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Chemotaxis of Adults of the Asiatic Citrus Psyllid, *Diaphorina citri* Kuwayama, to Volatile Terpenes Detected from Guava Leaves

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Abstract.- The Asiatic citrus psyllid, *Diaphorina citri* Kuwayama, is the most destructive insect pest of citrus. It causes severe damage to the crop by transmission of the pathogen of citrus Huanglongbing (HLB) disease. Previous researches have proven that the inter-planted guava trees, *Psidium guajava* L. (Myrtales: Myrtaceae), in citrus orchard showed positive effect on the reduction of psyllid infestation and the volatiles emitted from guava leaves exhibited repellent activities against the adult psyllids. To determine the active volatile compounds from guava leaves, present research deals with the detection and chemotaxis of volatile terpenes from guava leaves by GC-MS analysis and olfactometer bioassay. The GC-MS analysis of guava leaf volatiles revealed that 7 terpenes among the 21 volatile compounds were detected, *i.e.* α-pinene, sabinene, myrcene, β-pinene, β-ocimene, limonene and α-ocimene. The olfactometer bioassay with 3 commercial available volatile terpenes, limonene, α-pinene and β-pinene, against the adult psyllids showed that no significant chemotaxis effect was recorded when individual compounds or combination of (limonene + β-pinene) were tested. The combinations of α-pinene mixed with limonene, β pinene, or both of them at equal ratio, gave highly significant less chemotaxis response, 28.75%, 36.25% and 21.25% for female adults, respectively, indicating strong repellent activity of α-pinene combinations. The chemotaxis responses of male and female adult psyllids to the tested terpenes were similar. These results suggest that the blends of volatile terpenes from guava leaves contributed to the reduction of psyllid infestation in the guava-citrus intercropping system and might be developed as effective behavioral regulators of the adults of Asiatic citrus psyllid in practice.

Keywords: *Diaphorina citri, Psidium guajava,* volatile compounds, terpenes, insect behaviour.

INTRODUCTION

The Asiatic citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is globally known as the most serious pest of citrus because of its role in carrying the fastidious phloem-limited pathogen of huanglongbing (HLB) disease. Once the pathogen infects some trees, along with a relatively high psyllid population, the disease spreads quickly to other plants in orchards and the infected trees decline within few years (Yang *et al*., 2006). The combined presence of psyllid vector and HLB agent has been the limiting factor in worldwide citrus production. But the fact is that no effective management approaches to control HLB have yet been established (Bove, 2006; Frederic *et al*., 2010). The issue to control the population of citrus psyllids and the spread of HLB is still in great challenge.

Guava trees, *Psidium guajava* L. (Myrtaceae), interplanted with citrus are reported to be capable to reduce citrus psyllid infestation and consequently the incidence of HLB disease in orchards (Beattie *et al*., 2006). It has been experimentally confirmed that volatiles from guava leaves or leaf extracts inhibited the attraction of host citrus to adult psyllids (Zaka *et al*., 2010; Onagbola *et al*., 2011; Barman and Zeng, 2014). However, the mechanism of guava volatile effects on the host selection of citrus psyllid is still remained unknown. As a major component of flora volatiles, terpenoids are extensively targeted for the research of insect-plant interactions and were found to be important semiochemicals of plants to various insects (Aharoni *et al.*, 2005; Büchel *et al.*, 2014; Ekeh *et al.*, 2013; Kendra *et al.*, 2014; Muhlemann *et al.*, 2014; Sun *et al*., 2014). It is reasonable to presume that certain volatile terpenoids emitted by guava plants play the role in the reduction of adult citrus psyllids attacking citrus. For the purpose of

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verifying this presumption, the present research deals with the GC-MS identification of volatile terpenes emitted from guava leaves and the chemotaxis of adult citrus psyllids to three commercially available volatile terpene compounds detected from the guava leaves by method of olfactory bioassay. Understanding the chemotaxis of citrus psyllids to volatile terpenoids might lead to the elucidation of mechanism of guava volatile effects on the host selection of citrus psyllid, and to the development of natural protectants and novel measures for the management of the Asiatic citrus psyllid in practice.

MATERIALS AND METHODS

Insects

Adults of ACP were collected daily from 4-year-old sweet orange (*Citrus* × *aurantium* L.) trees in the botanical garden of South China Agricultural University. High psyllid populations were maintained by pruning the trees regularly to encourage new growth favoured by the psyllid. Adults were collected with mechanical aspirators each morning and held in small plastic cups for bioassay. Males and females were separated on the basis of their morphology. The tip of the abdomen of an adult male of ACP is bent upwards while the abdomen of the female is straight. Orange/yellow colour of female showed that it contains the eggs (Wenninger and Hall, 2008). After the experiment, all the psyllids were preserved in 70% ethanol and their sexes reconfirmed under the light laboratory microscope.

Plant material

Four-years-old guava (*P. guajava* L. cv. Pearl) trees in the university campus were used as the source of plant materials. These plants were regularly irrigated and fertilized and were visually free from any disease or pest. Fresh new shoots were collected for experiment use.

Detection of volatile compounds emitted from guava leaves

Volatile compounds released from about 200 g of guava leaves were collected for 3 h at (28°C±2) room temperature with a trap tenax tube (3 mm in diameter, 150 mm in length) by using the similar schedule and device as described by Deka and Bora (2014).

The volatiles collected were subjected to GC-MS (Shimadzu GC-17A with QP-5000, Japan) analysis. A capillary column of 30 m × 0.32 mm bonded with non-polar FSOT-RSL-200 fused silica with a film thickness of 0.25 µm (Biorad, Germany) was used. Analysis was carried out with helium carrier gas at flow rate of 1.0mL/min, splitless mode, 250°C injector temperature, 300°C interface-heating, 200°C ion source-heating, 70 eV EI-mode, and 41–450 amu scan-range. The temperature program was 40°C/5 min to 280°C/5 min, with a heating rate of 6°C/min. Mass spectra matching was accomplished with Chem-Station software (Agilent, Santa Clara, CA) and the NIST2010 Mass Spectral Library. A positive match required a spectral fit of ≥90. Verification of compound identification was obtained by comparison of their spectra to the retention times and mass spectra of standard compounds.

Olfactory bioassay

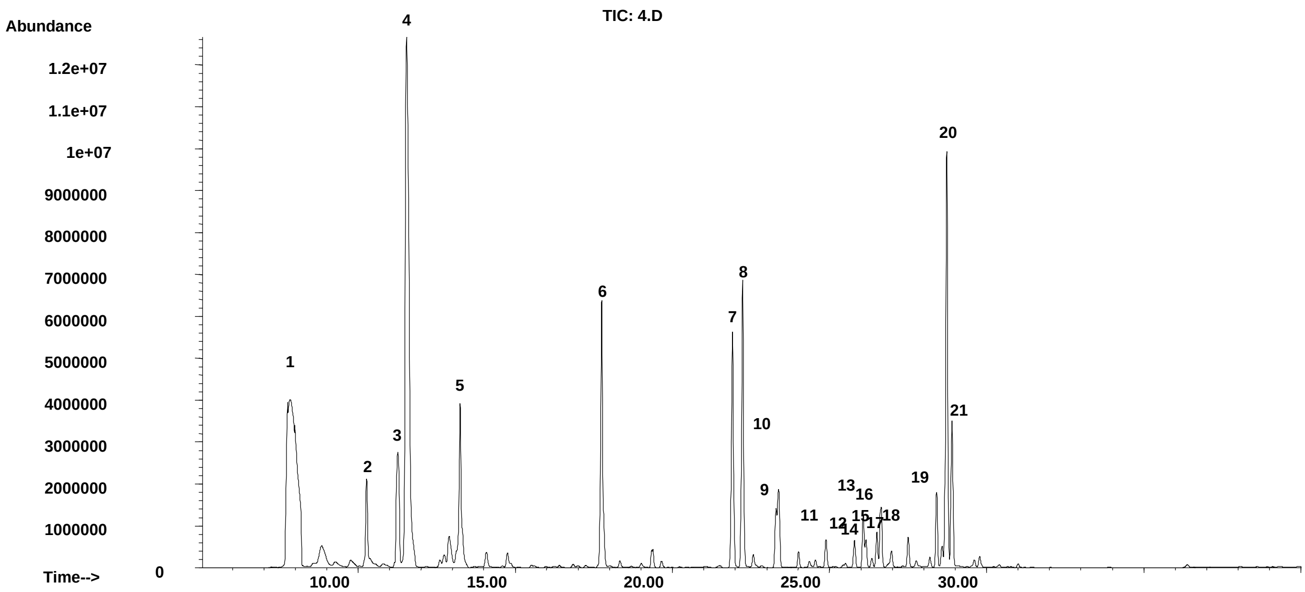
Olfactory bioassay was conducted following our previously established method (Zaka *et al*., 2010). Three commercially available volatile terpenoid compounds, *i.e.*, limonene, α-pinene and β-pinene, were ordered from product supplier of Alfa Aesar company in Beijing, China. The purity of these 3 products are 97%, 98% and 99%, respectively. Ten µl of each compound or their combinations at equal rate was used as odor source in each treatment. Each treatment was replicated 8 times and in each replication 10 single sex adult psyllids were tested.

The difference of psyllid number responded to test chemicals were analysed by PROC *T*- test using SAS V8.1 program.

RESULTS

Volatile terpenoid compounds emitted from guava leaves

As indicated by the peaks of total ion chromatograph (TIC) obtained by GC-MS analysis, more than 21 volatile compounds were emitted from 200 g guava leaves in 3 h under our experimental conditions (Fig. 1).



Seven volatile terpene compounds, *i.e.*, α-pinene, sabinene, myrcene, β-pinene, β-ocimene, limonene and α-ocimene, were detected (Table I). Among these compounds, limonene has the highest peak abundance, with a relative abundance of 53.80%; whereas β-pinene has the least peak abundance with its relative abundance of 2.13%.

Table I.- Volatile terpenes detected from guava leaves.

Peak No	RT (min)	Compound	CAS No	RA (%)
11	24.876	α-pinene	80-56-8	3.71
15	26.500	Sabinene	2009-00-9	4.55
16	26.641	Myrcene	123-35-3	6.96
17	26.967	β-pinene	127-91-3	2.13
19	28.406	β-ocimene	13877-91-3	9.83
20	28.732	Limonene	95327-98-3	53.80
21	28.895	α-ocimene	502-99-8	19.02

Note: RT and RA stand for peak retention time and relative peak abundance, respectively.

Chemotaxis of adult psyllids to volatile terpenes

Obviously, non significant percentage of female psyllids showed chemotaxis to individual tested terpenes of limonene (P=0.80183) or β-pinene (P=0.84361) (Table II). For α-pinene, slightly less than 50% (43.75% for female and 42.50% for male) psyllids showed chemotaxis to this single compound (P=0.0949), indicating very weak repellent activity against the adults of citrus psyllids.

Table II.- Olfactory response of citrus psyllids to volatile terpenes singles and in combinations.

Source of volatiles	% psyllid chemotaxis (Mean ± SE*)	
	Female	Male
Limonene	48.75 ± 0.48	46.25 ± 0.53
α –pinene	43.75 ± 0.32	42.50 ± 0.37
β -pinene	48.75 ± 0.61	45.00 ± 0.42
limonene + α-pinene	28.75 ± 0.44**	27.50 ± 0.41**
limonene + β-pinene	51.25 ± 0.30	45.00 ± 0.33
α-pinene + β-pinene	36.25 ± 0.46*	33.75 ± 0.53*
limonene + α-pinene + β-pinene	21.25 ± 0.40**	25.00 ± 0.46**

Note: treatment followed by * or ** means significant or highly significant difference from the respective control by the paired sample *t*-test at level 5% and 1%, respectively; n = 80 for each treatment in each comparison.

Olfactory responses of the all 4 combinations of the 3 terpene compounds were assayed. Results revealed that highly significant (P=0.00017) difference between the percentage of psyllids chemotaxis and non chemotaxis occurred in the test of (limonene + α-pinene + β-pinene) combination.

Far less than 50% (21.25% for female and 25.00% for male) of the adults moved toward the volatile source, indicating strong repellent activity of this combination against the citrus psyllids (Table II).

It was also found that combinations of (limonene + α-pinene) and (α-pinene + β-pinene) had activities at extent of highly significance (*P*=0.00192) and significance (*P*=0.01359), respectively; whereas the combination of (limonene + β-pinene) showed no significant activity (*P*=0.68453) against the adults of citrus psyllid. The percentage of chemotaxis psyllid in treatments of (limonene + α-pinene), (α-pinene + β-pinene) and (limonene + β-pinene) were 28.75, 36.25, 51.25 for female, and 27.50, 33.75, 45.00 for male, respectively (Table II).

The bioassay results might suggest that among the 3 tested terpenes, α-pinene is dominant for the reduction of psyllid chemotaxis, while limonene and β-pinene possess some extent of synergistic effect to α-pinene. Male and female adults statistically have similar chemotaxis response against the volatile compounds tested.

DISCUSSION

Guava is a mycorrhizotrophic and multipurpose plant highly suited to tropical and subtropical climates. Due to its economic importance as fresh fruit and food processing products, and its medicinal properties for certain types of diseases, including cancer, the chemistry of guava fruit has attracted extensive interest since last decade (Sharma *et al.*, 1999). Guava fruit contains more than 140 volatile compounds, including at least 43 esters, 37 terpenes, 18 aldehydes, 16 alcohols, 10 acids, 6 ketones, 4 furans and several miscellaneous compounds (Pino and Bent, 2013). Idestein and Schreier (1985) identified 154 volatile compounds obtained by high-vacuum distillation from guava fruit: quantitatively, C6 aldehydes and alcohols were predominant. Some of these aldehydes and alcohols are the so-called 'green leaf volatiles' that have repellent effects on oriental fruit flies (Jang and Light, 1991). A total of 181 volatile compounds from sour guava (*P. guineense* Swartz) fruit were identified, with major classes of esters (69.5%) and terpenes (60.8%), and major compounds of ethyl butyrate, ethyl hexanoate, β-caryophyllene and selin-11-en-4α-ol (Peralta-Bohorquez *et al.*, 2010). In the case of guava leaf volatile compounds, not many researches have been done so far. A volatile fraction rich in sesquiterpenes (particularly α-selinene, δ-selinene, and β-caryophyllene) was obtained by supercritical fluid extraction of guava leaves (Sagrero-Nieves *et al*. 1994). Ogunwande *et al.* (2005) identified 42 compounds in guava leaves after hydrodistillation (the most significant were limonene and β-caryophyllene). Seven sulfur volatiles of hydrogen sulfide, sulfur dioxide, methanethiol, dimethyl sulfide (DMS), dimethyl disulfide (DMDS), methional, and dimethyl trisulfide (DMTS), with 47 additional leaf volatiles from crushed and intact guava leaves (*P. guajava* L.) were identified (Rouseff *et al.*, 2008). DMDS is an insect toxic, defensive volatile produced only by wounded guava and, thus, may be the component responsible for the protective effect of guava against the HLB vector. Interestingly, 35 volatile components were identified from leaves of guava plants (*P. guajava* L.) grown on Jeju Island, South Korea, with terpene hydrocarbons and C6 compounds as the most abundant components, and α-pinene, β-caryophyllene, α-humulene, β-(Z)-ocimene, and (Z)-3-hexenal as the major components, and eamphene, sabinene, eucalyptol, α-terpinolene, δ-cadinene, and germacrene B as unique compounds in guava leaves (Lee *et al.*, 2011). In particular, sabinene, germacrene B, α-farnesene, and germacrene D were firstly reported from the leaves of guava plants. Similar results have been obtained from our present analysis of guava leaf volatiles. We found that more than 21 volatile compounds were emitted from the guava leaves in 3 h under laboratory temperature and atmospheric pressure conditions, and the terpene hydrocarbons, including α-pinene, sabinene, myrcene, β-pinene, β-ocimene, limonene and α-ocimene, were the major compounds. Although we have not found any sulfur compound emitted from the guava leaf under our test conditions, these results positively support our presumption that guava emits terpene compounds from leaves, which exhibited repellent activity against the Asiatic citrus psyllid. However, further study on the profile of guava leaf volatile compounds in fields is needed for better understanding of the chemical ecology of guava plants.

The pre-alighting discrimination of a potential host plant implies that the relevant characteristics are perceived by phytophagous insects at a distance. The semiochemicals from host and non-host plants are expected to influence host selection behavior of the insect. With psyllids, Horton and Landolt (2007) used a Y-tube olfactometer to show that males of the pear psylla, *Cacopsylla pyricola* (Förster) [Psyllidae], are attracted to volatile odors from female-infested or previously infested shoots. Terpenes are plant-derived chemicals with potent insect antifeedant and toxic properties to herbivorous insects (Gonzalez-Comoma *et al.*, 2005). Limonene alone or with other monoterpenes is deterrent (Ntiamoah *et al.*, 1996; Ntiamoah and Borden, 1996) or repellent (Peterson *et al.*, 1994; Nehlin *et al.*, 1994; Ibrahim *et al.*, 2001) toward insects. Carvone has been reported to inhibit the feeding of pales weevil (*Hylobius pales*) on *Pinus strobus* seedlings (Salom *et al.*, 1996), of pine weevil (*H. abietis*, Coleoptera: Curculionidae) on Scots pine (*P. sylvestris*) (Klepzig and Schlyter, 1999; Schlyter *et al.*, 2004), and of the slug *Arion lusitanicus* on lettuce (Frank *et al.*, 2002). Tripathi *et al.* (2003) reported feeding deterrence and contact and fumigant toxicity of carvone against stored product beetles, whereas Ouden *et al.* (1993) found a short-term oviposition repellence of carvone against cabbage root fly (*Delia radicum*). The α-pinene is a bicyclic terpene found in many essential oils including those from pine, piper, rosemary, and lavender, and possesses various biological activities such as repellent and antifeedant activities against the tobacco cutworm, *Spodoptera litura* (Fan *et al.*, 2011), silverfish (Wang *et al.*, 2006) and insecticidal activity towards a few stored product pests. Contrary, α-pinene was also reported to possess attraction activity to certain insects (Polandet *et al.*, 2004). Our results showed that tested with single compounds, except for α-pinene, which exhibited a very weak repellent activity against the adults of citrus psyllids, non significant repellent activity can be seen for limonene and β-pinene. When tested in combination, all combinations of α-pinene mixed with other compounds showed highly significant to significant repellent activities against the adults of citrus psyllids. These results indicated that α-pinene is dominant for the reduction of psyllid chemotaxis and may be synergistically enhanced by other volatile terpenes such as limonene. Although further studies are required to determine the other active volatile compounds from guava leaves, our results confirmed that the mixture of volatile terpene compounds, instead of single compound, emitted from guava leaves exhibited repellent (negative chemotaxis) activity against adult citrus psyllid and that these volatile compounds can be developed as natural protectants for the management of Asiatic citrus psyllid in citrus production.

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