptake from the soil (Albrecht et al., 2016). An increase in citric acid was also reported in the leaves from CLas-infected sweet oranges (Cevallos-Cevallos et al., 2011). It is also possible that the accumulation of these organic acids in citrus upon CLas infection could result from the induction of the tricarboxylic acid cycle.

‘Sugar Belle’ mandarin leaves were high in the phenolic amino acid (synephrine). Synephrine is synthesized from tyrosine, however its biosynthetic pathway is not fully understood (Bartley et al., 2010). An increase in synephrine, ornithine, citrulline, and proline was also observed in CLas-infected ‘Cleopatra’ mandarin leaves (Albrecht et al., 2016). In addition, an increase in synephrine in CLas-infected sweet orange fruits was reported (Chin et al., 2014). Synephrine has received great attention in medicine because of the similarity of its structure to several cold medicines and it is a vasoconstrictor and bronchiectatic agent (Bartley et al., 2010). The high level of synephrine in ‘Sugar Belle’ mandarin could contribute to its tolerance to CLas.

The current results showed that ‘Sugar Belle’ mandarin was high in sugar alcohols (*chiro*-inositol and *myo*-inositol). Because sugar alcohols can act as compatible solutes, it was hypothesized that they act as osmoprotectants in plants (Williamson et al., 2002). In addition to osmoprotection, sugar alcohols provide protection against photo-oxidative stress, and some sugar alcohols such as mannitol may play a role in plant-pathogen interactions (Williamson et al., 2002). An increase in *myo*-inositol and mannitol was observed in the leaves of CLas-infected sweet oranges (Cevallos-Cevallos et al., 2011). An increase in many sugar alcohols including mannitol, *chiro*-inositol, and *myo*-inositol was found in *D. citri*-infested citrus trees (non-published data). Because polyols have a high antioxidant capacity and can act as

osmoprotectants, high levels of these sugar alcohols in citrus trees could help them tolerate CLas infection.

Most sugar acids were found at high levels in ‘Sugar Belle’ mandarin. Saccharic acid and galactonic acid were increased in CLas-infected Cleopatra mandarin (*Citrus reticulata*) (Albrecht et al., 2016). Saccharic acid, galactaric acid, and arabino-hexaric acid were induced in *M. paniculata* upon infestation with *D. citri* (non-published data). In addition, an increase in feruloyl-derivatives of galactaric and glucaric acids was also observed in leaves from sweet oranges infected with CLas (Hijaz et al., 2013). Several defensive roles have been assigned to the production of oligomeric galacturonic acid in plants including activation of guanine-proteins, influx of calcium, induction of oxidative bursts, and induction of kinases and phospholipase C (Fürstenberg-Hägg et al., 2013). Galacturonic acid oligomers are produced through the hydrolysis of plant cell walls by polygalacturonases and pectic lyase. These enzymes are activated by jasmonic acid (Fürstenberg-Hägg et al., 2013). Our results together with the previous results indicate that sugar acids may play a role in citrus-CLas interaction.

In this study, we explored the polar and volatile leaf metabolites of ‘Sugar Belle’ mandarin and found that they were high in many metabolites that are implicated in plant defense against pathogen attacks. Future studies should also investigate other physical properties such as the sieve tube and sieve plate structure to determine if there are any structural differences among CLas-tolerant and susceptible cultivars. In addition, the tolerance of newly released hybrids should be evaluated using grafting/psyllid inoculation under controlled greenhouse conditions to generate faster and more accurate results.

The fact thatHLBdisease has killed millions of citrus trees and decreased citrus production in Florida, encouraged citrus breeders to identify citrus cultivars that could be tolerant to HLB. These tolerant cultivars could be bred with others to develop commercial tolerant cultivars in order to replace the traditional susceptible cultivars, especially the sweet oranges and grapefruits. Tolerant citrus cultivars would reduce the cost of citrus production by decreasing the use of insecticide and would be an eco-friendly solutions.

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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Figure 1: Pedigree of ‘Sugar Belle’ mandarin (LB8-9) and its parents.

Figure 2. Principal components analysis (A), its associated loading plot (B) and Hierarchical cluster analysis (HCA) and heat-map (C) using the concentration of all detected leaf volatiles in different citrus cultivars using GC-MS ($n=7$). In heat-map (C), rows represent the individual volatile compounds abundance and columns represent different cultivars. Volatiles and cultivars are organized using two-way HCA based on similarities using Bary-Curtis test (paired-groups). Cells are colored based on the abundances, red represents high abundances and green represents low abundances. Abbreviations: DG; ‘Duncan’ grapefruit, DT; ‘Dancy’ tangerine, CM; ‘Clementine’ mandarin, MT; ‘Minneola’ tangelo, and SB; ‘Sugar Belle’ mandarin

Figure 3. Chromatographic and spectral characteristics of γ -terpinene, *p*-cymene, thymol, and its methyl ether detected in different citrus cultivars. GC-MS chromatograms of γ -terpinene (A), *p*-cymene (B), thymol (C), thymol methyl ether (D) and their mass spectra (E, F, G, and H, respectively), obtained using GC-MS operating in full scan mode in hexane extract of different citrus cultivars leaves. (I) suggested biosynthesis pathway of thymol in citrus plants.

Figure 4. Total peaks area (A) and hierarchical cluster analysis (HCA) and heat-map (B) of different leaf volatile groups detected in different citrus cultivars ($n=7$). In A, different letters indicate statistically significant differences among the studied cultivars. In B, rows represent the volatile groups abundance and columns represent different cultivars. Volatile groups and cultivars are organized using two-way HCA based on similarities using Bary-Curtis test (paired-groups). Cells are colored based on

the abundances, red represents high abundances and green represents low abundances. Abbreviations: DG; ‘Duncan’ grapefruit, DT; ‘Dancy’ tangerine, CM; ‘Clementine’ mandarin, MT; ‘Minneola’ tangelo, SB; ‘Sugar Belle’ mandarin, and nd; non-detected groups.

Figure 5. Principal components analysis (A), its associated loading plot (B) and Hierarchical cluster analysis (HCA) and heat-map (C) using the concentration of all detected leaf non-volatile metabolites in different citrus cultivars using GC-MS ($n=7$). In heat-map (C), rows represent the individual compounds abundance and columns represent different cultivars. Metabolites and cultivars are organized using two-way HCA based on similarities using Bary-Curtis test (paired-groups). Cells are colored based on the abundances, red represents high abundances and green represents low abundances. Abbreviations: DG; ‘Duncan’ grapefruit, DT; ‘Dancy’ tangerine, CM; ‘Clementine’ mandarin, MT; ‘Minneola’ tangelo, SB; ‘Sugar Belle’ mandarin, and nd; non-detected compound.

Figure 6. Total peaks area (A) and hierarchical cluster analysis (HCA) and heat-map (B) of different leaf non-volatile metabolites groups detected in different citrus cultivars ($n=7$). In A, ‘ns’ indicate no statistically differences among the studied cultivars. In B, rows represent the metabolite groups abundance and columns represent different cultivars. Metabolites groups and cultivars are organized using two-way HCA based on similarities using Bary-Curtis test (paired-groups). Cells are colored based on the abundances, red represents high abundances and green represents low abundances. Abbreviations: DG; ‘Duncan’ grapefruit, DT; ‘Dancy’ tangerine, CM; ‘Clementine’ mandarin, MT; ‘Minneola’ tangelo, SB; ‘Sugar Belle’ mandarin.

Contribution

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

Table 1. Citrus cultivars used in this study

Cultivar	Genotype	HLB-susceptibility	References
‘Duncan’ grapefruit	<i>Citrus paradisi</i>	Susceptible	Folimonova et al., 2009
‘Dancy’ Tangerine	<i>Citrus reticulata</i>	Susceptible	McCollum et al 2016
‘Minneola’ tangelo	‘Duncan’ grapefruit x ‘Dancy’ tangerine	Susceptible	McCollum et al 2016
‘Clementine’ mandarin	<i>Citrus reticulata</i>	Susceptible	McCollum et al 2016
‘Sugar Belle’ mandarin	‘Clementine’ mandarin x ‘Minneola’ tangelo, ‘Duncan’ grapefruit x ‘Dancy’ tangerine	Tolerant	Dou and Gmitter, 2007; Stover, 2016

Table 2. Volatile organic compounds (VOCs) extracted by hexane from leaves of HLB-tolerant ‘Sugar Belle’ mandarin hybrid and its parents. VOC concentrations (mmol g⁻¹) * shown as means ± S.D. of 7 replicates analyzed by GC-MS.

Volatile	LRI ²	RT	‘Sugar Belle’ mandarin	‘Clementine’ mandarin	‘Minneola’ tangelo	‘Duncan’ Grapefruit	‘Dancy’ tangerine	p-value
α-thujene	898	7.70	1.30±0.82 ^a	0.25±0.10 ^a ^b	0.44±0.41 ^a ^b	0.16±0.03 ^b	0.68±0.99 ^{ab}	0.015
α-pinene ¹	909	7.85	4.19±1.80 ^b	1.19±0.41 ^b	2.05±1.58 ^b	0.42±0.15 ^b	13.82±9.38 ^a	0.0000
Sabinene ¹	962	8.75	0.34±0.13 ^b	23.83±8.92 ^a	0.40±0.29 ^b	5.41±3.26 ^b	0.40±0.12 ^b	0.0000
β-pinene ¹	966	8.87	3.07±1.37 ^{ab}	0.59±0.19 ^b	2.68±3.23 ^b	0.34±0.36 ^b	10.00±7.84 ^a	0.0003
β-myrcene ¹	985	9.12	0.82±0.52 ^b	3.53±1.36 ^a	0.82±0.50 ^b	0.45±0.16 ^b	2.74±2.96 ^{ab}	0.0001
γ-carene ¹	1008	9.59	0.28±0.06 ^b	16.45±5.32 ^a	0.32±0.12 ^b	0.22±0.07 ^b	0.27±0.16 ^b	0.0000
α-terpinene ¹	1018	9.77	0.27±0.17 ^b	0.24±0.12 ^b	0.15±0.14 ^b	ND ⁵	0.59±0.57 ^a	0.0076
para-Cymene ¹	1026	9.95	1.98±0.84 ^a	0.14±0.04 ^b	0.71±0.50 ^b	ND ⁵	1.37±0.36 ^a	0.0000
d-Limonene ¹	1034	10.06	1.51±0.64 ^a	7.66±1.59 ^a	7.86±13.12 ^a	0.55±0.26 ^a	1.95±0.56 ^a	0.0615
β-phellandrene ¹	1037	10.10	0.07±0.04 ^b	0.03±0.01 ^b	0.06±0.04 ^b	0.03±0.00 ^b	0.47±0.50 ^a	0.0016
Z-β-ocimene ¹	1055	10.43	3.26±1.24 ^b	3.21±1.24 ^b	4.28±2.78 ^b	0.64±0.17 ^b	10.71±2.76 ^a	0.0000
γ-terpinene ¹	1071	10.75	10.85±4.57 ^a	1.11±0.12 ^b	5.21±3.54 ^{ab}	0.84±0.44 ^b	8.64±3.39 ^a	0.0000
Sabinene hydrate ¹	1081	10.97	0.21±0.09 ^b	1.36±0.53 ^a	0.18±0.09 ^b	0.20±0.03 ^b	0.48±0.33 ^b	0.0000
Unk Terp Alc 1	1087	11.10	0.75±0.18 ^{ab}	0.67±0.10 ^{ab}	0.85±0.27 ^{ab}	0.61±0.19 ^b	1.37±1.00 ^a	0.0420
Unk Terp Alc 2	1092	11.20	0.47±0.11 ^{ab}	0.39±0.07 ^b	0.51±0.15 ^{ab}	0.37±0.11 ^{ab}	0.84±0.61 ^a	0.0000
α-terpinolene ¹	1101	11.34	1.01±0.41 ^a	1.37±0.47 ^a	0.46±0.27 ^{ab}	0.06±0.01 ^b	1.39±0.45 ^a	0.0000
Linalool ¹	1114	11.62	71.04±34.68 ^a	24.99±9.36 ^b	26.40±18.29 ^b	2.13±1.42 ^b	108.47±20.92 ^a	0.0000
α-ocimene	1152	12.14	0.04±0.02 ^b	0.03±0.01 ^b	0.05±0.03 ^b	0.03±0.00 ^b	0.26±0.18 ^a	0.0000
allo-Ocimene ¹	1164	12.52	0.28±0.11 ^b	0.03±0.00 ^b	0.16±0.11 ^b	0.03±0.00 ^b	1.08±0.81 ^a	0.0000
Citronellal ¹	1178	12.75	0.03±0.01 ^a	0.74±0.72 ^a	2.88±5.29 ^a	0.18±0.16 ^a	0.03±0.01 ^a	0.1474
Nonanol ¹	1203	13.20	tr ⁴	tr ⁴	tr ⁴	ND ⁵	ND ⁵	ND ⁵
α-terpineol ¹	1236	13.71	0.64±0.28 ^b	1.54±0.61 ^a	0.51±0.34 ^b	0.24±0.05 ^b	1.69±1.01 ^a	0.0000
cis-Carveol ¹	1259	14.08	ND ⁵	ND ⁵	0.06±0.04 ^a	ND ⁵	0.06±0.02 ^a	0.1723
Nerol ¹	1264	14.35	ND ⁵	0.11±0.05 ^a	ND ⁵	ND ⁵	ND ⁵	0.0000
Thymol methyl ether	1268	14.42	0.03±0.01 ^b	ND ⁵	0.53±0.32 ^b	ND ⁵	2.15±0.72 ^a	0.0000
Neral ¹	1280	14.59	0.44±0.12 ^a	0.63±0.10 ^a	0.37±0.08 ^a	0.32±0.09 ^a	0.50±0.48 ^a	0.0588
Geraniol ¹	1292	14.80	ND ⁵	3.92±4.20 ^a	ND ⁵	ND ⁵	0.04±0.05 ^b	0.0008
Geranial ¹	1308	15.82	1.04±0.26 ^{ab}	2.23±1.52 ^a	0.93±0.30 ^b	0.64±0.22 ^b	1.20±0.84 ^{ab}	0.012
Thymol ¹	1324	15.58	2.53±1.15 ^a	0.12±0.02 ^b	0.77±0.68 ^b	0.09±0.03 ^b	2.53±0.48 ^a	0.0000
δ-elemene	1363	16.57	1.46±0.39 ^b	1.18±0.19 ^b	1.39±0.82 ^b	1.14±0.35 ^b	13.68±12.70 ^a	0.0006
Chavicol	1375	17.00	0.10±0.03 ^a	0.10±0.02 ^a	0.10±0.05 ^a	0.08±0.02 ^a	0.08±0.07 ^a	0.8153
Geranyl acetate ¹	1384	17.26	ND ⁵	0.36±0.20 ^a	0.05±0.02 ^b	ND ⁵	0.08±0.08 ^b	0.0000
β-elemene	1400	17.56	4.17±2.06 ^a	0.13±0.03 ^b	0.79±0.56 ^b	1.26±0.75 ^b	0.81±0.29 ^b	0.0000
Me Anthranilate ¹	1424	17.97	0.06±0.02 ^a	ND ⁵	1.95±3.88 ^a	ND ⁵	0.07±0.02 ^a	0.1698
r-β-caryophyllene ¹	1461	18.18	1.58±0.69 ^a	1.42±0.70 ^a	0.39±0.15 ^b	0.82±0.53 ^b	1.14±0.45 ^{ab}	0.0011
γ-elemene	1473	18.30	ND ⁵	0.08±0.02 ^b	0.22±0.14 ^b	0.05±0.02 ^b	3.94±3.29 ^a	0.0000
trans-β-farnesene ¹	1505	18.66	0.11±0.02 ^b	0.62±0.25 ^a	0.11±0.06 ^b	0.18±0.10 ^b	0.10±0.02 ^b	0.0000
α-humulene ¹	1512	18.80	0.50±0.22 ^a	0.16±0.08 ^b	0.18±0.10 ^b	0.24±0.12 ^b	0.66±0.49 ^a	0.0009
Et Cinnamate ¹	1516	19.05	0.09±0.07 ^b	0.32±0.09 ^{ab}	0.12±0.10 ^{ab}	0.10±0.08 ^{ab}	1.25±1.72 ^a	0.038
α-farnesene ¹	1536	19.56	ND ⁵	0.62±0.50 ^b	0.46±0.42 ^b	ND ⁵	8.11±4.96 ^a	0.0000
β-bisabolene ¹	1541	19.69	0.15±0.03 ^a	0.16±0.02 ^a	0.13±0.04 ^a	0.12±0.02 ^a	0.19±0.10 ^a	0.1077
δ-cadinene	1547	19.84	ND ⁵	ND ⁵	0.64±1.18 ^{ab}	ND ⁵	2.99±3.37 ^a	0.0031
BA, 4-ethoxy ethyl ester ³	1548	19.91	1.71±0.46 ^a	1.62±0.24 ^a	1.22±0.66 ^a	1.06±0.49 ^a	1.33±0.35 ^a	0.0540
Elemol	1566	20.43	0.50±0.65 ^a	1.53±0.24 ^a	0.72±0.96 ^a	0.84±0.66 ^a	2.82±5.77 ^a	0.4013
Nerolidol ¹	1570	20.60	ND ⁵	ND ⁵	ND ⁵	ND ⁵	3.09±3.03 ^a	0.0000
γ-gurjunene	1575	20.66	ND ⁵	ND ⁵	ND ⁵	ND ⁵	1.08±1.09 ^a	0.0003
Caryophyllene oxide ¹	1589	21.06	ND ⁵	ND ⁵	ND ⁵	ND ⁵	0.15±0.17 ^a	0.0145
Phytol	2049	28.64	0.65±0.33 ^a	0.53±0.55 ^a	0.170.18 ^a	0.35±0.27 ^a	0.99±1.30 ^a	0.1614

* Concentration was calculated from liner calibration curves of reference standards diluted in hexane and injected in the same manner and under the same GC-MS conditions as biological samples.

¹VOCs were confirmed by their retention times, mass spectra, and by comparison to authentic reference standards; others were tentatively identified first by matching their spectra with NIST 2011 and Wiley Flavor and Fragrance mass spectral databases, and then by their published linear retention indices.

²Experimental linear retention indices (LRI) were calculated based on *n*-alkanes (C₈-C₂₆) injected on Elite-5MS (30 m x 0.25 mm x 0.25 μm) under the same conditions as samples.

³BA: Benzoic acid

⁴tr: traces

⁵ND: non-detected compound

Means in the same row with the same superscript letters are not significantly different from each other using (Tukey's test, $p > 0.05$)

Table 3. Concentrations ($\mu\text{g g}^{-1}$)* of the major metabolites detected in leaves of HLB-tolerant ‘Sugar Belle’ mandarin hybrid and its parents using GC-MS ($n=7$).

Metabolite	LRI ²	RT	‘Sugar Belle’ mandarin	‘Clementine’ mandarin	‘Minneola’ tangelo	‘Duncan’ grapefruit	‘Dancy’ tangerine
Lactic acid ¹	1105	4.60	53.43±11.96 ^a	53.17±19.15 ^a	56.67±15.99 ^a	52.60±21.31 ^a	50.42±14.59 ^a
L-Alanine ¹	1128	5.05	55.83±32.09 ^b	92.33±30.58 ^{ab}	139.04±60.43 ^a	93.65±34.71 ^{ab}	94.71±34.38 ^{ab}
L -Valine ¹	1214	6.48	18.32±0.79 ^b	18.87±1.02 ^b	22.19±1.76 ^a	21.96±2.45 ^a	21.43±2.03 ^a
Benzoic acid ¹	1251	7.04	133.90±55.49 ^a	84.17±20.06 ^{ab}	58.91±10.63 ^b	90.91±50.71 ^{ab}	36.37±4.48 ^b
Phosphoric acid	1267	7.26	1837.77±614.47 ^a	1570.87±416.26 ^a	2015.23±834.01 ^a	1237.59±651.13 ^a	1327.74±426.86 ^a
L -Proline ¹	1290	7.58	740.21±342.53 ^b	1232.81±529.55 ^{ab}	1589.44±488.32 ^a	791.64±373.96 ^b	602.97±157.27 ^b
L -Glycine ¹	1295	7.66	20.58±0.97 ^b	34.56±4.45 ^b	102.57±29.27 ^a	31.47±5.10 ^b	24.55±5.15 ^b
Maleic acid ¹	1296	7.67	114.78±17.79 ^b	81.43±18.00 ^{b,c}	157.12±29.43 ^a	94.35±37.60 ^b	48.34±7.30 ^c
Succinic acid ¹	1307	7.82	42.71±6.30 ^b	57.84±10.02 ^a	45.46±6.11 ^{ab}	42.75±13.37 ^b	37.85±4.86 ^b
Propanoic acid, -1-oxo	1318	7.97	104.75±52.49 ^b	316.85±58.61 ^a	97.29±31.00 ^b	203.27±44.31 ^{ab}	239.56±135.21 ^a
L Serine ¹	1349	8.38	352.82±118.64 ^{bc}	596.81±254.61 ^{ab}	713.35±279.88 ^a	292.87±198.65 ^{bc}	230.32±59.06 ^c
L -Threonine ¹	1372	8.68	79.60±26.99 ^{ab}	69.30±14.24 ^{ab}	98.88±21.55 ^a	60.27±23.32 ^b	68.86±15.28 ^{ab}
Malic acid ¹	1483	10.05	3688.54±795.04 ^a	2754.63±417.41 ^{ab}	3931.31±1558.30 ^a	3207.29±532.03 ^{ab}	1761.04±790.70 ^b
L-Aspartic acid ¹	1514	10.41	182.30±21.60 ^b	130.52±12.04 ^b	489.16±129.79 ^a	110.13±29.14 ^b	151.58±37.81 ^b
γ -aminobutyric acid ¹	1522	10.51	623.72±172.86 ^a	709.20±150.05 ^a	876.50±207.16 ^a	642.59±311.66 ^a	708.48±122.29 ^a
Threonic acid ¹	1552	10.85	1044.07±211.72 ^a	631.18±208.13 ^b	515.75±88.39 ^b	636.43±271.01 ^b	741.39±281.17 ^{a,b}
Xylose ^{1,3}	1647; 1654	11.94; 12.02	135.48±25.20 ^b	140.94±29.78 ^b	211.62±44.35 ^a	110.29±39.85 ^b	110.18±19.89 ^b
p-Syneprhine ¹	1688	12.40	426.41±154.05 ^a	368.06±82.07 ^a	150.69±208.13 ^b	ND ^d	398.49±133.79 ^a
Xylitol ¹	1732	12.88	80.88±102.87 ^b	102.87±26.01 ^b	128.72±49.92 ^b	319.62±189.27 ^a	93.57±35.93 ^b
Sorbose ¹	1743	13.00	47.64±12.01 ^a	52.75±13.84 ^a	45.41±9.26 ^a	45.10±12.77 ^a	41.67±9.17 ^a
Ribonic acid	1755	13.13	824.51±202.69 ^b	694.04±131.16 ^b	831.83±181.91 ^b	1334.66±468.21 ^a	553.69±161.83 ^b
Citric acid ¹	1816	13.80	8516.26±1915.36 ^{bc}	10497.11±2494.74 ^{abc}	14203.18±6110.99 ^a	7004.71±3147.95 ^c	13687.68±4087.49 ^{ab}
Unk-pyranose	1844	14.10	198.73±46.69 ^{ab}	229.36±104.87 ^{ab}	307.03±78.63 ^a	118.38±20.86 ^b	225.01±134.23 ^{ab}
Quinic acid ¹	1854	14.21	21643.46±10066.36 ^a	15075.07±5695.15 ^a	21290.26±7204.89 ^a	23514.53±6967.37 ^a	12663.62±5310.40 ^a
Fructose ^{1,3}	1862; 1870	14.29; 14.38	1009.71±341.64 ^a	813.38±283.48 ^{ab}	498.04±155.15 ^b	544.86±139.00 ^b	676.96±196.75 ^{ab}
Mannose ^{1,3}	1878; 1898	14.47; 14.68	284.28±76.04 ^a	297.45±55.68 ^a	279.71±68.43 ^a	201.67±62.79	253.15±69.82
Glucose ^{1,3}	1886; 1901	14.55; 14.71	1678.09±356.03 ^a	1176.09±332.20 ^b	527.61±154.86 ^c	562.64±245.65 ^c	813.61±416.91 ^{bc}
Mannitol ^{1,3}	1913	14.84	54.99±5.45 ^a	52.26±6.56 ^a	49.60±2.77 ^a	50.37±5.36 ^a	53.57±5.71 ^a
Galacturonic ¹ acid	1918	14.90	50.80±13.43 ^a	47.03±10.53 ^a	43.87±14.37 ^a	46.74±15.72 ^a	47.64±18.53 ^a
<i>chiro</i> -Inositol ¹	1939	15.12	516.88±103.59 ^a	392.86±109.25 ^{ab}	320.55±92.35 ^b	303.11±97.48 ^b	267.02±116.34 ^b
Gluconic acid ¹	1975	15.50	78.50±38.12 ^b	77.75±29.95 ^b	84.32±21.98 ^b	196.18±110.85 ^a	42.72±16.49 ^b
Saccharic acid ¹	1991	15.67	452.64±84.49 ^a	384.63±117.12 ^{ab}	316.51±116.24 ^{ab}	279.06±133.10 ^b	318.42±123.38 ^{ab}
<i>scyllo</i> -Inositol ¹	2005	15.82	119.02±14.63 ^{bc}	123.13±33.55 ^{bc}	140.20±18.51 ^b	219.98±76.17 ^a	68.28±14.63 ^c
Galactaric acid	2017	15.95	104.57±28.73 ^a	52.37±14.07 ^b	51.56±17.31 ^b	42.41±17.71 ^b	48.29±17.54 ^b
Palmitic acid	2031	16.10	132.30±17.46 ^{ab}	93.03±19.43 ^b	177.21±73.95 ^a	84.63±24.83 ^b	95.22±28.47 ^b
<i>myo</i> -Inositol ¹	2059	16.40	209.25±28.65 ^a	226.13±42.41 ^a	136.60±36.21 ^b	169.43±72.73 ^{ab}	160.83±27.89 ^{ab}
Ferulic acid ¹	2078	16.60	825.26±529.10 ^a	450.72±242.05 ^{ab}	275.94±67.41 ^b	185.27±32.20 ^b	244.25±119.45 ^b
Arabino-hexaric acid	2082	16.64	1309.16±340.78 ^a	986.81±277.05 ^{ab}	765.49±324.52 ^{ab}	1103.12±796.57 ^{ab}	601.02±212.37 ^b
Caffeic acid	2126	17.10	173.69±112.47 ^a	99.05±18.62 ^{ab}	56.74±8.72 ^b	55.18±10.17 ^b	74.74±8.44 ^b
Stearic acid	2243	18.33	88.69±25.96 ^b	135.43±51.20 ^{ab}	225.78±119.49 ^a	114.64±73.79 ^b	94.92±33.68 ^b
Glycerol-glyceride	2304	18.97	130.60±21.8 ^a	123.78±22.20 ^a	124.27±23.82 ^a	107.53±19.88 ^a	102.88±32.73 ^a
Sucrose ¹	2597	22.0	1663.83±425.18 ^c	5000.66±1391.59 ^a	4519.83±678.42 ^a	2573.56±573.94 ^{bc}	3026.83±386.53 ^b

*Concentrations in $\mu\text{g g}^{-1}$ calculated based on calibration curves made from authentic standards derivatized and injected in the same manner and under the same GC-MS conditions as biological samples.

¹Polar leaf metabolites were confirmed by their retention times, mass spectra, and by comparison to authentic reference standards; others were tentatively identified first by matching their spectra with NIST 2011 and Wiley 9th Ed. mass spectral databases, and then by their published linear retention indices.

²Experimental linear retention indices (LRI) were calculated based on *n*-alkanes (C₈-C₂₆) injected on Phenomenex ZB-5MS (30 m x 0.25 mm x 0.25 µm) under the same conditions as samples.

³Compounds showing two RT and LRI values had two chromatographic peaks (as described by Villas-Bôas et al. 2006).

⁴ND: non-detected compound

Means in the same row with the same superscript letters are not significantly different from each other using (Tukey's test, $p > 0.05$)

Figure1

Figure2

Figure3

Figure4

A

B

Figure5

Figure6