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Differences in Selection Behaviors and Chemical Cues of adult Asian Citrus Psyllids, *Diaphorina citri*, on Healthy and Huanglongbing-Infected Young Shoots of Citrus Plants

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Abstract

The pathogen *Candidatus* Liberibacter asiaticus (Las) causes devastating citrus huanglongbing in many parts of Asia. The host selection behavior of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Sternorrhyncha: Psyllidae), the only known Las vector, is critical to its epidemics. Las-infected citrus plants attract adult psyllids by altering their color and chemical cues. However, it is unclear whether any changes in selection behavior or chemical cues occur in young shoots. Selection behavior bioassays showed the number of adults and eggs on Las-infected shoots was significantly higher than on healthy shoots. Significantly more adults responded to the odor of Las-infected shoots than to that of uninfected shoots. GC-MS analysis of volatile constituents showed that the quantitatively dominant constituents were β-elemene and β-phellandrene in both samples, (+)-epi-bicyclosesquiphellandrene was only detected in Las-infected shoots, but ocimene, germacrene D, δ-cadinene, d-longifolene, and α-sinensal were detected only in healthy shoots. Analysis of the concentrations of surface-soluble carbohydrates indicated that the levels of sucrose, fructose, and glucose were significantly higher on the surfaces of Las-infected shoots than on the surfaces of healthy shoots. Alteration of volatile compounds and soluble surface carbohydrates of young shoots after Las infection could be attributed to the preference of Asian citrus psyllids for Las-infected young shoots. The knowledge obtained from this investigation may contribute to novel control measures for plant diseases transmitted by insect vectors.

Keywords: Asian citrus psyllid, huanglongbing (HLB), host selection behavior, volatile compounds, soluble carbohydrates

1. Introduction

Citrus huanglongbing (HLB), also known as citrus greening disease, is a devastating gram-negative phloem-restricted bacterial disease. It has a profound impact on the citrus industry worldwide. The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is the only known vector of *Candidatus* Liberibacter asiaticus (Las), which causes citrus huanglongbing in Asian countries (Halbert & Manjunath, 2004; Yang et al., 2006). Las-infected trees produce poorly colored, lopsided, bitter-tasting, and unmarketable fruit, and trees may die within a few years. This causes heavy economic losses in citrus-producing areas around the world (Bové, 2006). The host selection behavior of *D. citri* is critical to the spread of HLB in the field (Tiwari et al., 2010). *D. citri* adults and fourth- to fifth-instar nymphs can transmit Las after feeding on an infected plant for 30 minutes or longer. *D. citri* adults may move to uninfected trees from after feeding (Pelz-Stelinski et al., 2010; Capoor et al., 1974; Inoue et al., 2009). This vector-pathogen mutualism plays an important role in facilitating the spread of the pathogen.

Many pathogens produce morphological and chemical changes in their hosts. For example, infection with Las causes citrus trees to develop yellow shoots and deformed fruits and produce bitter compounds limonin and nomilin in its juice in both otherwise asymptomatic and symptomatic fruits. More methyl salicylate is released from infected trees than from uninfected trees. This attracts citrus psyllids to Las-infected trees and facilitates the spread of the pathogen (Mann et al., 2012; Baldwin et al., 2010). Similarly, apple trees infected with *Candidatus* Phytoplasma mali produce small and tasteless fruits and release more β-caryophyllene than uninfected trees. Apple psyllids (*Cacopsylla picta*) are attracted to the volatile compounds emitted by infected apple plants (Mayer et al., 2008).

Odors and surface compounds from plants mediate the processes by which phytophagous insects find and accept their hosts. The color and volatile compounds emitted by young shoots may play an important role in the processes by which the Asian citrus psyllid locates and evaluates host plants. *D. citri* infection is correlated with olfactory and visual cues, affecting at least these two sensory modalities (Wenninger et al., 2009; Sanchez, 2008). Tukey (1970) reported that the chemicals present on the surfaces of plants might directly affect host acceptance by phytophagous insects and influence feeding and oviposition.

Oviposition and development of *D. citri* occur only on new flush shoots of rutaceous host plants (Hall & Albrigo, 2007; Patt & Sétamou, 2010). Recent studies have shown that infection with Las can induce changes in the volatile compounds emitted and nutritional composition of host plants. *D. citri* had different responses to odors from the young shoots of different host plants (Mann et al., 2012; Patt & Sétamou, 2010). However, the host selection behaviors and chemical cues of adult *D. citri* on Las-infected and uninfected young shoots have yet to be evaluated. The differences in the host preference behavior of adult Asian citrus psyllids could be attributable to differences in volatile compounds and nutritional components of young shoots. For this reason, in the present study, oviposition preference and responses of psyllids to healthy and infected young citrus shoots were evaluated in cage tests and Y-tube olfactometer tests, respectively. The volatile compounds emitted by young shoots of infected and uninfected plants were collected directly using solid-phase micro-extraction (SPME) and analyzed using gas chromatograph-mass spectrometry (GC-MS). Soluble carbohydrates on surfaces of infected and uninfected young citrus shoots were extracted using methanol. The present work was performed to assess differences in host preference behavior of *D. citri* adults between healthy and Las-infected shoots and to assess differences in volatile compounds and soluble surface carbohydrates on Las-infected shoots and uninfected counterparts.

2. Materials and Methods

2.1 Insects and Host Plants

D. citri adults were obtained from *Murraya exotica* L. (Rutaceae) on the campus of South China Agricultural University (23°16′N, 113°35′E) and reared for several generations on healthy *M. exotica* in an insectary. The methods of rearing were as described by Wenninger and Hall (2007). The insectary was maintained at 28±1°C and 50–60% RH with a 14:10 h light:dark photoperiod. All psyllids used in the experiments, regardless of mating status, were 5–7 days posteclosion.

Twenty healthy 4 year-old *M. exotica* trees and 20 healthy “Shatangju” mandarin (*C. reticulata*) trees were obtained from the botanical gardens of South China Agricultural University (SCAU) in Guangzhou. Las-infected *C. reticulate* trees were cultivated in SCAU’s huanglongbing research laboratory. DNA from all trees was checked for the presence of Las by quantitative real-time polymerase chain reaction (qPCR). The qPCR procedures and conditions were as described by Li et al. (2006). All experiments were conducted from June to September of 2012. One to three branches with 10–12 cm long new flush shoots on each obtained trees were selected in experiments.

2.2 Bioassay of Young Shoot Preference and Odor Response

Selection preference experiments were conducted in observation cages (40×40×60 cm). Mixed populations of 15 male and 15 female psyllids were released into the cages. Three uninfected and three Las-infected young shoots (10 cm in length) were randomly placed in the cages with 50 mL conical flasks moistened on the inside with tap water. The cages were maintained at 28±1°C and 50–60% RH with a 14:10 h light:dark photoperiod. The number of adults settling on each young shoot was recorded after 4 h, 8 h, 12 h, 16 h, 20 h, and 24 h. Each assay was performed in triplicate.

Oviposition preference experiments were performed on healthy and Las-infected young citrus shoots. Several sexually mature male and female psyllids were reared on healthy *M. exotica* and released into plastic tubes (50 mL) for natural mating. The psyllid pairs that mated successfully were selected and then mated females were transferred to young shoots with gauze nets (20 cm long, 15 cm wide) for egg laying and allowed to remain there for 24 h. There was one young shoots per mated female. The females were removed after egg laying for 24h, and citrus plants with eggs were maintained at 28±1°C and 50–60% RH with a 14:10 h light:dark photoperiod for 5 days to allow hatching. The first instar larvae was counted. Each assay was performed five times.

The odor responses of *D. citri* adults to healthy and Las-infected young shoots were evaluated in a glass Y-tube olfactometer (Blackmer et al., 2004; Zaka et al., 2010). The arms from Y-tube were 21.0 cm long with an internal diameter of 2.5 cm, and the angle between two the arms was 75°C. Test odors were produced by single young citrus shoots (10–12 cm long) that had been placed within a taste flask connected to the air-line system. The young shoots had not been removed from the citrus plants. Odors from Las-infected plants passed through one arm of the Y-tube, and odors from healthy plants passed through the other. *D. citri* were sexed and deprived of food for about 4 h before the experiment. They were tested individually. Tests were conducted between 13:00 and 16:00 at 24–27°C and 50–65% RH, with an airflow velocity in both arms at a rate of 50 mL/min. Illumination was provided by overhead fluorescent lights at 900 lux. Each psyllid was given 5 min to crawl into one of the Y-tube arms and individuals remaining in a particular arm for ≥60 s were scored as having made a selection. Psyllids that did not respond were discarded and replaced with new individuals until 30 responsive psyllids. The arms of the Y-tube were reversed every five psyllids to prevent directional bias. After ten psyllids were tested, the entire Y-tube and its components were washed with hot water and detergent, rinsed with 95% ethanol, and air-dried. Before the tests with young shoots odors, preliminary tests were conducted to determine whether the two arms of the Y-tube olfactometer were balanced. Both arms of the olfactometer were kept blank, and the rest of the procedure was performed as described above. There were 10 psyllids per replicate for a total of three replicates.

2.3 Detection of Volatile Compounds from Citrus Young Shoots

Volatile compounds emitted by Las-infected and uninfected young shoots were collected using SPME technique as described by Katoka et al. (2000). Young shoots (30 g of fresh weight) were placed in a 300 mL sample bottle, which was sealed with a septum-type cap. A 100 μm PDMS fiber was used to extract headspace volatiles from healthy and Las-infected shoots. The extraction time was 40 min. In order to remove contaminants, the fibers were treated at the injection port of GC before experiments, aging temperature and time were 250°C and 2 h. Three Las-infected citrus plants and three similar citrus plants were sampled.

After extraction, the fibers were inserted directly into an Agilent 6890 Series gas chromatograph equipped with 30 m×0.25 mm-ID, 0.25 mm film thickness DB-5 capillary column and a mass selection detector under the following conditions: Injector: 250°C, split ratio 30:1; temperature program: 50°C (5 min) and then 5°C/min to 250°C. The mass spectrum detector parameters were as follows: electron impact detector (EI), detection port temperature: 250°C; ionization energy: 70 ev; mass range scanned 40–500 mass units. The mass spectra was matched with NIST and Willey standard spectra. A positive match required a spectral fit of ≥90, mass spectra and retention times were compared to those of standard compounds. The relative content of each compound was calculated using the area normalization method.

2.4 Measurement of Soluble Carbohydrate Content on Surface of Citrus Young Shoots

Surface soluble carbohydrates of Las-infected and uninfected young shoots were extracted using a technique described by Derridj et al. (1996). Methanol was used as a solvent in the extraction of soluble carbohydrates from the surfaces of young shoots. Young shoots were removed for chemical extraction at the end of the light period, which was 2 h before sunset. At this time, the concentration of nutrients, especially carbohydrates, in leaf tissues is high (Kalt-Torres & Huber, 1987). Young shoots (30 g fresh weight) were gently washed in 100 mL of methanol for 1 minute. The solutions were sieved using 0.45 μm (pore diameter) Whatman filter paper, and then concentrated to dryness on a rotary evaporator at 36°C for pentane and 60°C for methanol. The samples were diluted by adding 2 mL ultrapure water, and then transferred to a 2 mL centrifuge tube and centrifuged at 13,000 rpm for 10 minutes at 4°C. The samples were filtered through Sep-Pak®1cc (100 mg) C18 column. Then 20 μL of sample was injected into an Agilent 1100LC High Performance Liquid Chromatography (HPLC) apparatus. The carbohydrates were separated using an Agilent NH2 column (4.6×150 mm) that had been heated to 35°C with a mobile phase that comprised acetonitrile and water (75:25) at a flow rate of 1 mL min-1. Each assay was performed three times.

Analytical-grade sucrose, glucose, and fructose were diluted in ultrapure water and used to prepare standard solutions (30 mg/mL). These were sequentially diluted for mixed solutions of sucrose, glucose and fructose at five levels (2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, and 10 mg/mL). These samples were analyzed by HPLC, and the area under each peak was determined using a chromatographic data system. Mass concentration of standard samples was plotted on horizontal ordinate and peak areas of standard samples were plotted on vertical coordinates to produce the calibration curves. The concentrations of surface soluble carbohydrates (μg/g) were calculated by using these calibration curves.

2.5 Statistical Analyses

The data were analyzed using a paired-samples *T test* with the program SPSS (version 17.0, Norman et al, ORD, U.S.). Paired-samples *T test*s were used to analyze data sets at $\alpha=0.05$, including differences between the relative number of *D. citri* adults landing on Las-infected and uninfected young shoots at the same time; the number of eggs on Las-infected and healthy young shoots; the relative number of psyllids responding to either arm of the Y-tube olfactometer; differences between volatiles from Las-infected and healthy young citrus shoots; differences in the concentrations of soluble surface carbohydrates between Las-infected and uninfected young shoots. Data were subjected to arcsine transformation before analysis.

3. Results

3.1 Selection Behavior of Adults and Number of Psyllid Eggs on Young Citrus Shoots

In the cage test, all psyllids preferred to land on Las-infected young shoots rather than healthy ones. The number of *D. citri* adults on Las-infected shoots was significantly higher than on uninfected shoots at 8 h, 12 h, 16 h and 20h. The average percentage of adults landing on Las-infected and healthy shoots during test time were 32.59% and 17.78%, respectively (Figure 1).

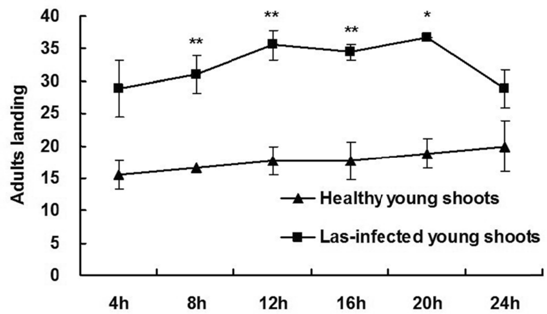

Figure 1. *D. citri* adults landing on young citrus shoots from healthy and Las-infected plants

Data was evaluated by *T-test*; ** *P*<0.01; * *P*<0.05.

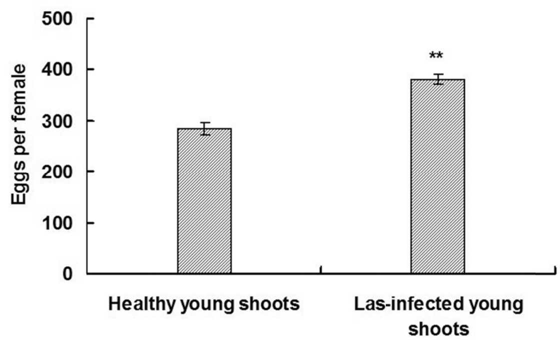

Figure 2. Oviposition preferences of *D. citri* adults

Data was evaluated by *T-test*; ** *P*<0.01.

Profoundly significant differences were observed between the number of eggs laid on Las-infected and uninfected young shoots. The average number of eggs laid on Las-infected and uninfected young shoots was 381.6 and 284.2, respectively (Figure 2).

3.2 Response of Psyllids to the Odor of Young Shoots

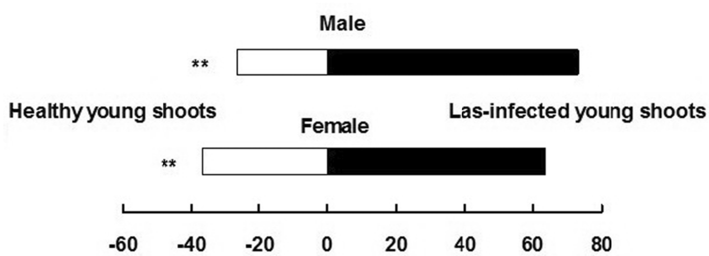

Figure 3. Response of *D. citri* to odors from young shoots from healthy and Las-infected plants

Data was evaluated by *T-test*; ** *P*<0.01.

Significantly more *D. citri* males and females were attracted to the odor of Las-infected young shoots than to those of healthy shoots. The relative number of male and female *D. citri* moving toward Las-infected young shoots was 73.33% and 63.33%, respectively. The relative number of male and female *D. citri* moving toward healthy young shoots was 26.67% and 36.67%, respectively (Figure 3).

3.3 Volatile Profiles of Las-infected and Uninfected Young Shoots

Fifteen volatile compounds were identified in samples collected from young shoots of Las-infected and uninfected citrus plants (Figure 4). The odor samples were composed primarily of monoterpenes and sesquiterpenes. The primary constituents were β-elemene and β-phellandrene of both samples. However, (+)-epi-bicyclosesquiphellandrene was only detected in samples from Las-infected shoots, and ocimene, germacrene D, δ-cadinene, d-longifolene, and α-sinensal were only detected in healthy shoots, but the chemicals of germacrene D and (+)-epi-bicyclosesquiphellandrene had the same retention time (Figure 4).

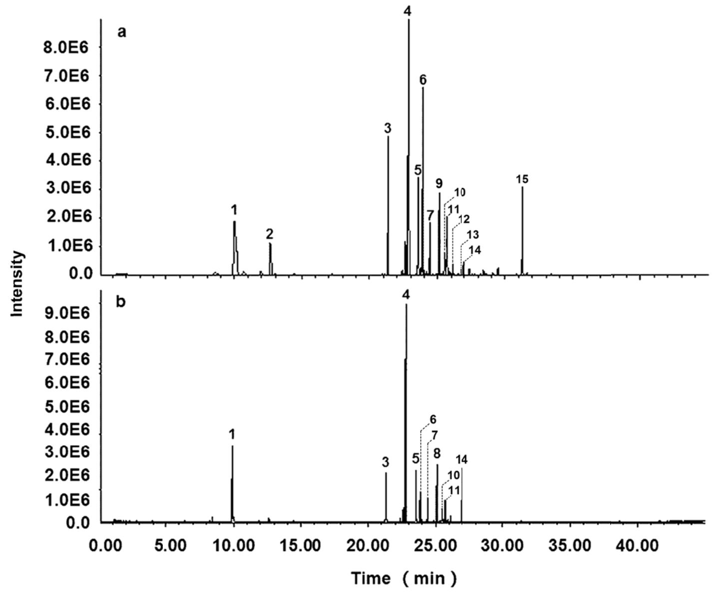

Figure 4. Volatile compounds emitted by young shoots of healthy (a) and Las-infected plants (b)

1: β-phellandrene; 2: Ocimene; 3: (3R-trans)-4-ethenyl-4-methyl-3-(1-Met-hylethenyl)-1-(1-methylethyl)-Cyclohexene; 4: β-elemene; 5: β-caryophyllene; 6: γ-elemene; 7: α-caryophyllene; 8:(+)-Epi-bicyclosesquiphellandrene; 9: Germacrene D; 10: Germacrene B; 11: α-farnesene; 12: δ-cadinene; 13: d-longifolene; 14: Elixene; 15: α-sinensal.

3.4 Concentrations of Soluble Carbohydrates on the Surfaces of Las-infected and Uninfected Young Shoots

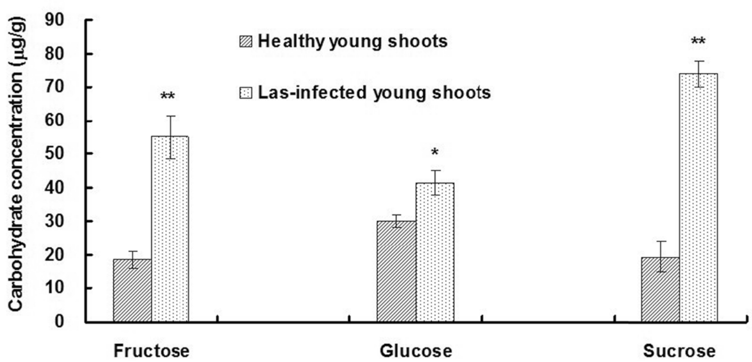

Figure 5. Carbohydrate concentrations in healthy and Las-infected young shoots as extracted by methanol

Data was evaluated by *T-test*; ** $P<0.01$; * $P<0.05$.

The chemicals of fructose and sucrose concentrations were differed profoundly significantly between infected and uninfected shoots, and glucose concentration on Las-infected young shoots was significantly higher than on uninfected shoots. The concentrations of fructose, glucose, and sucrose were 55.02, 41.40, and 73.88 µg/g on Las-infected shoots respectively, but on healthy young shoots, fructose, glucose, and sucrose concentrations were 18.62, 30.12, and 19.40 µg/g, respectively (Figure 5).

4. Discussion

Interactions among vector insects, pathogens, and host plants are complex, diverse, and crucial to the population dynamics of media insects and pathogen epidemics (Colvin et al., 2006; Stout et al., 2006). Jiu et al. (2007) found that invasive B biotype whiteflies that had fed on tobacco plants infected with the *tobacco curly shoot virus* (TbCSV) and or *tomato yellow leaf curl China virus* (TYLCCNV) tobacco plants had significantly more pronounced fecundity and longevity than those that had feed on healthy plants. This accelerated the growth and development of the B biotype whitefly population. Other previous studies have indicated that apple plants infected with *Candidatus* Phytoplasma mali were more attractive to apple psyllids (*Cacopsylla picta*) than uninfected plants were (Mayer et al., 2008). Similarly, *D. citri* adults were initially attracted to Las-infected plants even if they subsequently moved to uninfected plants after landing (Mann et al., 2012). The same results were observed in the present study. There were significantly more adults and eggs on Las-infected young shoots than on healthy ones, although the number of *D. citri* adults landing on Las-infected young shoots decreased after 24 h. The olfactometer results showed that odors from Las-infected young shoots were more attractive to *D. citri* adults than those from healthy counterparts. Infected citrus plants accumulate starch in the phloem, which leads to blockage of phloem and sub-optimal quality for feeding by *D. citri* (Kim et al., 2009; Folimonova & Achor, 2010). For this reason, after the initial attraction to the odors produced by Las-infected young shoots, *D. citri* might move to uninfected ones. The leathered surfaces of Las-infected citrus plants was not found to have any effect on choice of host plants by Asian citrus psyllids, although psyllids spent more time on Las-infected plants because of the longer time needed to penetrate the parenchyma and reach the phloem (Cen et al., 2012). Inoue et al. (2009) found that a large proportion (88%) of *D. citri* adults contained the bacterium (Las) after an access period of 24 h. The movement of *D. citri* adults infected with Las can facilitate the spread of the pathogen.

D. citri, finding a host is a complex process. Psyllids may use volatile compound cues from both host plants and conspecifics during host plant detection (Wenninger et al., 2008; Horton & Landolt, 2007). In the present study, nine compounds were found to be common in both infected and uninfected young shoots. β-elemene and β-phellandrene were the primary constituents of both samples, but only (+)-epi-bicyclosesquiphellandrene was detected in Las-infected young shoots. Patt and Sétamou (2010) reported that the quantitatively dominant constituents of the volatiles emitted by young shoots of the grapefruit cultivar Rio Red, Meyer lemon (Citrus×limon L. Burm.f.), and *M. paniculata* were (E)-β-ocimene, linalool, linalyl acetate, and β-caryophyllene. This difference from the present results may have been caused by different materials for experiments. Mann et al. (2012) found that Las caused changes in the concentrations of volatile profiles from old leaves of citrus plants. The results of the present work showed that changes in the concentrations of volatile profiles also took place on young citrus shoots. Volatile composition, proportionality, and concentration might change volatile cues from host plants during the search for host plants (Bengtsson et al., 2006). The antennal response of *D. citri* to blends was somewhat higher than to individual compounds (Wenninger et al., 2009). This indicated that two or more volatile compounds might act synergistically and be more stimulatory to *D. citri* than individual compounds. Further research is needed to determine the class, proportion, and concentration of active compounds from Las-infected young shoots capable of attracting *D. citri* and their efficacy.

The ratios of leaf surface soluble carbohydrates (glucose, sucrose, and fructose) profile vary with corn leaf age and growing conditions. Insects probably detected chemicals on the surfaces of leaves and used them as cues for feeding and oviposition (Fiala et al., 1990; Derridj et al., 1996). Mann et al. (2012) found that Las-infected citrus plants had lower levels of nitrogen, phosphorus, sulfur, zinc, and iron and higher levels of potassium and boron than uninfected plants. The results of the present work showed more soluble carbohydrates (fructose, glucose, and sucrose) on the surfaces of Las-infected young shoots than on uninfected ones, so Asian citrus psyllids might accept host plants using gustatory cues after landing. The number of eggs on Las-infected young shoots was significantly higher than on healthy shoots. Infection of citrus with Las may disrupt the nutrient balance of host plants. More soluble compounds may penetrate the surfaces of the leaves of plants, which might provide a good environment for the laying of eggs. *D. citri* acquire most of their nutrients from the phloem of host plants (Bonani et al., 2010). Therefore, further studies are needed to determine the contents of soluble carbohydrates inside citrus leaves and their relationship with oviposition in *D. citri* adults.

In summary, infection of citrus plants with Las not only changed the concentration of volatile compounds and nutritional elements but also changed the concentrations of soluble carbohydrates from the surfaces of host plants. This is the first reported of this. The alteration of the concentrations of volatile compounds and surface soluble carbohydrates in young citrus shoots after Las infection might be attributable to the preference of adult Asian citrus psyllids for infected shoots. Examination of odors and surface soluble carbohydrates of healthy and Las-infected citrus young shoots might lead to novel control measures for insect-vector-transmitted plant diseases.

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