

Herbivory by the insect *Diaphorina citri* induces greater change in citrus plant volatile profile than does infection by the bacterium, *Candidatus Liberibacter asiaticus*

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The volatile organic compound (VOC) profile in plant leaves often changes after biotic and abiotic stresses. Monitoring changes in VOCs in plant leaves could provide valuable information about multitrophic interactions. In the current study, we investigated the effect of Asian citrus psyllid (ACP) infestation, citrus greening pathogen (*Candidatus Liberibacter asiaticus* [CLas]) infection, and simultaneous attack by ACP and CLas on the VOC content of citrus leaves. Leaf volatiles were extracted using hexane and analyzed with gas chromatography-mass spectrometry (GC-MS). Although ACP is a phloem-sucking insect that causes minimal damage to plant tissues, the relative amount of 21 out of the 27 VOCs increased 2- to 10-fold in ACP-infested plants. The relative amount of *d*-limonene, β -phelandrene, citronellal, and undecanal were increased 4- to 20- fold in CLas-infected plants. A principle component analysis (PCA) and cluster analysis (CA) showed that VOC patterns of ACP-infested and CLas-infected plants were different from each other and were also different from the controls, while the VOC pattern of double-attacked plants was more like that of the controls than that of ACP-infested or CLas-infected plants. VOC amounts from leaves were compromised when plants were attacked by ACP and CLas. The results of this study showed that a simple direct extraction of citrus leaf volatiles could be successfully used to discriminate between healthy and CLas-infected plants. Information about the effects of insect and pathogen attack on the VOC content profile of plants might contribute to a better understanding of biotic stress.

Introduction

The main functions of plant volatiles are to protect plants from insect and pathogen attack, attract pollinators and other beneficial animals, and serve as communication signals within plant and among plants.¹ Both biotic and abiotic stress can trigger plant volatiles.²

Plant volatiles are synthesized and stored in different sites such as glandular trichomes, secreting ducts and cavities, secretory cells in flowers and root, and in extra-floral nectar.³ In *Citrus* *sp* L. species, essential oils are present in special oil glands in leaves, flowers, peel, juice vesicles, and seeds.⁴ The composition of citrus leaf oils is mainly stable and shows small variation from one season to another.⁵ In addition, it is slightly affected by the rootstock.⁴

The Asian citrus psyllid (ACP), *Diaphorina citri* (Kuwayama) (Hemiptera: Psyllidae), is the main vector of the citrus greening pathogen (*Candidatus Liberibacter asiaticus* [CLas]), a phloem-limited, uncultivable, and gram-negative bacterium.⁶ Recent

studies using 16S rRNA gene sequencing showed that 3 species of the bacterium, *Candidatus Liberibacter*, have been implicated in citrus greening: asiaticus (CLas) (in Asia, North America, and Brazil), africanus (CLaf) (in Africa), and americanus (CLam) (in Brazil).⁷ CLas and CLam are transmitted by ACP, while CLaf is transmitted by the African citrus psyllid *Trioza erytrea* (Del Guercio) (Hemiptera: Triozidae).⁸ The ACP transmits the HLB pathogen during feeding activities on citrus phloem sap.

Most studies on the insect-plant interactions focused on volatiles released from herbivore-damaged plants.⁹ Rupturing of the storage glands by insect feeding causes immediate release of stored volatiles.

Volatiles released from herbivore-damaged plants are distinctively different from those released from mechanically damaged plants.¹⁰ Mechanical damage of cotton leaf induces glands to release stored terpenes and induced emissions of green-leaf volatiles (GLVs). Some volatiles like indole and hexenyl acetate are released in higher levels in herbivore-damaged plants than

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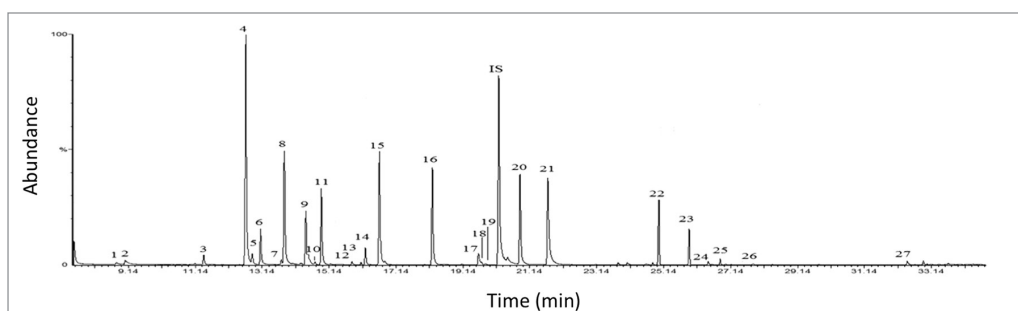


Figure 1. GC-MS chromatogram of healthy Valencia leaf hexane extract. The numbers above the peaks refer to the numbers listed in **Table 1**. IS: internal standard used to calculate the absolute amount of each compounds.

mechanically damaged plants.¹¹ Volatile emissions and plant defense are triggered by enzymes and elicitors from insect herbivores.^{10,12}

Volatiles from herbivore-attacked plant are released immediately or synthesized after several hours or even days after attack.⁹ In general, plant responses differentially to chewing insects than piercing-sucking insects. In the case of chewing insects, damaged tissue release GLVs that are produced immediately after insect damage.¹³ GLVs contain C6 aldehydes, alcohols, and esters such as hexanal, hexenol, and hexyl acetate, respectively. Piercing-sucking insects cause release of stored volatiles after enhancing by elicitors.

Volatiles released from herbivore-damaged plants may result in direct or indirect defense against herbivores; deterring herbivores as well as attracting natural enemies such as arthropod predators, parasitoids, and birds.¹⁴ Plant volatiles released by herbivore-damaged plant can also transmit information within plant and potentially among plants.¹ For example, laboratory studies showed that the expression of several genes involved in defense metabolism were elevated in *Phaseolus vulgaris* (lima bean) that was exposed to volatiles from herbivore-infested neighbors.¹⁵

Released volatiles are usually collected using a closed push/pull system. In this method, volatiles are pulled out through a trap that contains specific adsorbent. The collected volatiles are eluted with an organic solvent and analyzed using GC-MS.^{16–18} Although this procedure is simple and does not require sample cleanup, the type and the size of the adsorbent, collecting time, and collecting rate affect the quantity and quality of collected volatiles. Accordingly, the results obtained by this method may not reflect the actual released volatiles.

Studying the emission of plant volatiles in response to biotic stress is important because they are involved in direct and indirect defense. However, studying the total volatile contents of plant leaves after the attack might contribute to a better understanding of plant response to pathogen and insect attack, mechanism of volatile release, and the relationship between stored and released volatiles.

Because volatile extraction from plant leaves is difficult and require purification, in some studies volatile contents of plant leaves were collected from grounded samples using purge and trap apparatus¹⁹ or were extracted with solid phase micro-extraction (SPME).²⁰

Available information about the response of citrus leaf volatiles to ACP feeding and CLAs infection is limited and only 2 reports have considered the response of citrus leaf volatiles to CLAs infection. In first report, researchers used solid-phase microextraction (SPME) coupled with (GC-MS) to study the effect of CLAs on citrus leaf volatiles.²⁰ In second study, they used GC-MS to identify citrus leaf volatiles that were released in the headspace of CLAs-infected citrus trees and studied their effect on psyllid behavior.¹⁸ The objective of this study was to determine the effect of ACP infestation, CLAs infection, and the double attack of ACP and CLAs on the volatile content of citrus leaves.

Materials and Methods

Insect colonies and citrus tree maintenance

Field-collected ACP adults were used to establish insect colonies. Colonies were maintained in controlled growth rooms at $28 \pm 1^\circ\text{C}$, $60 \pm 2\%$ RH, and under an L16:D8 h photoperiod. Eight-month-old seedlings of Valencia sweet orange (*Citrus sinensis* (L.) Osbeck) were used in this study when we initiated the experiment. Trees were maintained in an insect-proof, AC-controlled greenhouse (28°C , 40% RH, L16:D8). To obtain infected trees with CLAs, 8-mo-old sweet orange seedlings from Valencia were grafted with 4 pieces of budwood sticks from a PCR-positive HLB source. Eight months later, the infection was confirmed using PCR as described by Tatineni et al.²¹ PCR was also used to check for the presence of CLAs in citrus trees and to insure that ACP colonies were negative from CLAs.

Tree exposure to Asian citrus psyllid (*Diaphorina citri*)

Eighteen-month-old healthy or CLAs-infected Valencia seedlings were experimentally exposed to healthy psyllids. Each tree was challenged with 50 adults and individually caged using insect rearing cages ($30 \times 15.5 \times 15$ inches). Exposed trees were kept in growth rooms under the condition as described before. Five months post exposure; 6 mature leaves were collected from each tree from different positions for further analysis. Control (healthy) and CLAs-infected trees without any exposure to ACP were also kept under the same condition in separate cages. For each of the 4 treatments, 6 replications have been used.

Extraction of citrus leaf volatiles

Leaves were ground to a fine powder with liquid nitrogen and 100 mg was transferred to a 1.5 ml micro-centrifuge tube.

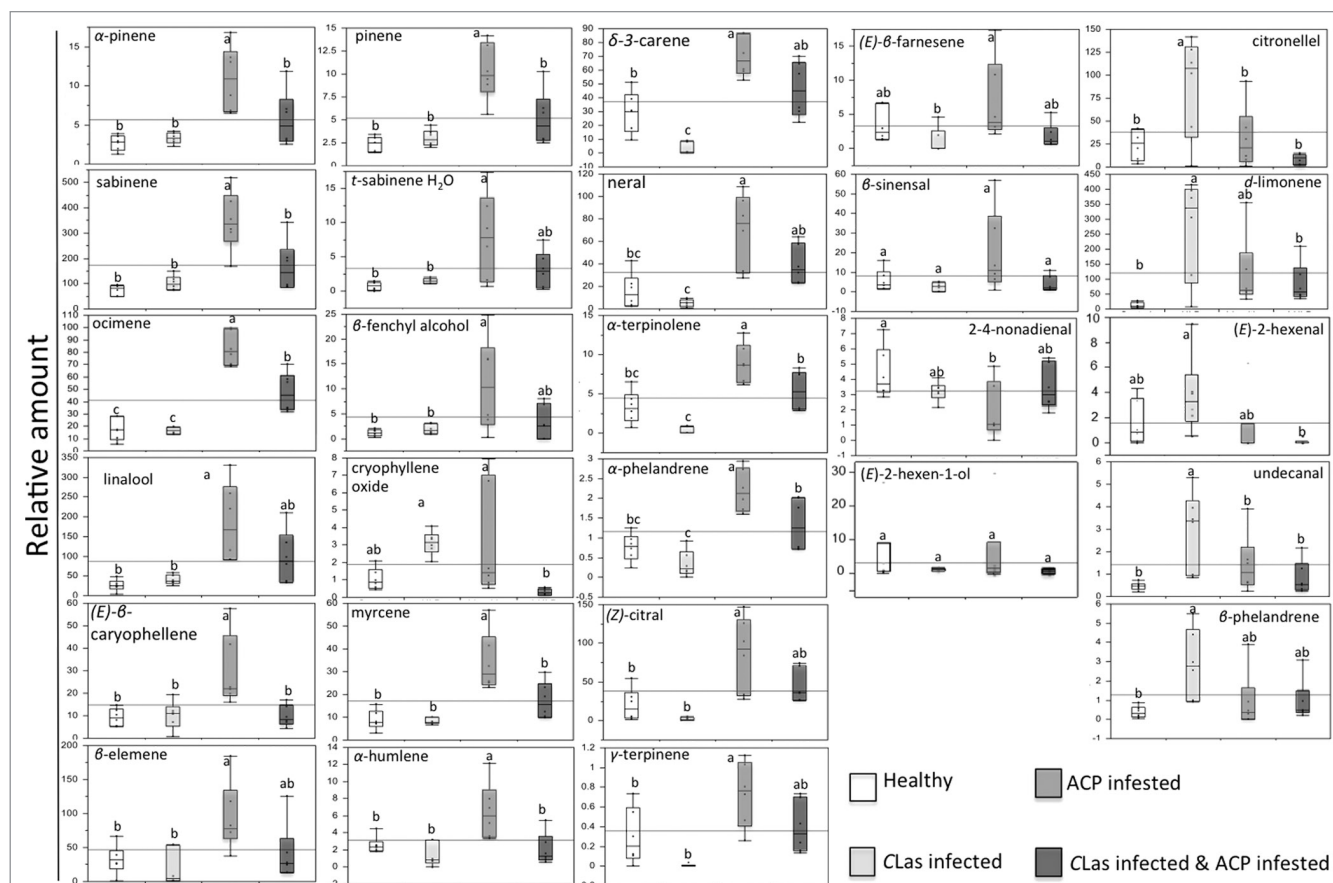


Figure 2. Relative amount of volatile compounds in different treatment. The peak areas were normalized using internal standard (*trans, trans*-2,4-nonadienal). Horizontal thick lines indicate the medians; boxes show the interquartile ranges including 50% of the values; whiskers reflect the highest and the lowest number of visits; and different letters indicate statistically significant different treatments ($P < 0.05$).

A 0.5-ml aliquot of hexane (Sigma) was added to the samples and vortexed for 30 sec. Samples were left on ice for 10 min and the vortexing was repeated 2 times. At the end of the extraction, samples were removed from the ice and centrifuged at 12,000 rpm for 1 min. A 0.2-ml aliquot of the organic layer was spiked with *trans, trans*-2,4-nonadienal (Sigma) as internal standard at a final concentration of 200 ppm, and 1 μ l of the spiked sample was injected into the GC-MS running in the full scan mode. Overall, 6 plants and 6 leaves from each were used for each treatment and two runs/replicate were performed.

GC-MS analysis

Citrus leaf volatiles were analyzed using a Clarus 500 GC-MS system (Perkin Elmer) fitted with an HP-5MS column (cross-linked 5% Ph Me siloxane, 50 min \times 0.22 mm \times 0.025 μ m film thickness). The flow rate for the helium carrier gas was 0.7 ml/min. The following GC temperature program was used: hold at 50 $^{\circ}$ C for 3 min, then increase to 200 $^{\circ}$ C at a rate of 5 $^{\circ}$ C/min, increase further to 250 $^{\circ}$ C at 10 $^{\circ}$ C/min, and finally hold at 250 $^{\circ}$ C for 2 min. The injector and the detector temperatures were 250 $^{\circ}$ C and 180 $^{\circ}$ C, respectively.

Peak identification and quantification

GC-MS chromatograms were analyzed using TurboMass software version 5.4.2 (Perkin Elmer). Peak identifications were

achieved using NIST (Natl. Inst. of Standards and Technology) and Wiley 9th edition (John Wiley and Sons, Inc.), mass spectra database libraries and linear retention index (LRI). The LRI values were calculated using a calibration curve generated by injecting a mixture of alkane (C8–C18) under previously described conditions. The percentage peak areas were calculated by dividing the peak area of each compound by the total area. To fairly compare the relative amount of each compound in different treatments, we normalized the peak areas using *trans, trans*-2,4-nonadienal as the internal standard (IS). The compounds' amounts were normalized by dividing their peak areas by IS peak area.

Statistical analyses

Statistical analyses were performed using JMP version 9.0 (SAS Institute Inc.). Data were normally distributed. Analysis of variance (ANOVA) was used to compare estimated volatiles in different treatments as a percentage (proportion) or normalized (amounts). Post hoc pairwise comparisons between treatments were performed with the Tukey honestly significant different test. Principal component analysis (PCA) and cluster analysis (CA) were used to discriminate among treatments. The PCA and CA were performed using the normalized data of individual VOCs and 3 main groups. PCA was used to summarize the pattern of correlation among the detected compounds in the

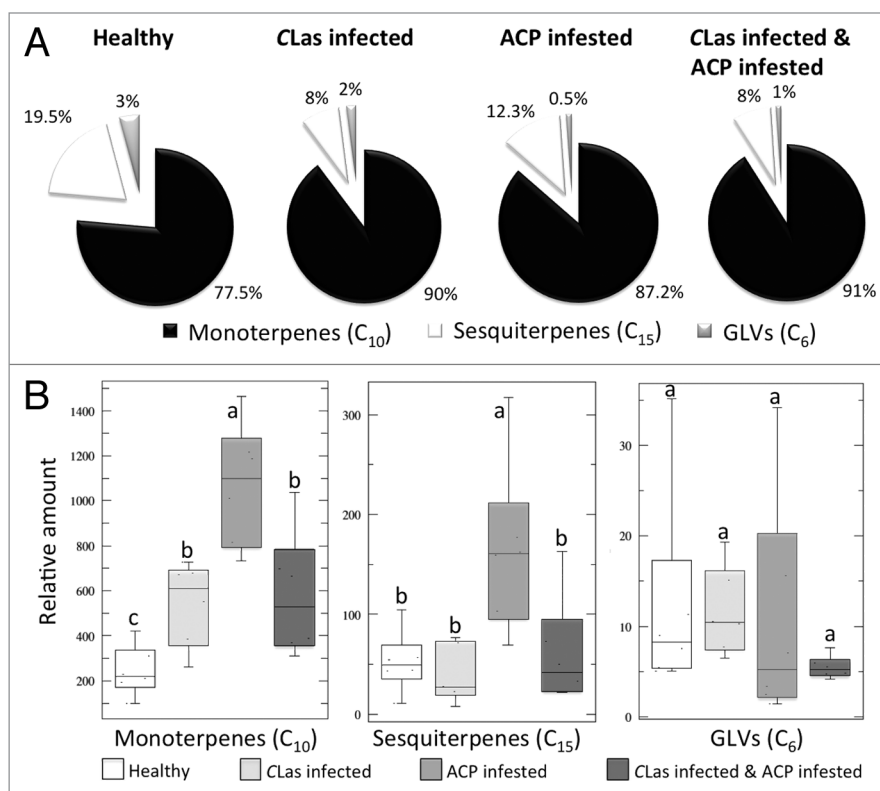


Figure 3. Proportions (percentages) and relative amount of volatile compounds categorized into groups in hexane extract of Valencia leaves. **(A)** Proportions (percentage). Number followed by each compound represents its percentage. **(B)** Relative amounts. Horizontal lines indicate the medians; boxes show the interquartile ranges including 50% of the values; whiskers reflect the highest and the lowest number of visits; and different letters indicate statistically significantly different treatments ($P < 0.05$).

different treatments. CA based on the Mahalanobi's squared distance between groups from the discriminant function analysis (DFA), with 95% confidence, was used to construct the similarity dendrograms.

Results

Volatile profile in Valencia orange leaf

In order to study the effect of ACP-infestation and/or CLas infection on the composition of volatile components in citrus leaves, we used hexane to extract the total volatiles. The composition of Valencia leaf individual volatiles is presented in Table 1 and the corresponding chromatogram is shown in (Fig. 1). Overall, 27 compounds were detected and identified in Valencia leaf. The main components were sabinene 26.2%, β -elemene 11%, δ -3-carene 9.1%, linalool 8.4%, citronellal 7.4%, ocimene 5.7%, (*Z*)-citral 6.3%, and neral 4.4% (Table 1).

Citrus responses systemically to biotic stress

In order to examine whether citrus plants respond systemically to the infection with CLas, the infestation with ACP or both together, leaves from different locations in plants were analyzed. No changes in volatile profile or volatile relative amounts were found among all tested leaves within each treatment. Additionally, for CLas-infected plants, no differences were found

between symptomatic and asymptomatic leaves (data non shown). Data were pooled in further analyses. This result suggests a systematic response within the plant to the biotic stresses.

Infection with CLas and/or infestation with ACP alter the volatile proportions profile

To compare the profile of different treatments, we calculated the proportion of each compound to all others for each treatment. The volatile profile of CLas-infected plants was more affected than that of ACP-infested or combined ACP and CLas. Four compounds were increased and 10 compounds were decreased in the profile of CLas-infected plants compared with control (healthy). The percentages of *d*-limonene, β -phellandrene, citronellal, and undecanal were significantly higher in leaves from CLas-infected plant. A dramatic increase in *d*-limonene percentage from 3.5 to 40.1% was observed. This observation could be used as a biomarker for fast identification of CLas-infected plants. A significant reduction in the percentage of (*E*)-2-hexen-1-ol, myrcene, α -phellandrene, δ -3-carene, γ -terpinene, α -terpinolene, 2,4-nonadienal, neral, (*Z*)-citral, and α -humulene was found in CLas-infected plants (Table 1).

The volatile profile of leaves from plants infested with ACP was relatively similar to the control profile with the exception of two compounds that were increased (linalool and *d*-limonene) and five compounds that were decreased [(*E*)-2-hexen-1-ol, (*E*)-2-hexenal, 2,4-nonadienal, citronellal, and δ -3-carene] (Table 1).

The volatile profile of attacked plants by ACP and CLas was more affected than ACP-infested plants and less affected than those infected by CLas. Seven compounds were decreased and two compounds were increased significantly within the profile. The percentages of the C₆-GLVs [(*E*)-2-hexen-1-ol, (*E*)-2-hexenal, and 2,4-nonadienal], citronellal, (*E*)- β -caryophyllene, α -humulene, and caryophyllene oxide were significantly lower, while the percentage of linalool and *d*-limonene was higher in the double-attacked plants (Table 1).

Infection with CLas and/or infestation with ACP alter volatile amounts

The intensive increase in *d*-limonene percentage (3.5 to 40%) in the CLas-infected plants affected the proportion of compounds to each other. Subsequently, the profile of CLas-infected looked significantly different from those of the healthy or the ACP-infested plants. To compare the relative amount of each compound in different treatments, we used the normalized data as described above. Unlike the profiles, we found that most compounds responded to the infestation with ACP, while only a few of them responded to the infection with CLas. The compounds

Table 1. Proportions (percentages) of VOCs detected in healthy, Clas-infected, ACP-infected, and double-attacked Valencia leaves

Group(1)	Compound	RI	Volatile proportion to each other (%) (2)							
			Healthy	Clas-infected		ACP-infested		Double-attacked		
C ₆ -GLVs										
Alc.	(E)-2-hexen-1-ol	825	0.9	0.9 ± 0.2 ^a	0.3	0.3 ± 0.1 ^b	0.2	0.2 ± 0.0 ^b	0.2	0.2 ± 0.1 ^b
Ald.	(E)-2-hexenal	834	2.1	0.4 ± 0.2 ^a	1.6	0.5 ± 0.2 ^a	0.3	0.0 ± 0.0 ^b	0.8	0.0 ± 0.0 ^b
	2,4-nonadienal	1222		0.6 ± 0.2 ^b		0.2 ± 0.2 ^b		0.7 ± 0.5 ^b		
	undecanal	1230		0.2 ± 0.1 ^b		0.5 ± 0.2 ^a		0.1 ± 0.1 ^b		0.1 ± 0.1 ^b
Total C ₆ -GLVs			3.0		1.9		0.5		1.0	
Monoterpenes (C ₁₀)										
Mt. alc.	β-fenchyl alcohol	1224	8.8	0.4 ± 0.3 ^a	7.6	0.3 ± 0.2 ^a	15.4	0.8 ± 0.7 ^a	15.0	0.4 ± 0.4 ^a
	Linalool	1113		8.4 ± 1.8 ^b		7.3 ± 3.6 ^b		14.6 ± 5.4 ^a		14.6 ± 4.9 ^a
Mt. ald.	Citronellal	1171	18.1	7.4 ± 1.8 ^b	15.1	14.0 ± 4.0 ^a	15.2	2.5 ± 2.2 ^c	15.8	1.7 ± 1.5 ^c
	Neral	1267		4.4 ± 3.2 ^a		0.8 ± 0.4 ^b		5.7 ± 2.5 ^a		6.6 ± 2.1 ^a
	(Z)-citral	1297		6.3 ± 4.4 ^a		0.3 ± 0.4 ^b		7.0 ± 3.6 ^a		7.5 ± 2.6 ^a
Mt. est.	t-sabinene H2O	1082	0.2	0.2 ± 0.2 ^b	0.3	0.3 ± 0.1 ^b	0.6	0.6 ± 0.2 ^a	0.5	0.5 ± 0.1 ^a
Mt. hd.	α-pinene	919	50.4	0.9 ± 0.2 ^a	66.9	0.6 ± 0.3 ^a	56.0	0.9 ± 0.2 ^a	59.6	0.8 ± 0.1 ^a
	Sabinene	965		26.2 ± 10.2 ^a		19.8 ± 13.1 ^a		27.8 ± 2.9 ^a		25.0 ± 2.7 ^a
	Pinene	973		0.8 ± 0.3 ^a		0.6 ± 0.4 ^a		0.8 ± 0.1 ^a		0.8 ± 0.1 ^a
	Myrcene	982		2.8 ± 0.6 ^a		1.5 ± 0.4 ^b		2.7 ± 0.8 ^a		2.7 ± 0.3 ^a
	α-phellandrene	1005		0.2 ± 0.1 ^a		0.1 ± 0.0 ^b		0.2 ± 0.0 ^a		0.2 ± 0.1 ^a
	δ-3-carene	1008		9.1 ± 1.9 ^a		0.7 ± 1.1 ^c		6.0 ± 2.3 ^b		7.4 ± 1.7 ^{ab}
	d-limonene	1032		3.5 ± 1.7 ^b		40.1 ± 22.1 ^a		9.7 ± 8.9 ^b		13.8 ± 7.8 ^b
	β-phellandrene	1042		0.1 ± 0.1 ^b		0.4 ± 0.2 ^a		0.1 ± 0.1 ^b		0.1 ± 0.1 ^b
	Ocimene	1049		5.7 ± 2.1 ^{ab}		3.0 ± 1.3 ^b		7.0 ± 2.1 ^a		7.9 ± 1.7 ^a
	γ-terpinene	1093		0.1 ± 0.1 ^a		0.0 ± 0.0 ^b		0.1 ± 0.0 ^{ab}		0.1 ± 0.0 ^{ab}
	α-terpinolene	1098		1.0 ± 0.3 ^a		0.1 ± 0.1 ^b		0.7 ± 0.2 ^a		0.8 ± 0.2 ^a
Total monoterpenes			77.5		90		87.2		91	
Sesquiterpenes (C ₁₅)										
Sqt. hd.	β-elemene	1419	19.1	11.6 ± 9.0 ^a	7.7	4.8 ± 6.9 ^a	12.1	7.3 ± 2.4 ^a	8.1	5.5 ± 2.8 ^a
	(E)-β-caryophyllene	1452		3.0 ± 0.6 ^a		1.8 ± 0.9 ^{ab}		2.4 ± 1.0 ^{ab}		1.6 ± 0.3 ^b
	(E)-β-farnesene	1473		1.4 ± 1.5 ^a		0.3 ± 0.5 ^a		0.5 ± 0.3 ^a		0.2 ± 0.1 ^a
	α-humulene	1486		0.9 ± 0.4 ^a		0.3 ± 0.4 ^b		0.5 ± 0.1 ^{ab}		0.3 ± 0.1 ^b
	β-sinensal	1691		2.2 ± 2.1 ^a		0.5 ± 0.5 ^a		1.4 ± 1.2 ^a		0.5 ± 0.4 ^a
Sqt. ox.	caryophyllene oxide	1521	0.4	0.4 ± 0.3 ^{ab}	0.5	0.5 ± 0.2 ^a	0.2	0.2 ± 0.2 ^{bc}	0	0.0 ± 0.0 ^c
Total sesquiterpenes			19.5		8		12.3		8.1	

(1) Abbreviations of groups: Alc, Alcohol; Ald, Aldehyde; Mt. alc, Monoterpene alcohol; Mt. ald., Monoterpene aldehyde; Mt. est., Monoterpene esters; Mt. hd., Monoterpene hydrocarbon; Sqt. hd., Sesquiterpene hydrocarbon; Sqt. ox., Sesquiterpene oxide. (2) Numbers that share the same superscript letters, in the same row, are not statistically different at 95% level of confidence.

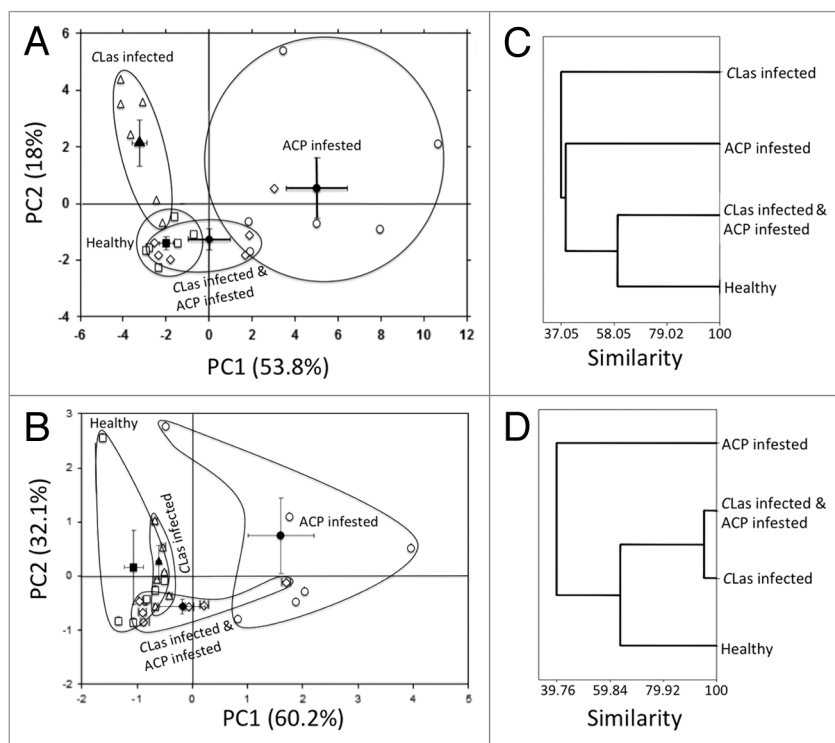


Figure 4. Principal components and cluster analysis of volatile organic compounds in different treatments (A) Principal component analysis of individual components. (B) Principle component analysis of volatile compounds categorized into main groups (monoterpenes, sesquiterpenes, and green leaf volatiles). (C): Cluster analysis using the individual compounds. (D) Cluster analysis of volatile compounds categorized into main groups (monoterpenes, sesquiterpenes, and green leaf volatiles).

were divided into two groups depending on their response to ACP or CLas as described below.

Specific response to ACP

The first group included 21 volatile compounds that were affected in ACP-infested plants (Fig. 2). Seventeen of these compounds were significantly higher. Fourteen out of the 17 were monoterpenes (α -pinene, sabinene, pinene, myrcene, ocimene, τ -sabinene H₂O, γ -terpinene, linalool, (*Z*)-citral, δ -3-carene, α -phellandrene, α -terpinolene, neral, and β -fenchyl alcohol) and the other 3 were sesquiterpenes (β -elemene, α -humulene, and (*E*)- β -caryophyllene). The abundance of these compounds increased between 2 to 10-fold (Fig. 2). None of these compounds were increased in CLas-infested plants. The amounts of these volatiles in CLas-infested trees that were also attacked by ACP were lower than those exposed to ACP only, and higher than those infected with CLas. This finding suggests a compromising effect due to the double attack (Fig. 2). The other 4 compounds displayed non-significant trends were caryophyllene oxide, β -sinensal, and (*E*)- β -farnesene (increased), and 2,4, non-adienal (decreased).

Specific response to CLas

Four volatiles were induced at significantly higher amounts in CLas-infested plants compared with the control (Fig. 2). These compounds were three monoterpenes (*d*-limonene, β -phellandrene, and citronellal) and an aldehyde (undecanal).

These compounds were higher in CLas-infested plants than ACP-infested plants. The amounts of β -phellandrene, citronellal, and undecanal were induced 4-fold, while *d*-limonene induced to more than 20-fold (Fig. 2). Surprisingly, the amount of δ -3-carene, known for its antimicrobial activity, was decreased significantly in CLas-infested plants. We also observed that the double attack compromised the amount of these compounds. As expected, the C₆-GLVs [(*E*)-2-hexenal and (*E*)-2-hexen-1-ol] were not significantly affected.

The effect of CLas and/or infestation with ACP on volatiles as categories

The 27 detected compounds were classified into 3 main categories; C₆: GLVs, C₁₀: monoterpenes, and C₁₅: sesquiterpenes (Table 1 and Fig. 3). The percentages of these categories in healthy leaves were 3%, 77.5%, and 19.5%, respectively. These 3 categories consisted of 8 groups based on their functional group. C₆-GLVs included 2 groups: Alcoholic (0.9%) and aldehyde (2.1%) compounds. Monoterpenes include monoterpenes hydrocarbon (50.4%), monoterpenes aldehyde (18.1%), monoterpene alcohol (8.8%), and monoterpene esters (0.2%). The 2 functional groups of sesquiterpenes were sesquiterpene hydrocarbons (19.1%) and sesquiterpene oxide (0.4%).

The monoterpenes category was increased significantly in all treatments while both C₆-GLVs and sesquiterpenes were decreased (Fig. 3A).

The percentage of monoterpene hydrocarbon increased to 66.9, 56.0, and 59.6% in the CLas-infested, ACP-infested, and double-attacked leaves, respectively. These increases were due to the great increase in *d*-limonene and β -phellandrene in CLas-infested and *d*-limonene, sabinene, and ocimene in ACP-infested and double-attacked plants. Finally, the percentages of monoterpene alcohols, especially linalool, were increased in ACP-infested and double-attacked plants but remained constant in CLas-infested plants compared with the control.

Changes of the relative amounts of the main group due to CLas-infection and/or ACP-infestation are shown in Figure 3B. There was a significant increase in monoterpenes in all treatments compared with control. ACP-infested plants possessed a higher amount of monoterpenes than CLas-infested and double attacked plants. No significant differences in the amount of monoterpenes were observed between CLas infected and double attacked. Sesquiterpenes increased significantly only in ACP-infested plants. No significant differences among treatments were found in C₆-GLVs.

Clustering and multivariate analysis

PCA using the individual VOCs showed clear clustering among treatments and we were able to discriminate between CLas-infested and ACP-infested plants (Fig. 4A). The principal components (PC) 1 and 2 accounted for 71.1% of the variation. Pinene, α -pinene, sabinene, myrcene, α -phellandrene, ocimene,

α-sabinene H₂O, *α*-terpinolene, linalool, neral, and (*Z*)-citral were the compounds with the highest absolute loading values in PC1. While *d*-limonene, *β*-phellandrene, citronellal, undecanal, (*E*)-*β*-caryophyllene oxide, and (*E*)-*β*-caryophyllene were among the compounds with the highest absolute PC 2 loading. In addition, PCA using the three main groups of VOCs showed a clear discrimination between control and ACP-infested plants (Fig. 4B). The principal components (PC) 1 and 2 accounted 92.2% of the variation. Monoterpenes has the highest sum of the absolute PC 1 and 2 loading, followed by sesquiterpenes. Because VOC patterns of the samples in each treatment were clustered around their centroid, this supports our findings that plant responses to ACP and CLas attack were systematic.

Likewise PCA and CA using individual VOCs or main groups showed that VOCs profile in the leaves from double-attacked plants was closer to the profile of CLas-attacked plants than ACP-attacked plants (Fig. 4C and D). CA using the 3 main categories showed high similarity between CLas-infected and double-attacked plants (95.13%) (Fig. 4D). The ACP-infested plants were more distinguished from the control (39.76% similarity) than CLas-infected and double-attacked plants (63.45%) (Fig. 4D). CA analysis using individual volatiles showed that VOCs profile in the leaves from double-attacked plants was closer to the profile of CLas-attacked plants than ACP-attacked plants (Fig. 4C). VOCs in double-attacked plants were closer to the control (59.61% similarity) than the ACP-infested (39.01% similarity) or CLas-infected (37.05% similarity) (Fig. 4C).

Discussion

Twenty-seven VOCs were successfully identified and quantified. Most of the compounds detected in our study were reported to be released by citrus leaf.^{18,23} No significant changes in volatile profile or volatile relative amounts were found among leaves from different positions within the plant in each treatment suggesting a systemic response in citrus plants as previously described.²⁴

In our study, the major group of VOCs in leaf from healthy plants was terpenes (97%), while C6-GLVs represented only 3%. Terpenoids represent the largest VOCs in most plant leaves.^{9,25} Both monoterpenes and sesquiterpenes protect plants against biotic or abiotic stresses.^{24,26}

Plant infestation by herbivores induces compounds implicated in antixenosis or antibiosis,²⁷ and attracts natural enemies.²⁸ In addition, the infection with pathogens induces the production of antimicrobial compounds.²⁹ In general, terpenoids play an important role in plant-herbivore-pathogen interaction.³⁰ Many plants show high emission of monoterpenes after herbivory and/or pathogen attack. Monoterpenes may act as kairomones in attracting predators and parasitoids to attacked plants,¹⁴ as well as function as phytoalexins,³¹ affect oviposition behavior,^{32,33} and act as feeding deterrents for insects.³⁴ Monoterpenes and sesquiterpenes were found to be induced systemically in *Pinus sylvestris* by egg deposition of the sawfly *Diprion pini*.²⁴

As expected, in our results, there were no significant differences in the abundances of GLVs (C6) between ACP-infested and/or CLas-infected and control plants. In fact, the plant

bleeds GLVs instantaneously from disrupted tissue as a wounding response, but ACP causes minimal damage to plant tissue; therefore, no effect on GLVs was found.

Plant response for piercing-sucking insects such as aphids, mealy bugs, leafhoppers, and psyllids is different than that for chewing insects.³⁵ Although piercing-sucking insects do not cause significant cell damage, during their prolonged stylet interactions, injected elicitors might induce specific reactions.³⁶ In general, chewing insects induce the expression of jasmonic acid pathway (JA) regulated gene and wound-responsive gene while the piercing-sucking insects such as ACP induces salicylic acid (SA) signaling pathway³ and JA pathway.³⁶ In ACP-infested leaves, 17 compounds were significantly higher in ACP-infested plants. Attack with other species of psyllids induces many changes in leaf VOCs. For example, *Psylla pyricola* feeding causes simultaneous changes in the composition of released and stored VOCs in pear trees.³⁷ Relative abundance of secondary metabolite volatiles in the leaves of avocado was correlated positively with psyllid (*Trioza anceps*) attack.³⁸ Aphids also induce the release of VOCs including *α*-pinene, *β*-pinene, cymene, *α*-phellandrene, and *d*-limonene.³⁹

In our study, monoterpene alcohol in ACP-infested and double-attacked plant leaves was changed from 8.8% to 15.4%. The main volatile compound of monoterpene alcohol was linalool, which increased 7-fold compared with the control. In fact, linalool is a cue molecule that helps insects locate and recognize plants.⁴⁰ Linalool synthase in tomato was induced in leaf trichomes by spider mite infestation.⁴¹ Linalool and ocimene were induced in lima bean after spider mite attack.⁴² Ocimene was increased in ACP-infested and double-attacked but not in CLas-infested leaves as compared with the control. It seems that to induce ocimene, plants need several events such as several feeding periods by insect. A study supporting this idea showed that a single event of damage for *Lotus japonicus* plants did not induce the release of (*E*)-*β*-ocimene from the plants.⁴³ The monoterpene myrcene, was also increased in ACP-infested plants. This observation was found in *Arabidopsis* infested by *P. rapae*.⁴⁴ *α*-humulene, (*E*)-*β*-caryophyllene, and caryophyllene oxide were increased as a response to ACP-infestation. Both *α*-humulene and caryophyllene oxide were also induced in response to simultaneous infestation with sap-feeder insect, *Frankliniella occidentalis*, and chewing insect, *Heliothis virescens*.⁴⁵

Some induced volatiles in ACP-infested plants were reported to be biologically active and attractive for natural enemies such as sabinene and *α*-pinene.⁴⁶ (*E*)-*β*-caryophyllene was increased in ACP-infested plants. The release of (*E*)-*β*-caryophyllene attracts entomopathogenic nematodes to *Diabrotica virgifera* larvae around maize roots.⁴⁷

Plants infested with sap-feeder insects such as aphids are induced to release VOCs that attract parasitoid wasps⁴⁸ and determine aphid densities on host plants.⁴⁹ *β*-ocimene, linalool, *α*-farnesene, *β*-farnesene were induced by corn leaf aphid feeding.^{10,49} Our findings revealed that (*E*)-*β*-farnesene was higher in ACP-infested than CLas-infected plants and control. (*E*)-*β*-farnesene is an aphid alarm pheromone and is also released from aphid-infested plants to call parasitoids.⁴⁸

α -pinene was found to repel spruce beetle from *Pinus resinosa* plants and enhance predator's attraction.⁵⁰ In our study, α -pinene increased 5-fold in ACP-infested plants compared with control. The monoterpenes *p*-cymene, α -terpinene, and α -phellandrene repelled *Bemisia tabaci* when these compounds were applied on tomato.⁵¹ β -sinensal was increased (4-fold) in ACP-infested plants. β -sinensal represents 9.4% of compounds in sandalwood oil. This oil produced mortality and deterrent effects against two-spotted spider mite, *Tetranychus urticae* Koch.⁵² (*E*)- β -caryophyllene which increased 3-fold in ACP-infested plants compared with control was reported as attractant to newly emerged females of phloem-sap psyllid, *Cacopsylla picta*, the vector of *Candidatus* Phytoplasma mali in apple.⁵³ Sabinene was the highest compound in healthy trees and increased in all treatments especially in ACP-infested trees (more than 4-fold) and in double-attacked trees (more than 2-fold). (*E*)-ocimene, *d*-limonene, myrcene, and sabinene were the main volatiles produced by the new shoots of citrus that attract ACP.²³

The infection with plant pathogens induces the production of plant volatiles that possess a powerful antimicrobial activity to inhibit the movement of the pathogens within plant tissues.^{3,54} Although, the percentage profile of VOCs in CLas-infected plants was highly affected, only 4 compounds were increased in leaves from CLas-infected plants. These compounds include 3 monoterpenes (*d*-limonene, β -phellandrene, and citronellal) and an aldehyde (undecanal).

The accumulated compounds could play an important role in plant-pathogen interactions. In vitro studies showed that tomato leaf volatiles, including β -phellandrene and other compounds inhibited *Botrytis cinerea* fungus.⁵⁵ The antimicrobial activity of *d*-limonene was also assessed against 8 organisms.⁵⁶ It has been found that CLas-infected citrus plants released less *d*-limonene than healthy plants.¹⁸ In current study, we showed an accumulation of *d*-limonene in leaves from CLas-infected plants. It could be a result of the induction of its biosynthesis and/or inhibition of its release. Theoretically, the level of any volatile in plant leaf is controlled by the rate of its formation and the rate of its loss (release or catabolism).⁵⁷ Volatiles could be released from storage glands or biosynthesized de novo following insect damage.⁵⁸

A previous study showed that CLas-infected citrus plants were initially more attractive to *D. citri* adults than non-infected plants. However, after probing, psyllids moved to non-infected plants and settled.¹⁸ Volatile cues may be implicated in the initial movement of psyllids to CLas-infected plants; while the arrestment is determined by the nutritional status of the host, a phenomenon was reported in other vector-borne diseases.^{17,59} The results of the previous studies together indicate that the infection makes plants more attractive to insect-vectors by suppressing the plants basal defense, releasing attractive volatiles, or by inhibiting the release of deterrent volatiles. This will help in continuously spreading the disease by the vector. From the evolutionary view, there is a two-edge benefit for both the releaser and

the receiver. First, for the releaser, it is a kind of resistance and may be used to invite natural enemies of ACP; second, for the receiver (ACP), this will direct the adults of insects to infest new trees, subsequently, less competition and increased distribution.

The accumulation of volatiles in citrus leaves induced by ACP attack was compromised when citrus plants were attacked simultaneously by CLas and ACP. It has been shown in other systems that the double biotic stress may compromise the plant response.⁴⁵ Plants attacked by more than 1 attacker produce some compounds that may induce a pathway and reduce the activity of another pathway and cause synergistic or antagonistic effects on other pathways. Pathogen-inducible SA causes down-regulation of the JA/ethylene-regulated defense-response genes, as well as JA-regulated wound responses and secondary metabolite accumulation.^{60,61}

CA analysis using individual volatiles or main groups showed that VOCs profile in the leaves from double-attacked plants was closer to the profile of CLas-attacked plants than ACP-attacked plants. PCA of normalized data using individual volatiles or main group showed that double-attacked plants were closer to control than other treatments. These results prove that CLas infection compromises the responses of plant to ACP for its benefit.

Our findings suggest that CLas-infected and double-attacked plants might be more susceptible to other insects and pathogens since some biologically important compounds were decreased. These compounds possess antimicrobial activity or attraction to natural enemies. (*E*)- β -farnesene, the natural enemies attractant, decreased 3-fold in CLas-infected. (*E*)-2-hexenal and (*E*)-2-nonenal have an antimicrobial effect and are related to plant hypersensitive response.⁶² Although, (*E*)-2-hexenal increased in CLas-infected plants and possesses high antibacterial properties even in low concentration,⁶² it decreased severely in CLas-infected after attacking with ACP. (*E*)-2-hexenal and δ -3-carene, which has been reported as an antibacterial compound,⁶³ were reduced significantly in the infected plants with CLas. This suggests that the double attack of CLas and ACP in citrus makes plants more susceptible to other pests and pathogens.

Studying the effect of CLas and ACP in citrus plant volatile contents may lead to producing transgenic plants that over- or down-express certain compounds to enhance citrus resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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