Volatile compounds of Persian and Mexican lime associated with HLB (Huanglongbing) symptoms

Compuestos volátiles del limón persa y limón mexicano asociados con síntomas de HLB (Huanglongbing)

ESTRELLA MENDOZA-PEÑA[1], JUAN CIBRIÁN-TOVAR[2], JULIO VELÁZQUEZ-GONZÁLEZ[3], FELIPE TAFOYA-RANGEL[4] and AUSENCIO AZUARA-DOMÍNGUEZ[5]

Abstract: The bacterium *Candidatus* Liberibacter asiaticus is the pathogen that causes the disease known as Huanglongbing (HLB) in citrus. During the course of the disease, the bacterium affects citrus plant phloem tissues, but their leaves remain asymptomatic for HLB for months to years after initial infection. This limits the early detection and control of the bacterium in infected trees. Therefore, in order to design a diagnostic strategy for HLB, the aim of this study was to quantify the abundance and concentration of the volatile compounds released from young shoots of the Persian lemon (*Citrus latifolia* Tanaka) and the Mexican lemon [*Citrus aurantifolia* (Christm.) Swingle] with and without HLB symptoms. The volatiles emitted by young shoots were captured by Solid Phase Microextraction (SPME) and analyzed in a gas chromatograph coupled to a mass selective detector (CG / MS). The results clearly indicate that young shoots with and without HLB symptoms released different abundances and concentrations of volatile compounds. The compounds: D-limonene, β-ocimene, and caryophyllene were collected at higher concentrations in the young shoots of both lemon species with HLB symptoms. This result shows the feasibility of designing a strategy for early detection of the disease in different species of lemon through recognition of patterns and concentrations of volatile compounds released from infected trees.

Key words: *Diaphorina citri*, new shoots, D-Limonene, β-Ocimene, caryophyllene.

Resumen: La bacteria *Candidatus* Liberibacter asiaticus es el patógeno causante de la enfermedad conocida como huanglongbing (HLB) en los cítricos. Ésta afecta los tejidos del floema y los síntomas de la enfermedad no aparecen en las hojas durante meses o años después de la infección inicial, lo cual limita la detección temprana y el control de la bacteria en árboles infectados. Por lo anterior, con el fin de diseñar una estrategia de diagnóstico del HLB, en esta investigación se cuantificó la abundancia y concentración de los compuestos volátiles liberados de los brotes jóvenes del limón persa (*Citrus latifolia* Tanaka) y limón mexicano [*Citrus aurantifolia* (Christm.) Swingle] con y sin síntomas de HLB. Los volátiles fueron capturados por micro extracción en fase sólida (SPME) y analizados en un cromatógrafo de gases acoplado a un detector selectivo de masas (CG / MS). Los brotes jóvenes con y sin síntomas de HLB liberaron diferente abundancia y concentración de compuestos volátiles. Los compuestos: D-limoneno, β-ocimeno y cariofileno fueron recolectados en mayor concentración en los brotes de ambas especies de limón con síntomas de HLB. Este resultado indica la factibilidad de diseñar una estrategia para la detección temprana de la enfermedad en las diferentes especies de limón a través del patrón y concentración de los compuestos volátiles de los árboles infectados.

Palabras clave: *Diaphorina citri*, brotes nuevos, D-Limoneno, β-Ocimeno, cariofileno.

Introduction

The Asian citrus psyllid, *Diaphorina citri* (Kuwayama, 1908) (Hemiptera: Psyllidae), is the vector of the bacteria *Candidatus* Liberibacter asiaticus (Jagoueix *et al.* 1994), which produce the disease known as Huanglongbing or HLB in the genus *Citrus* (Garnier *et al.* 2000; Halbert and Núñez 2004). In citrus, *D. citri* develops of its biological cycle exclusively in the vegetative young shoots under five centimeters long (Fernández and Miranda 2005). It has been determined that *D. citri* locates its host through its volatile compounds (Wenninger *et al.* 2009; Patt and Sétamou 2010). The hosts of *D. citri* release different numbers of volatile (Wenninger *et al.* 2009; Patt and Sétamou 2010; Robbins *et al.* 2012). Attributed to the plant-insect and plant-pathogen interactions (Patt and Sétamou 2010; Hare 2011). Mann *et al.* (2012) determined that *C.* Liberibacter asiaticus induces volatile compounds in *C. aurantium* and *C. sinensis* young shoots. Also, *D. citri* has a higher response to volatile produced by infested young shoots than those by asymptomatic young shoots of both Rutaceae´s species. The detection and understanding of the role that volatiles play in the chemical ecology of *D. citri* can be used to formulate management strategies for this insect, as well as in the production of resistant or tolerant cultivars through traditional and genetic engineering (Robbins *et al.* 2012). The detection of trees infected with *C.* Liberibacter asiaticus is done by visual inspection of the symptoms, electron microscopy, polymerase chain reaction (PCR), and real-time PCR (Salcedo *et al.* 2010). However, the use of these techniques is limited because the bacteria is in low concentrations in the infected trees and symptoms of HLB do not appear on leaves for months to years after initial infection (Gottwald 2010). This increases the possibility of misdiagnosis and the need to seek new alternatives to detect *C.* Liberibacter asiaticus in citrus more efficiently

[1] Maestra en ciencias, Colegio de Postgraduados, Montecillo, Estado de México, 56230, México, *etoile_dao@yahoo.com.mx*. [2] Doctor en ciencias, Colegio de Postgraduados, Montecillo, Estado de México, 56230, México, *zacarias1999@gmail.com*. [3] Doctor en Ciencias, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Campo Experimental Delicias, Carretera Delicias-Rosales Km 2,5, Chihuahua, 56576, México, *ibtjul2@gmail.com*. [4] Doctor en Ciencias, Universidad Autónoma de Aguascalientes, Aguascalientes, Aguascalientes, 20131, México, *ftafoy@correo.uaa.mx*. [5] Doctor en Ciencias, Tecnológico Nacional de México/I.T. de Cd. Victoria, Ciudad Victoria, Tamaulipas, 87010, México, *azuarad@gmail.com*. Corresponding autor: Ausencio Azuara-Domínguez. Doctor en ciencias, Tecnológico Nacional de México/I.T. de Cd. Victoria, Ciudad Victoria, Tamaulipas, 87010, México, *azuarad@gmail.com*.

(Pacheco *et al.* 2012). In this report, the aim was to quantify the abundance and concentration of the volatile compounds released from the young shoots of Persian lemon (*C. latifolia* Tanaka) and Mexican lemon [*Citrus aurantifolia* (Christm.) Swingle] with and without HLB symptoms.

Materials and methods

Plant material. Young shoots were cut from eight-year-old trees in the flowering stage (January to March in 2013). Samples of Persian lemon tree shoots were collected in Santiago Ixcuintla, Nayarit and samples of Mexican lemon tree shoots were collected in Tecomán, Colima. In both species of lemon, shoots of 1 to 3 cm were collected following the sampling methodology described by the National Agroalimentary Health and Safety Service (Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria-SENASICA 2010). Shoots with symptoms were collected from trees with mature leaves with asymmetric mottling, green islands and marked positive for HLB by SENASICA (SENASICA 2010). The healthy shoots were collected in commercial plots with trees without report or HLB symptoms. The collected shoots were covered with bee wax in order to avoid water loss or aroma change (Flamini *et al*. 2007). Then, they were covered with a paper towel, deposited in a polyethylene bag with a sealing device and transported, in a cooler gel cooler, to the laboratory of the Autonomous University of Aguascalientes in Aguascalientes, Mexico (SENASICA 2010). In the laboratory, the wax layer was removed and the shoots without the presence of nymphs or eggs of *D. citri* were selected.

Collection of volatile compounds. The volatile compounds were sampled from: T1) asymptomatic Persian lime (*C. latifolia*) young shoots (BAA), T2) symptomatic Persian lime young shoots (AAS), T3 asymptomatic Mexican lime (*C. aurantifolia*) young shoots, T4) symptomatic Mexican lime young shoots, and T5) a vial without plant sample (control). Each treatment consisted of ten replicates and the experiment was repeated five times. The collection was done using the solid-phase microextraction (SPME) technique using a polydimethylsiloxane fiber (PDMS-DVB, Supelco, Bellefonte, PA). The wax was removed from the stem base and weighed one gram of plant samples (young shoots). The sample was maintained at room temperature for 20 seconds and placed in a 10 ml glass vial with a polyethylene cap (Sigma-Aldrich®, Toluca, Mexico). Then, the fiber was introduced into the vial and the volatile compounds were captured for 60 minutes. The fiber was removed and placed in the injection port of a gas chromatograph (Agilent 6850 Series II; Agilent, Foster City, CA) linked to a mass selective detector (model 5975C, Agilent, Foster City, CA). The chromatography analyses were done with the instrumentation method described by Patt and Sétamou (2010), using a 30 m DB-WAX column, 0.320 mm internal diameter and 0.25 µm film thickness (Agilent®, Folsom, CA, USA).

Identification and quantification of the volatile compounds. The compounds were identified based on the retention time and mass spectrum of the standard library compounds NIST 2002 version (National Institute of Standard and Technology). The abundance and concentration of each compound were determined by contrasting the area of the volatile compounds collected from the young shoots against the area of the pure standard of D-limonene, 6 % pure (Sigma-Aldrich) (González-Palomares *et al.* 2009; Lin *et al.* 2010).

Data analysis. To determine statistical differences, we applied a Student's t-test for independent samples to the concentration value of the volatiles collected in both types of the two species of lemon young shoots. All statistical tests were considered as two-tailed, and the significance level was set at the value of $P < 0.05$. The continuous variable in this study showed a non-normal distribution by Kolmogorov-Smirnov (Chakravarti *et al*. 1967) and Shapiro-Wilk tests (Shapiro and Wilk 1965). Hence, statistical analysis was performed with the variable after logarithmic transformation of data. Analyses were performed in the 2004 version SAS statistical package (SAS 2004).

Results

In asymptomatic and symptomatic Persian lime tree shoots 29 and 21 volatile compounds, respectively, were collected (Table 1). In both types of shoots, 16 compounds were identified in common. In contrast, in asymptomatic Mexican lime tree shoots, 13 compounds were identified. While in symptomatic shoots of the same lemon species, eight volatiles were collected. In both types of buds, five compounds were identified in common. In asymptomatic and symptomatic lemon tree shoots of both species the following compounds were collected: D-limonene, caryophyllene, and β-ocimene in different amounts and concentrations (Table 2).

Furthermore, analyses of the amount and concentration of the volatiles collected showed statistically significant differences between the two lemon species. The citronellal, β-elemene, α-terpineol, Isogeraniol and lavandulol compounds were more abundant and in higher concentration in asymptomatic Persian lime tree shoots (Fig. 1; $P < 0.05$ and $P < 0.005$ by Student's t-test). In contrast, β-myrcene compounds, D-limonene, β-ocimene, γ-elemene, β-citral and geraniol were collected in higher concentration in symptomatic than in asymptomatic shoots of the same lemon tree species. In the case of the volatiles collected in the Mexican lemon tree, the elemene compound was collected in higher concentration in asymptomatic tree shoots. While in this same lemon species, compounds: D-limonene, caryophyllene and β-farnesene were collected in greater concentration in symptomatic than in asymptomatic tree shoots (Fig. 2; $P < 0.05$, $P < 0.005$ and $P < 0.001$ by Student's t-test). The volatile compound D-limonene was present in both species of lemon tree. However, this compound was collected in higher concentration (13.33 mg / mL) in symptomatic Mexican lemon tree shoots.

Discussion

Different authors have analyzed the volatile compounds released by the host plants of *D. citri* (Wenninger *et al.* 2009; Patt and Sétamou 2010; Robbins *et al.* 2012). In this report, volatiles were collected in Persian lime and Mexican lime tree shoots. In both lime, asymptomatic and symptomatic shoots released different numbers of volatile. Asymptomatic and symptomatic shoots released similar compounds but in

Table 1. Abundance of volatile compounds emitted by young shoots asymptomatic and symptomatic of *Citrus latifolia* and *Citrus aurantifolia*.

Compound	Tr (min)	Abundance (%)			
		C. latifolia		*C. aurantifolia*	
		SA	SS	SA	SS
α-pineno	3.55	0.53	0.50		
Sabinene	5.42	0.59			
β-thujene	5.42	3.44			
β-myrcene	6.38	0.52	4.88		
β-pinene	6.38				2.62
D-limonene	7.22	33.38	45.73	21.62	80.78
β-phellandrene	7.44	0.89	0.63		
Eucaliptol	7.50	1.58	2.10		
γ-terpinene	8.38		0.27		
β-ocimene	8.53	7.25	10.82	1.62	7.30
σ-elemene	14.00				0.81
δ-elemene	14.02	1.09		5.51	
Carene	14.06	0.78			
Terpineol	14.07	0.48			
Citronellal	14.30	1.58	0.34		
β-citronellal	14.31	1.01			
Linalool	16.06	1.03		1.12	
α-bergamotene	16.84	0.96		2.50	
Elemene	16.94			6.35	1.32
Methylgeranate	16.94		0.35		
β-elemene	16.95	1.55	0.85		
Caryophyllene	17.11	0.54	0.86	1.63	2.63
γ-elemene	18.09	0.35	1.40	6.06	
α-cariofileno	18.80			1.13	
β-citral	19.13	10.18	12.64		
α-terpineol	19.58	1.39	0.26		
D-germacrene	19.70	0.69	1.63	3.31	
α-bisabolene	20.10			1.40	
β-bisabolene	20.10	2.61			1.64
Neryl acetate	20.14		1.24		
Citral	20.27	8.20		46.37	
α-citral	20.27		5.74		
α-farnesene	20.56	2.45			
β-farnesene	20.58			1.38	2.90
Citronellol	21.12		0.64		
β-citronellol	21.12	1.06			
Perillal	21.40	0.47			
Isogeraniol	22.04	1.76	0.34		
Geraniol	22.87	3.11	4.99		
Lavandulol	22.87	10.53	3.81		

Tr = Retention time. SA = Shoots asymptomatic. SS = Shoots symptomatic.

different amounts and concentrations. This may be attributed to the plant-insect and plant-pathogen interactions (Patt and Sétamou 2010; Hare 2011). Mann *et al.* (2012) determined that *C.* Liberibacter *asiaticus* induces the release of β-ocimene and D-limonene in *C. aurantium* and *C. sinensis* young shoots. And that the higher percentage found in *D. citri* responds to these volatile compounds emitted from asymptomatic tree shoots of both species of Rutaceae. In this study, β-myrcene, D-limonene, β-ocimene, γ-elemene, β-citral, geraniol, caryophyllene, and β-farnesene had higher concentrations in shoots from trees with symptoms of Huanglongbing. This is of biological significance and could be used for early

Table 2. Concentration of volatile compounds emitted by young shoots asymptomatic and symptomatic of *Citrus latifolia* and *Citrus aurantifolia*.

Compound	Concentration (µg/mL)			
	C. latifolia		*C. aurantifolia*	
	SA	SS	SA	SS
α-pinene	0.08	0.08		
Sabinene	0.08			
β-thujene	0.49			
β-myrcene	0.07	0.81		
β-pinene				0.07
D-limonene	4.76	7.55	0.91	13.33
β-phellandrene	0.13	0.10		
Eucaliptol	0.23	0.35		
γ-terpinene		0.04		
β-ocimene	0.96	1.51	0.07	0.21
σ-elemene				0.02
δ-elemene	0.16		0.23	
Carene	0.11			
Terpineol	0.07			
Citronellal	0.23	0.06		
β-citronellal	0.14			
Linalool	0.15		0.05	
α-bergamotene	0.14		0.11	
Elemene			0.27	0.04
Methylgeranate		0.06		
β-elemene	0.22	0.14		
Caryophyllene	0.08	0.14	0.00	0.17
γ-elemene	0.05	0.23	0.26	
α-caryophyllene			0.05	
β-citral	1.45	2.09		
α-terpineol	0.20	0.04		
D-germacrene	0.10	0.27	0.14	
α-bisabolene			0.06	
β-bisabolene	0.37			0.05
Neryl acetate		0.20		
Citral	1.17		1.96	
α-citral		0.95		
α-farnesene	0.35			
β-farnesene			0.00	0.18
Citronellol		0.11		
β-citronellol	0.15			
Perillal	0.07			
Isogeraniol	0.25	0.06		
Geraniol	0.44	0.82		
Lavandulol	1.50	0.63		

SA = Shoots asymptomatic. SS = Shoots symptomatic.

detection of the disease in different species of lemon trees. For example, when growing *Capsicum annuum* L. it has been determined that infected, healthy and mechanically damaged *Coletotrichum* spp. fruits emit different numbers of volatile compounds. Such information is used in rating their quality in the commercialization of these vegetables (In-Kyung *et al.* 2007).

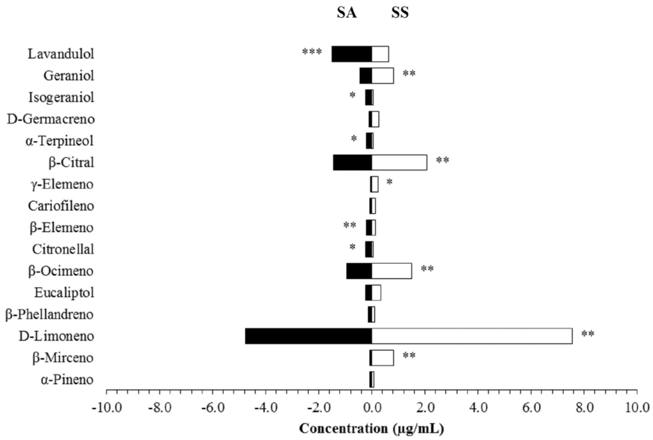

Figure 1. Analysis of the concentration of volatile compounds collected in asymptomatic (SA) and symptomatic (SS) *Citrus latifolia* trees shoots. Significantly different from the corresponding control value: * P<0.05, ** P<0.005 and *** P<0.001.

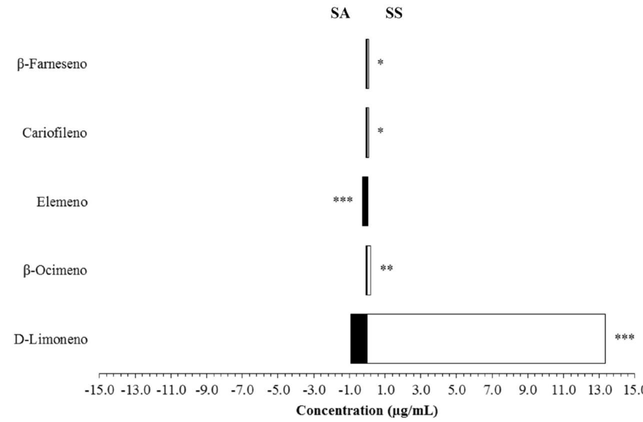

Figure 2. Analysis of the concentration of volatile compounds collected in asymptomatic (SA) and symptomatic (SS) *Citrus aurantifolia* trees shoots. Significantly different from the corresponding control value: * P<0.05, ** P<0.005 and *** P<0.001.

Conclusions

The young shoots with and without HLB symptoms released volatile compounds. The compounds D-limonene, β-ocimene and caryophyllene were collected in greater concentrations in the infected tree young shoots in both species of lemon tree. These results indicate the feasibility of early detection of trees infected with this bacterium.

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