



Volatile metabolites of willows determining host discrimination by adult *Plagioder a versicolora*

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Abstract *Plagioder a versicolora* Laicharting is a highly damaging leaf beetle foraging on willow leaves. In willow germplasm collections, observation has shown that *Salix suchowensis* Cheng was severely foraged by this leaf beetle while *Salix triandra* L. was damage free or only slightly damaged. Results of olfactometer bioassays show that the headspace volatiles from leaves of *S. triandra* significantly repelled adult beetles, suggesting that this species produces volatile repellents against *P. versicolora*. *S. suchowensis* had no effect on the beetles. Gas chromatography-mass spectrometry was carried out to profile the headspace volatile organic compounds and 23 compounds from leaves of the alternate species in significantly different concentrations were detected. The effects of 20 chemical analogs on host discrimination were examined. Olfactory response to these chemicals showed that *o*-cymene, a *S. suchowensis* specific constituent, significantly attracted adult *P. versicolora*. In contrast, *cis*-3-hexenyl acetate, a constituent concentrated more in *S. triandra* than in *S. suchowensis*, significantly repelled beetles. Mixing *o*-cymene and *cis*-3-hexenyl

acetate in comparable concentrations as in the volatiles of *S. suchowensis* demonstrated that the latter could mask the attracting effect of the former, causing a neutral response by adult beetles to leaves of *S. suchowensis* against clean air. In addition, chemical analogs have the same effect as plants when resembling volatile organic compounds in real samples. Two volatile metabolites were detected triggering host discrimination by one of the most damaging insect pests to host and non-host willows. The two metabolites are of considerable potential for use as olfactory signs in managing the beetles.

Keywords Herbivore insect · Olfactory response · Volatile organic compounds · *Cis*-3-hexenyl acetate · *O*-cymene

Introduction

Plagioder a versicolora Laicharting (willow beetle) feeds exclusively on leaves of species of Salicaceae and is the most damaging herbivore insect on *Salix suchowensis* Cheng as well as on many other willow species (Ishihara and Ohgushi 2008). Willow beetles forage on young leaves and cause bud death (Wade and Breden 1986). Several studies have shown that food preference of the beetles was significantly impacted by many host factors. For instance, secondary metabolites of *Salix integra* Thunb., chlorogenic acid and 3,5-dicaffeoyl quinic acid, were identified as antifeedants that would inhibit attack by insects and grazing animals, whereas 1,2-dilino-lenoyl-3-galactopyranosyl glycerol could stimulate trophic behavior, the position occupied in the food chain, of the beetles (Jassbi 2003). Raupp (1985) reported that leaf toughness affected the morphology, feeding behavior, and ultimately shaped the spatiotemporal distribution of *P. versicolora*.

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Adult willow beetles preferentially feed on young leaves of Salicaceae species (Raupp and Denno 1983), leading to an increase in mortality of willows due to necrosis of apical buds. Leaf age has also been reported to affect the choice of oviposition for female adults (King et al. 1998).

How do insects discriminate their preferred food resource? Conchou et al. (2019) proposed that herbivore selection of plants relies heavily on olfactory clues, although they also have visionary, mechanoreception and gustation mechanisms (Miller and Strickler 1984). Multiple lines of evidence support this hypothesis. For example, host selection by the spittle bug (*Mahanarva spectabilis* Distant) was affected by several volatile organic compounds (VOCs) emitted from forage grasses (Silva et al. 2019). Whereas the strawberry leaf beetle (*Galerucella vittaticollis* Baly) used a component in the headspace volatiles of several host plants as olfactory clues (Hori et al. 2006). For the pollen beetle *Meligethes aeneus* Fab., they were more attracted by volatiles from their most preferred host, rapeseed, *Brassica napus* L. (Ruther and Thiemann 1997). Plant VOCs are important to insects for finding hosts and for discerning non-hosts. Non-host plants succeed in affecting herbivore behavior in two possible ways. First, the VOCs mask those of host plants so that efficiency of host finding is greatly reduced, which was the case for the carrot fly (Uvah and Coaker 1984), several beetle species (McNair et al. 2000; Zhang and Schlyter 2004; Hori et al. 2006; Kerr et al. 2017) and some moth species (Asare-Bediako et al. 2010; Jactel et al. 2011). Secondly, VOCs released from non-host plants contain deterrent chemicals, thus insects are repelled (Nottingham and Hardie 1993) or normal development is disturbed after landing on the plants (Nishida et al. 1990; McNair et al. 2000; Wang et al. 2016).

For *P. versicolora*, it was shown that it could determine host status by odor (Yoneya et al. 2009). Through years of empirical observation, we found that infestation by willow beetles was severe on germplasm collections of *Salix suchowensis*, while the collections of *Salix triandra* L. were pest free. In this study, olfactory response tests were carried out with adult *P. versicolora* with leaves of *S. suchowensis* and *S. triandra*. Subsequently, VOCs emitted from the leaves of the alternate species were analyzed by gas chromatography mass spectrometry (GC-MS), and effects on hosts for the VOCs with different concentrations in the two species were examined with chemical analogs. We aimed to identify the VOCs underlying host discrimination for adult *P. versicolora* in host and non-host willows.

Materials and methods

A large germplasm collection of *S. suchowensis* and *S. triandra* maintained at the Baima Forest Farm in Nanjing, Jiangsu

Province. Cuttings propagated with clone “DB134” of *S. triandra* and “XY12” of *S. suchowensis* were produced in the greenhouse of the Nanjing Forestry University in February 2018. Leaves were collected from the top first node to the top fifth node on the shoots in June 2018. Adult *P. versicolora* were collected from the willow plantation at Baima Forest Farm (118°48' E, 32°04' N) and reared in an incubator at 27 °C, 65% ± 10% relative humidity, and under 16 h light, 8 h dark photoperiod. All adults were from the following generations of the initial collection. The duration of development from egg to adult emergence is approximately three weeks. Emerging beetles were provided with poplar leaves for 4–6 days of adult sexual maturation. Before the tests, the adults were starved for 12 h, and both male and females were tested.

Analyzing the headspace volatile organic compounds

Headspace VOCs emitted from leaves of *S. suchowensis* and *S. triandra* were extracted using solid-phase micro extraction (SPME) in the headspace mode. Separation and identification of the constituents were carried out by GC-MS. For volatile extraction, 2-g fresh leaves were cut into pieces, and placed into a 50 mL vial. The headspace of the vial was extracted with SPME (65 µm PDMS/DVB, Supelco Inc., Bellefonte, PA., USA) for one hour at 50 °C, which was based on a series of preliminary experiments.

The GC-MS (Trace ISQ-LT; Thermo Fisher Scientific Co., Waltham, MA, USA) was equipped with a DB-5 column (30 m × 0.25 mm × 0.25 µm, Agilent J & W) and helium was used as the carrier gas at a column head speed of 1 mL/min. GC was set for splitless injection. The temperature program of the column oven was 40 °C (2-min hold), 6 °C min⁻¹ to 250 °C (5-min hold). The MS parameters were set at ionization energy of 70 eV and a scan range of 45–450 U. Six replicates were used to conduct qualitative and quantitative analyses of leaf volatiles of the two willow species.

Olfactometer bioassays

The olfactory responses of adult willow beetles to leaf VOCs were investigated with a glass Y-tube olfactometer (8 cm long × 0.5 cm radius, 75 °Y angle). The stem of the Y-tube was connected to a rubber hose where the test beetles were released. Each arm was connected to a 50 mL air chamber, an airflow (500 mL/min) was introduced into each chamber through activated charcoal and purified-water using an air pump. All tests were performed under ambient temperatures at 25 ± 1 °C and relative humidity of 80 % ± 10 % (Koschier et al. 2017).

Beetles were placed into the stem of the olfactometer, with each arm defined a “finishing line” at 3 cm from the

end of the long stem. The selection behavior was recorded by observing if a beetle crossed the finishing line of the corresponding Y-tube glass connecting to the sample vial within 5 min. If a beetle did not cross the finishing line within 5 min, it was recorded as ‘pest did not make a selection’. The blend and control arms were exchanged after every 20 individual insects, and the glass Y-tube was washed in 75% ethanol and oven-dried at 90 °C after testing every 40 insects. The host-selection behavior was estimated by the excess proportion index (*EPI*) (Hori et al. 2006):

$$EPI = \frac{N_t - N_c}{N_t + N_c} \quad (1)$$

where N_t = number of insects in a treated sample side, and N_c = number of insects in a control sample side. To give a meaningful explanation of *EPI*, we defined the EPI_0 as follows:

$$EPI_0 = \frac{N_{c1} - N_{c2}}{N_{c1} + N_{c2}} \quad (2)$$

where N_{c1} = number of insects in one control sample and N_{c2} = number of insects in the other control sample. With *EPI* significantly lower than EPI_0 = repel; *EPI* showed no significance with EPI_0 = no preference; and *EPI* significantly higher than EPI_0 = attract.

In bioassay 1, the olfactory response of adult *P. versicolora* to odors of 2-g leaf samples was compared against clean air. For each sample, 40 insects were tested with five replicates for each test. In addition, to demonstrate preference of beetles between the willow species, a four-choice olfactory bioassay (bioassay 2) was conducted with the two willows and two controls. The olfactometer was designed on the basis of the glass Y-tube olfactometer; the two long arms of the Y-tubes were connected with openings at the connection point. This is similar to bioassay 1.

In bioassay 3, olfactometer bioassays were carried out with chemical analogs of the headspace VOCs. These were purchased from ChengShao Biological Technology Co., Ltd., Shanghai, China and Wuhan Yuanchen Technology Development Co., Ltd., Wuhan, China. The chemicals were dissolved separately in liquid paraffin and used in bioassay 2. In each treatment, the volume of solution

applied to filter paper was calculated based on the concentration of the corresponding compound in *S. suchowensis* or *S. triandra*. The filter paper with solution was placed in the blend-side of the container, whereas the filter paper with an equal volume of control solution (liquid paraffin) was placed in the control-side of the container. For each chemical analog, 40 insects were tested, with each test having five replicates.

Statistical analyses

Chi-square test was used to evaluate the difference in number of insects choosing between the blend arm and the control arm for data in both bioassays 1 and 3. One-way ANOVA was conducted to identify whether the numbers were significantly different among the two willow species treatments and the two controls in bioassay 2. The difference in relative abundance of plant volatiles was also determined using the Chi-square test. One-way ANOVA was identified whether the *EPI* values were significantly different between the odor sources. All analyses were carried out using SPSS 19.0, SPSS Inc.

Results

Olfactometer bioassays with leaves of *S. suchowensis* and *S. triandra*

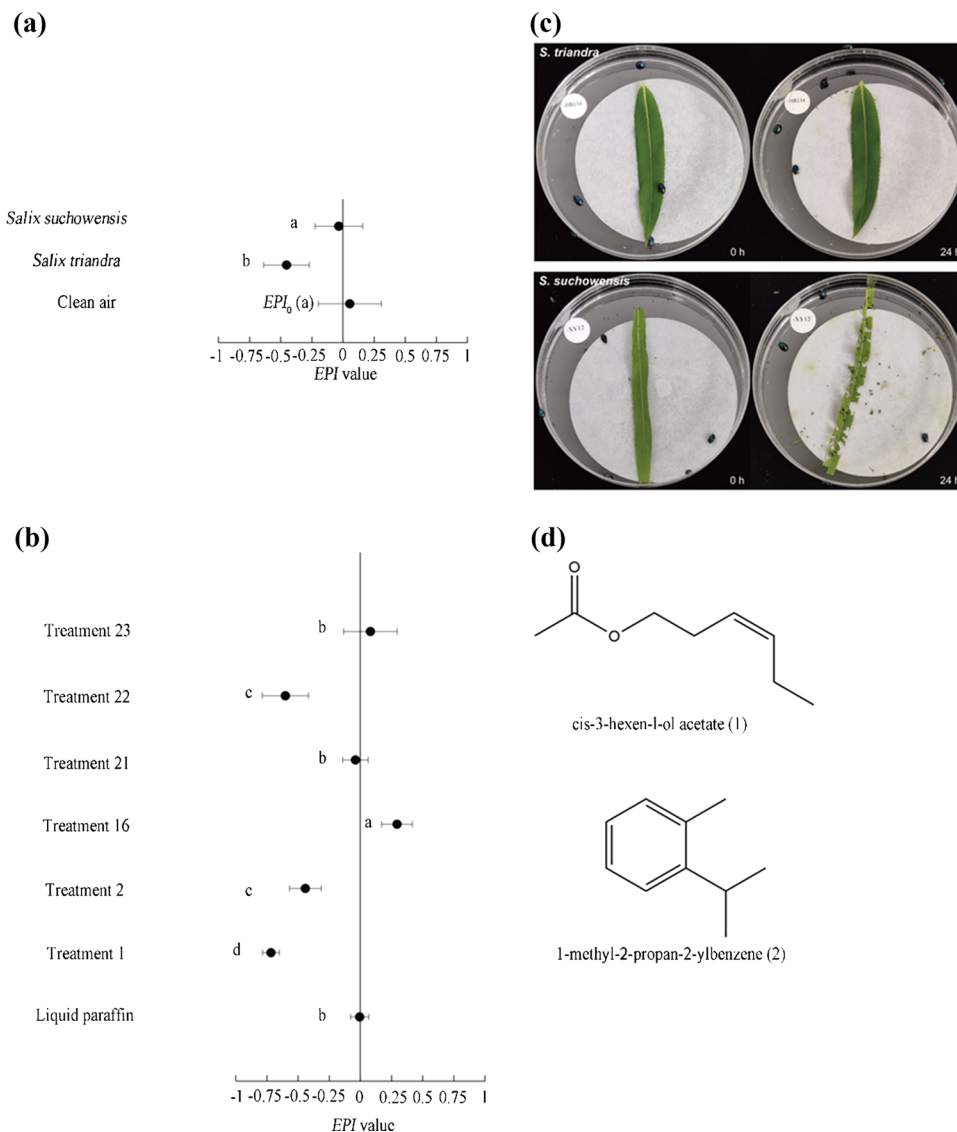
The response by *P. versicolora* to VOCs of willow leaves was tested in bioassays 1 and 2. For *S. triandra* vs. clean air, the selection rate indicated that the beetles significantly preferred clean air over the airborne substances (Table 1), with an *EPI* value of -0.45 ± 0.18 (Fig. 1a), while the EPI_0 of bioassay 1 was -0.01 ± 0.17 (Fig. 1a), meaning that the beetles were significantly repelled by *S. triandra* VOCs (Table 1). For *S. suchowensis* vs. clean air, the selection rates showed no significant difference (Table 1), and the *EPI* value was -0.03 ± 0.19 (Fig. 1a), which was also insignificantly different compared with the EPI_0 (Fig. 1a). Therefore, somewhat unexpectedly, the olfactometer bioassay showed no preference for *S. suchowensis* by adult *P. versicolora*, so that plant odors of *S. suchowensis* might not attract the beetles, although it is a host plant in the field.

Table 1 Olfactory response of adult *Plagioderma versicolora* to willow leaves and clean air in bioassay 1 (Mean \pm SD)

Group		Pests that did not make a selection	Pests that made a selection		<i>P</i> value
Treatment arm	Control arm		Treatment	Control	
Clean air	Clean air	2.4 \pm 0.5 (40)	19.8 \pm 2.9	17.8 \pm 2.7	0.295
<i>Salix triandra</i>	Clean air	2.0 \pm 1.6 (40)	10.4 \pm 2.3	27.6 \pm 3.0	***
<i>Salix suchowensis</i>	Clean air	3.6 \pm 0.9 (40)	17.6 \pm 3.2	18.8 \pm 3.7	0.599

****P* < 0.001, indicates significant difference

Fig. 1 **a** Host-selection behavior of *Plagioderia versicolora* to olfactory cues from leaves of the two willow species. Note: X-axis shows the *EPI* values. Different letters on the left of the horizontal bars indicate significant difference at $P < 0.05$ between the corresponding leaf samples and clean air. **b** Selection behavior of *Plagioderia versicolora* to olfactory cues in different treatments. Note: X-axis shows the *EPI* values. Different letters on the left of the horizontal bars indicate significant difference at $P < 0.05$ between the corresponding treatment and the control. **c** Damage on leaves of *Salix triandra* and *Salix suchowensis* after foraging by adult *Plagioderia versicolora* for 24 hours. **d** The molecular structure of the detected volatile repellent and attractant



Subsequently, *S. triandra*, *S. suchowensis* and clean air were compared simultaneously, the selection rate indicated preference by the beetles as: *S. triandra* < *S. suchowensis* = clean air (Table 2).

Table 2 Olfactory response of adult *Plagioderia versicolora* to willow leaves of two species and clean air simultaneously in bioassay 2. (Mean \pm SD)

Pests that made a selection				Pests that did not make a selection
Treatment arm 1	Treatment arm 2	Control arm 1	Control arm 2	
<i>Salix suchowensis</i>	<i>Salix triandra</i>	Clean air	Clean air	2.6 \pm 1.5
11.6 \pm 1.8 ^a	3.4 \pm 1.9 ^b	10.2 \pm 1.5 ^a	12.2 \pm 2.7 ^a	

Different letters following the selection numbers of each arm indicate significant difference at $P < 0.05$.

Identification of headspace VOCs from leaves of *S. suchowensis* and *S. triandra*

To detect the VOCs triggering host discrimination for adult willow beetles, the headspace VOCs emitted from *S. suchowensis* and *S. triandra* leaves were analyzed by GC-MS. Twenty compounds were identified, 14 present in *S. triandra* and 11 in *S. suchowensis* (Table 3). For compounds detected from *S. triandra*, nine were species-specific

Table 3 Headspace volatiles of *Salix suchowensis* and *Salix triandra* detected by SPME-GC-MS analysis

Compounds	Cas	Relative abundance (%)		
		<i>Salix triandra</i>	<i>Salix suchowensis</i>	Identification
<i>cis</i> -3-hexenyl acetate	3681-71-8	44.68±3.28 ^a	7.52±1.58 ^b	STD, RI, MS
<i>cis</i> -3-hexenyl isovalerate	35154-45-1	31.88±2.32 ^a	52.18±4.02 ^b	STD, RI, MS
farnesane	3891-98-3	9.07±1.21 ^a	2.74±0.35 ^b	STD, RI, MS
<i>cis</i> -3-hexenyl 2-methylbutanoate	53398-85-9	3.26±0.57 ^a	7.16±0.32 ^b	STD, RI, MS
<i>cis</i> -3-hexenyl angelate	84060-80-0	2.96±0.36	NF	RI, MS
2-hexyl-1-decanol	2425-77-6	2.38±0.26	NF	STD, RI, MS
<i>n</i> -tetradecane	629-62-9	2.37±0.35 ^a	1.36±0.47 ^b	STD, RI, MS
<i>cis</i> -7-hexadecenal	56797-40-1	2.24±0.38	NF	RI, MS
<i>trans</i> -3-hexen-1-ol	928-97-2	2.18±0.45	NF	STD, RI, MS
2-butyl-1-octanol	3913-2-8	1.68±0.23	NF	STD, RI, MS
1,5-dimethyl-1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalene	66552-62-3	1.14±0.32	NF	RI, MS
<i>cis</i> -3-hexen-1-yl caproate	31501-11-8	1.06±0.35	NF	STD, RI, MS
<i>trans</i> -4,8-dimethyl-1,3,7-nonatriene	19945-61-0	0.65±0.30	NF	RI, MS
α-farnesene	502-61-4	0.55±0.27	NF	RI, MS
<i>p</i> -cymene	99-87-6	NF	13.42±0.95	STD, RI, MS
<i>o</i> -cymene	527-84-4	NF	10.60±0.82	STD, RI, MS
phytol	150-86-7	NF	7.03±0.64	STD, RI, MS
2-methylbutan-2-ylbenzene	2049-95-8	NF	4.89±0.70	STD, RI, MS
<i>n</i> -octadecyne	629-89-0	NF	2.78±0.41	STD, RI, MS
<i>n</i> -dodecan	112-40-3	NF	1.59±0.35	STD, RI, MS
Unknown chemical 1	–	2.36±0.39	NF	MS
Unknown chemical 2	–	1.55±0.24 ^a	1.55±0.32 ^a	MS
Unknown chemical 3	–	NF	4.87±0.35	MS

Different letters following the concentrations of each compound indicate significant difference at $P < 0.05$ between the two species, NF = not found.

MS comparison of mass spectra with those of NIST2014, RI comparison of retention index with those reported in the literature (To obtain RI, saturated n-alkane standard solution (C7–C40 at a concentration of 1000 mg/L in hexane) was run under the same analytical conditions as the samples and thus the calculated RI were compared with those in the literature. <https://webbook.nist.gov/>), STD comparison of retention time and mass spectra of available standards

(Table 3) and were abundant as *cis*-3-hexenyl acetate, *cis*-3-hexenyl isovalerate, farnesane and *cis*-3-hexenyl 2-methylbutanoate, while the remaining constituents were trace components. For the compounds detected in *S. suchowensis*, six were species-specific (Table 3).

Effects of VOC analogs on finding hosts

To analyze the effects of the emitted compounds on host finding or host identification, 15 commercially available standards were purchased. With these chemicals, 23 treatments were carried out in bioassay 3 and details are shown in Table 4.

Olfactory response to these analogs showed that adult *P. versicolora* were significantly repelled in treatment 1 and treatment 2 (Table 5), because both their numbers for choosing were significantly lower than the control. The *EPI* value was significantly lower ($P < 0.001$) than *EPI*₀ of bioassay 2

(-0.01 ± 0.07) (Fig. 1b) and also showed beetle repellence in treatments 1 and 2. However, the *EPI* value of treatment 1 was -0.72 ± 0.12 (Fig. 1b), which is significantly lower ($P = 0.03$) than treatment 2 (-0.44 ± 0.13) (Fig. 1b), indicating that the repelling effect decreases with a decrease in *cis*-3-hexenyl acetate. In contrast to treatments 1 and 2, the beetles exhibited significant preference to treatment 16 (Table 5). The *EPI* value of treatment 16 was 0.29 ± 0.12 (Fig. 1b), significantly higher ($P = 0.02$) than the *EPI*₀, demonstrating that *o*-cymene had significant effects on attracting adult *P. versicolora*.

In Table 3, VOCs profiling shows that *S. suchowensis* possessed both the repellent (*cis*-3-hexenyl acetate) and the species-specific attractant (*o*-cymene) but concentration of the repellent was much lower than in *S. triandra*. We mixed the two treatments for *S. suchowensis* in treatment 21 (Table 4) and the selection rates vs. control are shown in Table 5. There was no significant difference observed. The

Table 4 Solutions of chemical analogs and treatments in olfactory response tests in bioassay 3

Treatment	Chemical	Concentration (mg/L)
1	<i>cis</i> -3-hexenyl acetate	1.80
2	<i>cis</i> -3-hexenyl acetate	0.30
3	<i>cis</i> -3-hexenyl isovalerate	0.90
4	<i>cis</i> -3-hexenyl isovalerate	1.50
5	farnesane	0.40
6	farnesane	0.10
7	<i>cis</i> -3-hexenyl 2-methylbutanoate	0.10
8	<i>cis</i> -3-hexenyl 2-methylbutanoate	0.20
9	<i>n</i> -tetradecane	0.10
10	<i>n</i> -tetradecane	0.05
11	2-hexyl-1-decanol	0.10
12	<i>trans</i> -3-hexen-1-ol	0.10
13	2-butyl-1-octanol	0.10
14	<i>cis</i> -3-hexen-1-yl caproate	0.05
15	<i>p</i> -cymene	0.40
16	<i>o</i> -cymene	0.30
17	phytol	0.20
18	2-methylbutan-2-ylbenzene	0.10
19	<i>n</i> -octadecyne	0.10
20	<i>n</i> -dodecan	0.05
21*	Dissolved 1 ml treatment 2 and 1 ml treatment 16	
22*	Dissolved treatment 1, 3, 5, 7, 9, 11, 12, 13 and 14, each of their volume is 1 mL.	
23*	Dissolved treatment 2, 4, 6, 8, 10, 15, 16, 17, 18, 19 and 20, each of their volume is 1 mL.	

Treatment 21* was used to test the masking effect of *cis*-3-hexenyl acetate over *o*-cymene. In which, *cis*-3-hexenyl acetate is in 1.80 mg/L and *o*-cymene is in 0.30 mg/L. Treatment 22* was used to test the effect of resembling analogs for leaves of *Salix triandra*, in which, *cis*-3-hexenyl acetate is in 1.80 mg/L, *cis*-3-hexenyl isovalerate is in 0.90 mg/L, farnesane is in 0.40 mg/L, *cis*-3-hexenyl 2-methylbutanoate is in 0.10 mg/L, *n*-tetradecane is in 0.10 mg/L, *trans*-3-hexen-1-ol is in 0.10 mg/L, 2-butyl-1-octanol is in 0.10 mg/L and *cis*-3-hexen-1-yl caproate is in 0.05 mg/L. Treatment 23* was used to test the effect of resembling analogs for leaves of *Salix suchowensis*, in which, *cis*-3-hexenyl acetate is in 0.30 mg/L, *cis*-3-hexenyl isovalerate is in 1.50 mg/L, farnesane is in 0.10 mg/L, *cis*-3-hexenyl 2-methylbutanoate is in 0.20 mg/L, *n*-tetradecane is in 0.05 mg/L, *p*-cymene is in 0.40 mg/L, *o*-cymene is in 0.30 mg/L, phytol is in 0.20 mg/L, 2-methylbutan-2-ylbenzene is in 0.10 mg/L, *n*-octadecyne is in 0.10 mg/L and *n*-dodecan is in 0.05 mg/L.

EPI value of treatment 21 (-0.04 ± 0.15) is also not significantly different ($P = 0.716$) from the *EPI*₀ of bioassay 2 (-0.01 ± 0.07) (Fig. 1b), indicating the effect of the volatile attractant was masked by the repellent. Moreover, blends of VOCs resembling the compounds in the leaf samples of *S. triandra* and *S. suchowensis* were prepared as treatments 22 and 23, respectively. The number of beetles attracted to

treatment 22 was significantly different compared to those attracted to the solvent (Table 5). The *EPI*₀ was significantly higher than the *EPI* of treatment 22 (Fig. 1b; Table 5), indicating that the higher repulsive power of the blend was similar to the smells of *S. triandra*. However, treatment 23 failed in influencing the selection behavior of the beetles (Table 5; Fig. 1b), indicating that the beetles showed no preference to the blend, which was similar to the odors of *S. suchowensis*.

Discussion

Through years of empirical observation in the field, we found that *S. suchowensis* was a favorable host for the leaf beetle, *P. versicolora*, while *S. triandra* was a non-host plant. On a macro level, studies have shown that the preference of oligophagous herbivores, especially those feeding on plants of the same genus, were strongly affected by whether the plants were native species or not. Native species were more readily threatened by biotic stresses, while introduced species were more tolerant (Agrawal et al. 2005; Richard et al. 2019). In this study, *S. suchowensis* is a native species of Jiangsu province in southern China but *S. triandra* germplasm was collected in Heilongjiang province in northern China. Our study confirmed that adult *P. versicolora* was repelled by the chemicals from leaves of *S. triandra*, partially explaining why it is a non-host plant. For *S. suchowensis*, the olfactory bioassay indicated no preference by adult beetles for this favored host compared with clean air, although it produced volatile attractants. Subsequent analyses showed that the effect of the attractant was masked by the endogenous repellent. Therefore, locating host plants might not be triggered by olfactory signals. Enclosed starved beetles with leaves of *S. triandra* and *S. suchowensis* were only observed occasionally on the former, while leaves of the latter were consumed rapidly (Fig. 1c). Since *S. triandra* produced more concentrated *cis*-3-hexenyl acetate than *S. suchowensis*, this chemical might act as an antifeedant. However, *S. triandra* might also synthesize other nonvolatile antifeedants yet to be discovered.

Plant secondary metabolites, including C6-C10 hydrocarbons, aldehydes, alcohols, esters and their acetates, commonly serve as olfactory signals to insects (Matsui 2006). Besides directly attracting or repelling herbivore insects (Mitchell and McCashin 1994; Müller and Hilker 2000; Samantaray and Korada 2016; Castro et al. 2017; Mbitsemunda and Karangwa 2017), they also affected mating and reproduction behavior (Tabashnik 1985; Shelly 2000; Geroftis et al. 2013; Molnár et al. 2017; Xu et al. 2017). As a typical VOC, *cis*-3-hexenyl acetate is widely known as an olfactory signal for herbivores (Sun et al. 2020), and also repelled male *Anoplophora glabripennis* Motschulsky (Nehme et al. 2009) and female *Cephus cinctus* Norton (Piesik et al. 2008)

Table 5 Olfactory response of adult *Plagioder versicolora* to chemical analogs in different treatments (Mean \pm SD)

Group	Group		Pests did not make a selection	Pests that made a selection		P value
	Treatment	Control		Treatment arm	Control arm	
Liquid paraffin	Liquid paraffin	Liquid paraffin	2.2 \pm 1.1 (40)	18.8 \pm 1.5	19.0 \pm 1.4	0.833
1	Liquid paraffin	Liquid paraffin	3.2 \pm 1.8 (40)	5.2 \pm 1.8	31.6 \pm 1.5	< 0.001
2	Liquid paraffin	Liquid paraffin	1.2 \pm 0.4 (40)	10.8 \pm 2.5	28.0 \pm 2.4	< 0.001
3	Liquid paraffin	Liquid paraffin	1.6 \pm 0.9 (40)	19.4 \pm 2.6	19.0 \pm 1.9	0.788
4	Liquid paraffin	Liquid paraffin	1.0 \pm 0.7 (40)	19.2 \pm 2.2	19.8 \pm 2.2	0.673
5	Liquid paraffin	Liquid paraffin	2.8 \pm 1.8 (40)	18.2 \pm 2.9	19.0 \pm 2.7	0.669
6	Liquid paraffin	Liquid paraffin	1.8 \pm 1.3 (40)	19.2 \pm 1.5	19.0 \pm 1.2	0.822
7	Liquid paraffin	Liquid paraffin	3.0 \pm 1.9 (40)	19.0 \pm 3.7	18.0 \pm 3.2	0.657
8	Liquid paraffin	Liquid paraffin	2.4 \pm 1.1 (40)	19.4 \pm 1.1	18.2 \pm 1.1	0.128
9	Liquid paraffin	Liquid paraffin	3.2 \pm 1.3 (40)	17.6 \pm 2.5	19.2 \pm 1.5	0.656
10	Liquid paraffin	Liquid paraffin	2.2 \pm 1.3 (40)	19.0 \pm 1.6	18.8 \pm 2.7	0.262
11	Liquid paraffin	Liquid paraffin	3.0 \pm 1.4 (40)	18.8 \pm 2.2	18.2 \pm 1.9	0.89
12	Liquid paraffin	Liquid paraffin	2.4 \pm 1.1 (40)	18.6 \pm 2.7	19.0 \pm 3.5	0.844
13	Liquid paraffin	Liquid paraffin	2.8 \pm 1.3 (40)	18.4 \pm 2.6	18.8 \pm 3.8	0.851
14	Liquid paraffin	Liquid paraffin	2.6 \pm 1.1 (40)	18.2 \pm 0.8	19.2 \pm 1.5	0.235
15	Liquid paraffin	Liquid paraffin	2.6 \pm 2.2 (40)	17.8 \pm 2.8	19.6 \pm 1.8	0.265
16	Liquid paraffin	Liquid paraffin	2.0 \pm 1.4 (40)	24.6 \pm 2.7	13.4 \pm 2.3	< 0.001
17	Liquid paraffin	Liquid paraffin	2.6 \pm 1.5 (40)	18.8 \pm 2.4	18.6 \pm 1.5	0.919
18	Liquid paraffin	Liquid paraffin	3.8 \pm 2.8 (40)	18.0 \pm 2.4	18.2 \pm 3.5	0.879
19	Liquid paraffin	Liquid paraffin	2.6 \pm 2.1 (40)	19.0 \pm 1.4	18.4 \pm 1.8	0.577
20	Liquid paraffin	Liquid paraffin	1.2 \pm 1.1 (40)	20.2 \pm 0.8	18.6 \pm 1.8	0.127
21	Liquid paraffin	Liquid paraffin	1.2 \pm 1.3 (40)	18.6 \pm 1.9	20.2 \pm 2.3	0.267
22	Liquid paraffin	Liquid paraffin	2.6 \pm 1.1 (40)	7.4 \pm 3.4	30.0 \pm 3.9	< 0.001
23	Liquid paraffin	Liquid paraffin	2.8 \pm 1.9 (40)	20.2 \pm 4.6	17.0 \pm 3.4	0.253

*** $P < 0.001$, indicates significant difference

in finding hosts. Other effects of *cis*-3-hexenyl acetate were also reported to be an inhibitor of pheromone response of spruce bark beetles (*Ips typographius* L.) (Zhang et al. 1999), while it enhanced activities of *Cotesia glomerata* L. and *C. plutellae* Kurdjumov, natural enemies of spruce bark beetles and thus reduced damage by the herbivores (Shiojiri et al. 2006). However, this chemical could also act as an attractant for some other insect pests in finding hosts (Schröder et al. 2015; Xin et al. 2017) and mates (Rouyar et al. 2015; Yu et al. 2015).

In this study, *cis*-3-hexenyl acetate was detected as a repellent (Fig. 1d) and the effect is directly proportionate to concentration: the higher level, the stronger repellency. Accumulated studies have revealed that concentration is a key factor determining the effect of VOCs. For instance, the essential oil of sweet wormwood (*Artemisia annua* L.) to two stored product insects: *Tribolium castaneum* Herbst and *Callosobruchus maculatus* L. worked at a 1% volume concentration but stronger repellency occurred when increased to 2% volume concentration (Tripathi et al. 2000). Rosemary oil effect on onion thrips (*Thrips tabaci* Linde.) was positively correlated with concentrations in the range of 0.1% to 10% (Koschier and Sedy 2003). Similar effects were observed for the silverleaf white fly,

Bemisia argentifolii Genn., where repellency increased with increased concentration (Zhang et al. 2004). The reduction in predation by a species of wolf-spider, *Pardosa pseudoannulata* Boesenberg, occurred with increasing plant essential oil concentration in the range of 100 ppm to 1000 ppm (Farid et al. 2019).

A constituent of plant oils, *O*-cymene, is recognized as a toxin to *Sitophilus zeamais*, *Tribolium confusum* (Tapondjou et al. 2005), and *Bemisia tabaci* (Bleeker et al. 2009). However, we found that *o*-cymene was an attractant (Fig. 1d) to adult *P. versicolora*. It is also noteworthy that *S. suchowensis* contains both repellent and attractant chemicals. Chemicals with opposite effects on insect behavior have been found in numerous plants such as in *Olea europaea* (Scarpati et al. 1993), *Solanum tuberosum* (Dickens 2000) and *Pastinaca sativa* (Carroll and Berenbaum 2002). Research has shown that functional chemicals should be mixed in the right ratio for insects to discern (Bruce and Pickett 2011).

Conclusions

Since the favored host resulted in a neutral response in the olfactory experiment, finding a favorable host by the leaf beetles was not triggered by olfactory signals but likely through visual ones. In contrast, the repellency properties of the non-host willow was unambiguously mediated by olfactory signals. The functional chemicals detected in this study not only improve our understanding of interactions between herbivore insects and plants but also have potential to control willow beetles.

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