The Inhibitory Effect of Powdery Mildew-Induced Volatiles from Rose on Host Selection Behavior of Beet Armyworm Moths (Lepidoptera: Noctuidae)[1]

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Abstract Plant-mediated indirect interactions (PMIIs) between phytopathogenic fungi and herbivorous insects on shared host plants occur in nature. Knowledge of PMIIs is critical in plant molecular breeding and integrated pest management. We studied the response and chemical mechanism of beet armyworm, *Spodoptera exigua* (Hübner), adults to rose plants, *Rosa chinensis* Jacquin, infected with rose powdery mildew, *Podosphaera pannosa* (Wallr.: Fr.) de Bary. Using gas chromatography-electroantennographic detection (GC-EAD) coupled with electroantennogram (EAG), we found that beet armyworm antennae responded to 8, 11, and 3 volatile organic compounds (VOCs) from noninfected roses, mildew-infected roses, and mildew alone, respectively. The EAG analyses showed 11 chemicals (e.g., limonene [1], 2-ethyl-1-hexanol [2], linalool [3], nonanal [4], (*E*)-β-caryophyllene [5], 1-dodecanol [7], *n*-hexadecane [9], 1-hexadecanol [11], methyl palmitate [12], 1-octadecanol [14], and *n*-butyl hexadecanoate [15]) elicited electrophysiological responses of beet armyworm antennae with significant dose-response relationships ($P < 0.05$). The EAG responses to the three chemicals (3, 11, and 15) were greater than that to the reference chemical [i.e., (*E*)-2-hexenal] at 0.5, 5.0, and 50.0 mg/ml. Olfactory and ovipositional behavior assays indicated that three chemicals (2, 3, and 5) significantly attracted beet armyworm females and four chemicals (7, 11, 14, and 15) strongly repelled females. Chemicals 2, 3, and 5 from healthy roses appear to be responsible for the attraction of beet armyworm moths to healthy roses, whereas chemicals 7, 11, 14, and 15 from mildew-infected roses play key roles in inhibiting attraction of moths. VOCs from mildew alone did not attract or repel beet armyworm moths.

Key Words plant-mediated indirect interaction, volatile organic compounds, *Rosa chinensis*, *Spodoptera exigua, Podosphaera pannosa*

Plant-mediated indirect interactions (PMIIs) between phytopathogenic fungi and herbivorous insects on shared host plants exist ubiquitously in the nature, as with interactions between host plants and fungi or between host plants and insects (Beck et al. 2018; Moran 1998; Rostás et al. 2003; Srisakrapikoop et al. 2020; Stout et al. 2006). PMIIs can be very strong if a host plant is shared by two pest organisms even if the pests are not closely related (e.g., the vascular wilt fungus,

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Verticillium dahliae Klebahn, and the spider mite, *Tetranychus urticae* Koch, on their shared host cotton plant, *Gossypium hirsutum* L. (Karban et al. 1987). The understanding of PMIIs is critical not only to assist in plant breeding but also in integrated pest management programs (Franco et al. 2017). However, research on the chemical mechanism involved in PMIIs is in its infancy.

In many cases, insect preference and performance can be negatively impacted by infection of host plant by a phytopathogenic fungus in a ternary relationship consisting of a fungus, a plant, and an insect (Simon and Hilker 2005; Stout et al. 2006). Phytopathogen infection results in activation of plant defense system, thus causing alterations of the plant in phenotype, nutritional composition, and secondary metabolites (e.g., volatile organic compounds [VOCs]) (Szendrei and Rodriguez-Saona 2010; Pierik et al. 2014) and, therefore, the insect on the shared host plant is significantly and detrimentally affected (Beck et al. 2018).

For insects in naturally complex ternary systems, VOCs released by their host plants play important roles in host searching, oviposition site selection, and other behaviors of the pest organism (Allmann and Baldwin 2010). Insects have evolved sophisticated olfactory sensory systems permitting them to detect, recognize, and develop a behavior according to a mixture of VOCs. Plant VOCs can be used as semiochemicals by the insects to recognize the health of the potential host plants and can elicit insect antennal electrophysiological and behavioral responses (Mas et al. 2020; Meza et al. 2020; Munro et al. 2020; Yang et al. 2019b). Plant VOCs change when the plant has been infected by a pathogen, and such pathogen-induced chemical signals impact the ability of gravid insects to find a site suitable for oviposition and, thus, serve to increase the potential fitness of their offspring on their eventual host plants (Pierik et al. 2014). Such interorganismal chemical communications are common in agricultural ecology (Srisakrapikoop et al. 2020).

Our previous studies have demonstrated that olfactory and ovipositional behaviors of insects on rose plants, *Rosa chinensis* Jacquin, are dramatically affected by infection of the plants by fungi. In olfactory bioassays, rose aphids, *Macrosiphum rosivorum* Zhang, were significantly attracted to the mixture of VOCs emitted from healthy rose plants than to plants that were treated with a mixture of toxins from the phytopathogenic fungus, *Alternaria alternata* (Fr.) Keissler (Yang et al. 2012). The VOCs from the infected roses played a key role in host selection by the aphids (Yang et al. 2015). In addition, these VOCs were identified using the method of gas chromatography coupled with mass spectrometry (GC-MS) (Yang et al. 2020). Similarly, the oviposition behavior of the beet armyworm, *Spodoptera exigua* (Hübner), adults on roses was inhibited by the mixture of the rose plant VOCs induced by the rose powdery mildew, *Podosphaera pannosa* (Wallr.: Fr.) de Bary (Yang et al. 2013). The moths preferred to oviposit on healthy rose leaves that were not sprayed with any other volatiles, while they were repelled from rose leaves sprayed with a volatile mixture obtained from *P. pannosa*-infected rose leaves. In that case, the mildew-induced rose VOCs served as semiochemicals to gravid moths to select an oviposition site on healthy roses rather than mildew-infected roses. These induced VOCs consisted mainly of 1-hexadecanol, 1-tetradecanol, and *n*-butyl hexadecanoate and were produced by infected rather than healthy roses (Yang et al. 2019a).

Because the mixtures were used only for olfactory and ovipositional bioassays in those studies, the identity of the chemicals used by the moths to recognize infected

versus healthy roses remains unknown, thus, obscuring our understanding of the chemical mechanisms in the inhibition of lack of attraction of beet armyworm adults to roses infected by the rose powdery mildew. Our objective in the present study was to compare the response of beet armyworm moths to the mono-chemicals in VOCs collected from healthy and *P. pannosa*-infected roses.

Materials and Methods

Plants. Rose plants of susceptible cultivar of *R. chinensis* cv. 'Movie Star' were grown in greenhouses used to produce cut rose flowers in Chenggong county, Yunnan Province, Southwest China. Healthy roses (i.e., control) were grown in a compartment of greenhouse without any phytopathogens and herbivorous insects at approximately 35°C and 95% relative humidity (RH) with a 12/12 h light–dark cycle. Treated rose plants were grown in another separate greenhouse compartment under the same conditions but were naturally infected with the mildew, *P. pannosa*. Plants were used in experiments about 24 d post infection. Rose plants aged about 2 years with 6 to 8 fully expanded leaves, but without buds or flowers, were chosen for the experiments.

Insects. Fourth- or fifth-instar beet armyworm, *S. exigua*, larvae were collected from the greenhouses for cut rose flower production and kept in transparent plastic cages ($60 \times 60 \times 60$ cm^3, with 0.2×0.2 mm^2 mesh holes) filled with a thin layer of soil for pupation. The larvae were fed with healthy rose leaves without any fungal infection at 27°C and 80% RH with a 16/8 h light–dark cycle. The sex of the insects was determined using stereoscopy (50×) at the pupal stage according to genital aperture (Yang et al. 2013). After emergence, pairs of male and female moths were bred separately in two containers on a 10% (w/v) sucrose solution. In experiments, eggs were laid on rose leaves in the cages and newly hatched larvae were reared with the same methods.

VOCs. The methods described by Yang et al. (2019b) were used to obtain mixtures of VOCs from healthy rose plants, rose plants infected with rose powdery mildew, and from the mildew alone. One-half of each mixture was analyzed by the GC-MS method so that the chemicals in each sample were determined qualitatively and quantitatively as reported in Yang et al. (2019a). At the same time, the other half of each mixture was used to perform the experiments described herein.

The dynamic headspace absorption method described by Giusto et al. (2010) with some modifications with different air fluxes and different sorbents was used to separately collect VOCs emitted from healthy and *P. pannosa*-infected rose twigs that were attached to intact and undamaged live plants. VOCs were in situ collected from nine healthy twigs without any symptoms of rose powdery mildew or other pests (determined by visual observation) between 6 and 10 August 2015. Each twig was enclosed in a polyethylene terephthalate bag (Nalophan; Kalle, Wiesbaden, Germany) to collect VOCs. Air was cleaned by passing through a charcoal filter and then deionized water. Pure air was then drawn into the bag at a flow rate of 600 ml/min and released through a trap containing 300 mg Tenax TA sorbent (80–100 mesh, Markes, Llantrisant, UK) in a glass cartridge at a flow rate of 500 ml/min. Because leaks in the bag could not be completely avoided, the difference between the inlet and outlet flow rates could ensure that the leaks were continually purged

during the whole collection. Thus, no outside air could enter the system. Another same cartridge containing Tenax TA (the first cartridge) was inserted into the system between the deionized water and the bag inlet to ensure no VOCs from elsewhere could enter the bag and be collected in the outlet cartridge (the second cartridge). Therefore, all of the VOCs trapped in the second cartridge were released by rose twigs. Each sample was collected for 24 h to exclude diurnal variations, and then VOCs trapped in the outlet cartridge were eluted with 250 μl of *n*-hexane (HPLC [high-performance liquid chromatogram] grade, Sigma-Aldrich, St. Louis, MO). The extract solution was stored at −80°C and concentrated to 25 μl under a gentle stream of pure N_2 before electrophysiological tests. VOCs were in situ collected from nine mildew-infected rose twigs following the same method at the same time and under the same conditions. The leaves on the selected twigs were completely covered with mycelia and spores of the mildew (determined by visual observation).

In addition, VOCs produced by rose powdery mildew itself were obtained using the method of solvent extraction. Nine rose powdery mildew samples (including mycelia and spores) were collected from infected rose leaves. Each sample was immediately steeped in *n*-hexane (HPLC grade; Thermo Fisher Scientific, Waltham, MA), transported to the laboratory in dry ice and then stored at −80°C. Before electrophysiological tests, VOCs were extracted from each mildew sample by leaving the sample to steep in the *n*-hexane for 4 h at room temperature and then the solution was filtered and concentrated to 250 μl under a gentle stream of pure N_2.

Coupled gas chromatography-electroantennographic detection (GC-EAD) analysis. To determine which individual volatile chemicals elicited antennal responses, the VOC mixtures separately obtained from healthy and mildew-infected rose plants and the mildew itself were analyzed using an Agilent Technologies HP 7890A gas chromatograph (GC) coupled with a flame ionization detector (FID) and an electroantennographic detector (EAD, Syntech, Germany). The GC was equipped with an HP-5MS column (30 m × 0.25 mm × 0.25 μm film). One microliter of each sample was injected with injector. The temperature program was 40°C for 1 min, ramped 3°C/min to 80°C, then 5°C/min to 260°C held for 15 min. Nitrogen was used as carrier gas (1.0 ml/min).

Antennae were excised from the heads of *S. exigua* moths. Each antenna was amputated at the base, and the tip of the antenna was removed. The dissected antenna was immediately attached to the antenna holder with two metal electrodes using conductive gel (Spectra 360, America) and then the electrode holder was inserted into the EAD probe. The antenna on the holder was positioned in the charcoal-filtered and humidified air stream that carried the VOCs eluted from the GC column. The effluent from the GC was split with half delivered to FID and the remainder conveyed to EAD. The antennal and FID signals were amplified and recorded simultaneously through a two-channel serial-bus acquisition controller (IDAC-2, Syntech, Germany) and analyzed with software (GC-EAD, version 4.6, Syntech, Germany). For each VOC mixture, electroantennograms (EAGs) were recorded from three kinds of antennae excised from unmated and mated female moths and male moths, separately. EAD-active chemicals were identified by comparing with the GC-MS results (Yang et al. 2019a) from the samples same as ones used in this work.

Comparative EAG responses to selected chemicals. The antennal receptivity of *S. exigua* moths to 15 selected chemicals was determined by EAG to further verify the mono-chemicals in the obtained VOC mixtures that may be potentially responsible for the moth avoidance of the mildew-infected roses. The antennae were removed from the base, and the tip of the antenna was removed. Each dissected antenna was immediately fastened with electrode gel (Spectra 360, America) onto two metal electrodes.

Each test chemical was diluted in hexane to concentrations of 0.5, 5.0, and 50.0 mg/ml for use in comparative EAG response tests. Ten microliters of each chemical solution was applied to a piece of filter paper (0.5×5 cm^2, NewStar, Hangzhou, China). The solvent was allowed to evaporate from the filter paper for 1 min, then the paper strip was placed inside a glass Pasteur pipet (15 cm in length, inner diameter of 0.6 cm) (Fisher Scientific, Pittsburgh, PA). The tip of the pipet was inserted about 3 mm into a small hole through the wall of a glass tube (0.8 cm diameter, 10 cm long) directed at the antenna. A continuous flow (850 ml/min) of charcoal-filtered and humidified air was provided by a stimulus controller (CS-05, Syntech, Germany) with a stimulus duration of 1 s. A 90-s interval between successive stimulations (three times for each compound) was allowed for antennal recovery. EAG responses to 10 μl of hexane were tested as a control. Responses to 10 μl of hexane and the reference chemical ([(*E*)-2-hexenal, a green leaf volatile], 10 μl of solution in hexane at a concentration of 50.0 mg/ml) were obtained before and after each concentration of each test chemical so that correction could be made in the event of loss of sensitivity of the preparation during the recording. It was assumed that the decrease in sensitivity was linear with time for the correction. EAG recordings were obtained from three antennae from different individuals of moths for each solution. Signals were stored and analyzed using EAD version 4.6 software (Syntech, Germany). The antennal receptivity to the chemicals was represented as relative values of EAG responses (R [%]) and calculated with the formula, $R\,(\%) = (A_t - A_c) / (A_r - A_c) \times 100\%$, where R (%), A_t, A_r, and A_c represent relative value of EAG response (%), and absolute values (mV) of EAG responses to a tested chemical, to the reference chemical [(*E*)-2-hexenal], and to control (hexane), respectively.

Y-Tube olfactory bioassays. Olfactory bioassays were conducted using a Y-tube olfactometer to confirm the bioactivities of gravid *S. exigua* moths to individual chemicals. The glass tube contained a stem that was 5 cm in diameter and 25 cm long with two arms that were 5 cm in diameter and 25 cm long. Chemicals were dissolved in hexane in concentrations of 0.5, 5.0, and 50.0 mg/ml. An aliquot (10 μl) of each test solution was applied to a filter paper strip (2×1 cm^2), and the solvent was allowed to evaporate (1 min) before inserting the strip into an odor-source glass bottle connected to 1 arm of the olfactometer. The control glass bottle connected to the other arm of the olfactometer contained a filter paper strip treated with 10 μl of hexane only. The airflow was charcoal-filtered to remove organic contaminants and then passed through a band of needle valves attached to flow meters that regulated the volume of airflow to 200 ml/min. The airflow passed through the odor source bottles, then into either side of the Y-tube, converging at the base of the tube. The Y-tube was positioned horizontally and covered by a black cloth. The female moths were provided with a 10% (w/v) sucrose solution prior to olfactory bioassays. The gravid moths were individually released at the base of the Y-tube and could either

walk or fly towards the source odors. After 15 min, a positive response was recorded if a female moved forward approximately 3 cm beyond either of the arm entrances. The positions of the two arms of the olfactometer were systematically switched after testing one moth to avoid positional bias. After two assays, the Y-tube was cleaned with 90% ethanol and placed into a drying oven (100°C) to dry for 15 min before the next assay. The bioassays were performed in the laboratory under 18 $\pm$ 3°C and 50–70% RH at night when the insects are active. There were 10 insects in each of three groups tested for each solution.

Dual-choice oviposition bioassay. The bioassay was performed to test the effects of the selected chemicals on oviposition behavior and performance. The chemicals were dissolved in a small amount of ethanol and diluted with deionized water at concentrations of 0.5, 5.5, 10.5, and 15.5 mg/ml. Five milliliters of the diluted solution of each chemical were evenly sprayed on the upper and lower sides of the healthy leaves of rose twigs (approximately 25 cm^2) using a new and completely cleaned sprayer similar to a perfume bottle. The other healthy twigs (controls) were sprayed with 5 ml of deionized water. The two bouquets of twigs (five twigs in each bouquet) in water were placed into a steel wire cage covered with a piece of black cloth and diagonally separated by approximately 50 cm. All the rose twigs were replaced every day. Four female and 4 male *S. exigua* moths were released into the cage. The females were allowed to voluntarily choose between the two types of twigs and to oviposit for 8 d at 27°C and 80% RH. The total number of eggs laid each day by 4 females was recorded using stereoscopy until the death of the moths in order to calculate activity index (*AI*%) of the chemicals with the formula, $AI\% = (N_T - N_C) / (N_T + N_C) \times 100\%$, where, N_T and N_C represent the total numbers of eggs on the rose leaves treated with the chemicals and on the controls, respectively. A large absolute value of *AI*% means strongly attractive or repellent activity of the chemical. Four replicates were conducted for each concentration of each chemical. Eggs laid elsewhere in the cage were excluded from analysis.

Data analysis. Statistical analysis was performed using SPSS version 21.0 (IBM-SPSS, Chicago, IL). Data on relative values of EAG responses to the selected chemicals were analyzed using the one-way analysis of variance. Results of oviposition preference and performance for the chemicals were analyzed using the independent samples Student's *t* test. Results of Y-tube olfactory bioassays were compared using a Pearson's χ^2 test according to Fisher's exact test (two-sided) to test the null hypothesis that there was no preference of *S. exigua* moths to odors of the selected chemicals. Individuals that did not make a choice were excluded from the statistical analysis. Significant differences were determined by Tukey's least significant difference posthoc test ($\alpha = 0.05$).

Results

GC-EAD analysis. Nine successful GC-EAD recordings were obtained from antennae of unmated and mated female and male *S. exigua* moths with three headspace VOC samples from healthy rose plants, three headspace samples from *P. pannosa*-infected rose plants, and three samples from the mildew (extracted with hexane) (Figs. 1–3). Overall, a total of 16 chemicals generated reliable EAD responses in antennae. Among them, 15 chemicals were identified by comparing

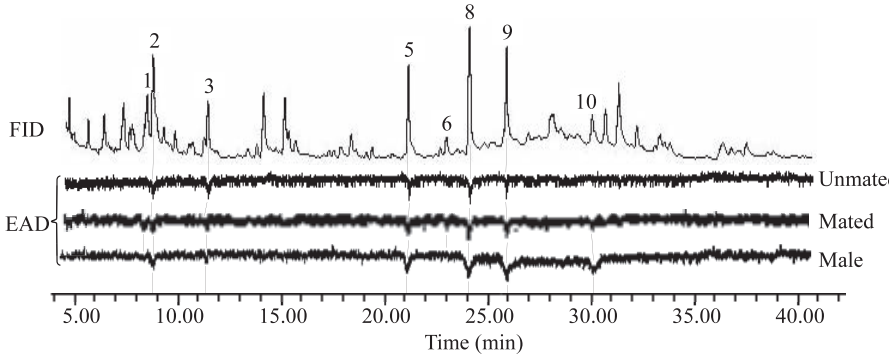

Fig. 1. Gas chromatography-electroantennographic detection (GC-EAD) responses of unmated and mated female moths and male moths of *S. exigua* to headspace volatile compounds collected from healthy rose plants. A dashed line below a peak of the representative flame ionization detector (FID) recording (upper trace) or three electro-antennographic detector (EAD) recordings (lower trace) indicates an EAD-active chemical listed in Table 1.

their retention times with the GC-MS results obtained from the same three types of the samples in our previous study (Yang et al. 2019a) (Table 1), including four terpenes (limonene, linalool, β-caryophyllene, and phytane), four alcohols (2-ethyl-1-hexanol, dodecanol, 1-hexadecanol, and 1-octadecanol), a phenolic compound

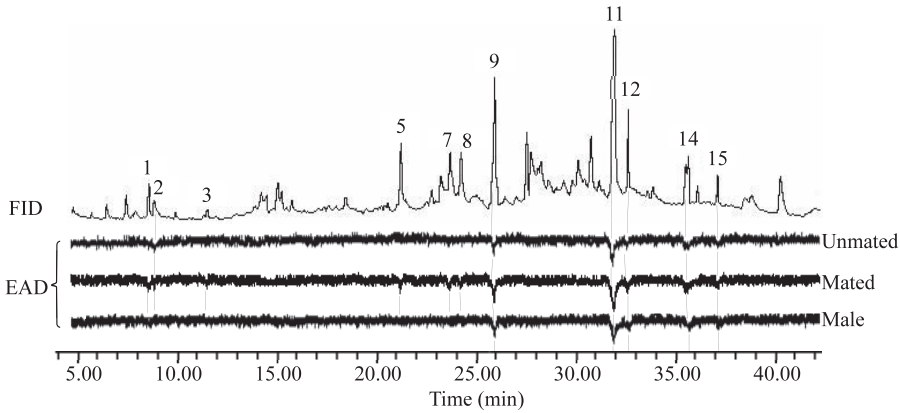

Fig. 2. Gas chromatography-electroantennographic detection (GC-EAD) responses of unmated and mated female moths and male moths of *S. exigua* to headspace volatile compounds collected from powdery mildew-infected rose plants. A dashed line below a peak of the representative flame ionization detector (FID) recording (upper trace) or three electroantennographic detector (EAD) recordings (lower trace) indicates an EAD-active chemical listed in Table 1.

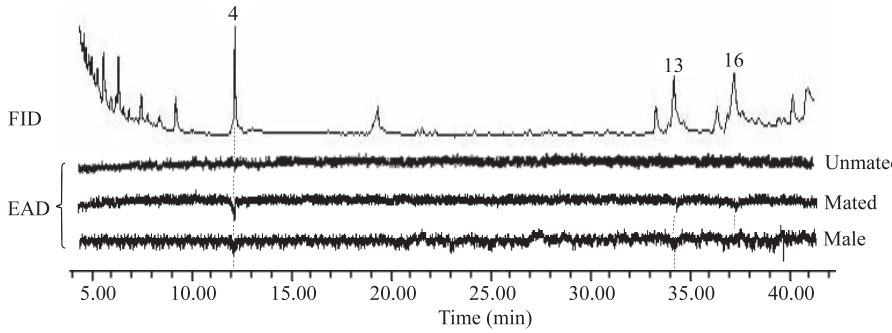

Fig. 3. Gas chromatography-electroantennographic detection (GC-EAD) responses of unmated and mated female moths and male moths of *S. exigua* to headspace volatile compounds collected from rose powdery mildew, *P. pannosa*. A dashed line below a peak of the representative flame ionization detector (FID) recording (upper trace) or three electroantennographic detector (EAD) recordings (lower trace) indicates an EAD-active chemical listed in Table 1.

(2, 4-di-tert-butylphenol), an aldehyde (nonanal), two carboxylic acids (palmitic acid and linolenic acid), two esters (methyl palmitate, and n-butyl hexadecanoate), and an alkane (n-hexadecane).

When the samples from healthy roses were analyzed by GC-EAD, eight chemicals in total were found that exhibited EAD activities (Table 1). Among them, five chemicals (numbers 2, 3, 5, 8, and 9 in Table 1) elicited antennal responses of unmated females, mated females, and male moths. Two other chemicals (1 and 6) from healthy roses elicited antennal responses from only mated female moths, and one chemical (10) from healthy roses elicited EAD responses from mated female and male moths but not from unmated moths. Five, eight, and six EAD-active chemicals were found in volatiles from healthy roses when the antennae from unmated female moths, mated moths, and male moths were assayed, respectively (Fig. 1; Table 1).

The electrophysiological responses of antennae were elicited by a total of 11 chemicals in volatiles from mildew-infected roses. Among them, five chemicals (9, 11, 12, 14, and 15) elicited EAD responses of the three kinds of moths. Unmated moths showed apparent EAD responses to six chemicals, mated moths to 11 chemicals, and male moths to five chemicals (Fig. 2; Table 1). Five EAD-active chemicals (7, 11, 12, 14, and 15) differed from the chemicals in healthy roses, that is, dodecanol, 1-hexadecanol, methyl palmitate, 1-octadecanol, n-butyl hexadecanoate.

Additionally, only three chemicals in the mildew itself were found that possessed EAD activity and only nonanal elicited EAD responses of antennae from the three kinds of moths (Fig. 3; Table 1). No EAD-active chemical was identified from both healthy or infected roses.

Compared with unmated female moths and male moths, mated female moths showed the strongest activity of EAD-responses to the two types of headspace

Table 1. A list of the EAD-active chemicals according to GC-EAD analysis (Figs. 1–3).

Peak No.*	Retention Time, min	Compound**	Sample***		
			Healthy Rose	Mildewed Rose	Mildew
1	8.526	Limonene	+	+	
2	8.842	2-Ethyl-1-hexanol	+	+	
3	11.504	Linalool	+	+	
4	12.156	Nonanal			+
5	21.041	(*E*)-β-Caryophyllene	+	+	
6	23.001	?	+		
7	23.663	Dodecanol		+	
8	24.026	2, 4-Di-*tert*-butylphenol	+	+	
9	26.050	n-Hexadecane	+	+	
10	30.023	Phytane	+		
11	31.985	1-Hexadecanol		+	
12	32.779	Methyl palmitate		+	
13	34.234	Palmitic acid			+
14	35.861	1-Octadecanol		+	
15	37.102	n-Butyl hexadecanoate		+	
16	37.317	Linolenic acid			+
Total			8	11	3

EAD, electroantennographic detector; GC-EAD, gas chromatography-electroantennographic detection.
* Numbers correspond to labeled peaks in Figs. 1–3.
** "?" means unidentified EAD-active compound.
*** "+" means the presence of EAD-active chemicals in plant or mildew volatiles based on the GC-EAD results.

VOCs separately from healthy and mildew-infected roses and the VOCs from the mildew according to the frequency of EAD responses (unmated females, 12; mated females, 22; males, 13) (Figs. 1–3).

Comparative EAG responses to the selected chemicals. The EAG responses of mated female *S. exigua* to the 15 selected chemicals with their EAD activity (with the exception of the chemical number 6, which could not be identified by GC-MS) were compared with the reference chemical, (*E*)-2-hexanal (Fig. 4). There were significant differences in electrophysiological responses of gravid *S. exigua* to the selected chemicals ($F = 21.538$, df = 14, 120, $P < 0.001$; $F = 24.757$, df = 14, 120, $P < 0.001$; and $F = 29.973$, df = 14, 120, $P < 0.001$ at concentrations of 0.5, 5.0, and 50.0 mg/ml, respectively). The gravid showed the strongest EAG response (the

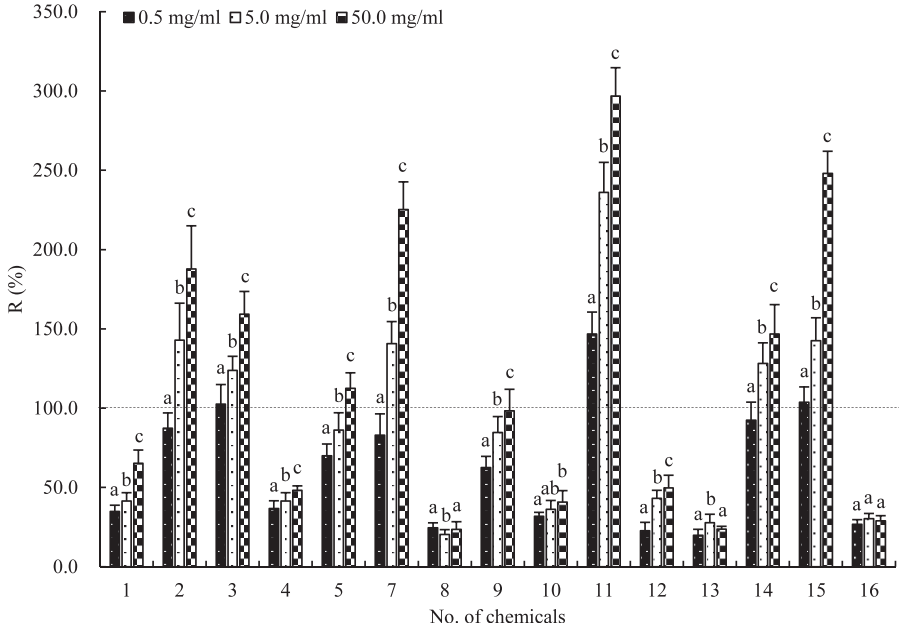

Fig. 4. Electroantennogram (EAG) responses (mean + standard deviation) of gravid *S. exigua* to 15 electroantennographic detector (EAD)-active chemicals at different concentrations. The chemicals were numbered according to Table 1. *R* (%) was calculated using the formula, $R\ (\%) = (A_t - A_c) / (A_r - A_c) \times 100\%$. *R* (%), A_t, A_r, and A_c represent relative value of EAG response (%), and absolute values (mV) of EAG responses to a tested chemical, to the reference chemical [(*E*)-2-hexenal, with a dashed line], and to the control (hexane), respectively. A larger value of *R* (%) means a stronger EAG response. The EAD-active chemical number 6 was not identified by gas chromatography coupled with mass spectrometry (GC-MS). Different letters above bars indicate significant differences among different concentrations (one-way analysis of variance followed by a post hoc test of Fisher's least significant difference [LSD], $P < 0.05$).

maximum > 200%) to 1-hexadecanol, followed by 1-dodecanol and n-butyl hexadecanoate. Moderate responses (>100%) were obtained with 2-ethyl-1-hexanol, linalool, 1-octadecanol, and (*E*)-β-caryophyllene. Responses to other selected chemicals (≤100%) were significantly lower or equivalent to the reference (*E*)-2-hexanal.

The EAG responses of gravid *S. exigua* to 11 of the selected chemicals (numbers 1–5, 7, 9, 11, 12, 14, and 15 in Table 1) exhibited dose-dependent characteristics. The relative values of EAG responses significantly increased with increasing concentrations of these 11 chemicals. The significant differences were observed in the relative values ($F = 5.563$; df = 3, 27; $P \leq 0.012$) when the

comparisons were made among different concentrations (0.5, 5.0, and 50.0 mg/ml). The most significant dosage-effect relationships were obtained by using the six chemicals to test EAG responses, including 2-ethyl-1-hexanol (chemical number 2 in Table 1; $F = 16.700$; df = 3, 27; $P < 0.001$), linalool (chemical 3; $F = 14.095$; df = 3, 27; $P = 0.001$), β-caryophyllene (chemical 5; $F = 11.210$; df = 3, 27; $P = 0.001$), dodecanol (chemical 7; $F = 17.005$; df = 3, 27; $P < 0.001$), 1-hexadecanol (chemical 11; $F = 26.079$; df = 3, 27; $P < 0.001$), and *n*-butyl hexadecanoate (chemical 15; $F = 24.357$; df = 3, 27; $P < 0.001$). Three chemicals (numbers 8, 13, and 16 in Table 1) did not exhibit dose-dependent characteristics.

Olfactory behavior of gravid *S. exigua* to the selected chemicals. The 15 EAD-active chemicals were used to test olfactory responses of gravid *S. exigua* using a Y-tube olfactometer (Fig. 5). The comparisons were performed between synthetic chemicals (T) at different concentrations (Fig. 5A, 0.5 mg/ml; B, 5.0 mg/ml; C, 50.0 mg/ml) and the control of hexane (CK). Among them, 12 chemicals exhibited their activities to olfactory behaviors of the females ($P < 0.05$ at one concentration at least). The other three (linolenic acid, palmitic acid, and phytane) exhibited slight attractive activities, but the differences in the percentages of the females were not significantly different ($P > 0.05$).

Additionally, 1-hexadecanol showed the strongest repellent activity against the *S. exigua* in olfactory bioassays. Repelling percentages reached 77.8% ($\chi^2 = 6.642$; df = 1; $P = 0.010$), 88.3% ($\chi^2 = 6.930$; df = 1; $P = 0.008$), and 90.7% ($\chi^2 = 14.157$; df = 1; $P \leq 0.0001$) at concentrations of 0.5, 5.0, and 50.0 mg/ml, respectively. The chemicals n-butyl hexadecanoate ([i] at 0.5 mg/ml: $\chi^2 = 4.561$; df = 1; $P = 0.033$; [ii] at 5.0 mg/ml: $\chi^2 = 4.128$; df = 1; $P = 0.042$; [iii] at 5.0 mg/ml: $\chi^2 = 6.853$; df = 1; $P = 0.009$), 1-dodecanol ([i] $\chi^2 = 4.162$; df = 1; $P = 0.041$; [ii] $\chi^2 = 4.905$; df = 1; $P = 0.027$; [iii] $\chi^2 = 3.844$; df = 1; $P = 0.050$), and 1-octadecanol ([i] $\chi^2 = 5.497$; df = 1; $P = 0.019$; [ii] $\chi^2 = 4.048$; df = 1; $P = 0.044$; [iii] $\chi^2 = 4.664$; df = 1; $P = 0.031$) also significantly repelled females. 2-ethyl-1-hexanol ([i] $\chi^2 = 4.306$; df = 1; $P = 0.038$; [ii] $\chi^2 = 6.602$; df = 1; $P = 0.010$; [iii] $\chi^2 = 4.008$; df = 1; $P = 0.045$), (*E*)-β-caryophyllene ([i] $\chi^2 = 5.686$; df = 1; $P = 0.017$; [ii] $\chi^2 = 4.164$; df = 1; $P = 0.041$; [iii] $\chi^2 = 7.580$; df = 1; $P = 0.006$), and linalool ([i] $\chi^2 = 4.464$; df = 1; $P = 0.035$; [ii] $\chi^2 = 5.875$; df = 1; $P = 0.015$; [iii] $\chi^2 = 8.396$; df = 1; $P = 0.004$) significantly attracted the females. The other five chemicals (methyl palmitate, n-hexadecane, 2, 4-di-tert-butylphenol, limonene, nonanal) were only weakly attractive or repellent but were statistically significant only at their higher or lower concentrations (Fig. 5).

Ovipositional behavior. Among the 15 EAD-active chemicals (Fig. 6), 2-ethyl-1-hexanol (number 2, in Table 1), linalool (number 3), and β-Caryophyllene (number 5) were strongly attractive in ovipositional behaviors of *S. exigua*. Their activity indices (*AI*%) increased significantly with increasing concentrations, demonstrating the obvious dose–response relationships. Chemical number 2 (2-ethyl-1-hexanol) at a concentration of 15.5 mg/ml stimulated the largest *AI* value of 73.1%, suggesting the most attractive activity ($t = -8.537$, df = 6, $P < 0.0001$, calculated with the number of eggs on treated roses and control roses), whereas linalool (number 3) at a low concentration of 5.5 mg/ml caused a large *AI* value of 64.8% ($t = -11.291$, df = 6, $P < 0.0001$). On the whole, the limonene (number 1), 2, 4-di-tert-bytylphenol (number 8), *n*-hexadecane (number 9), phytane (number 10), palmitic acid (number 15), and linolenic acid (number 16) showed only weakly attractive activity to the females (*AI*% $\leq$ 26.9%). No apparent dose–response relationships

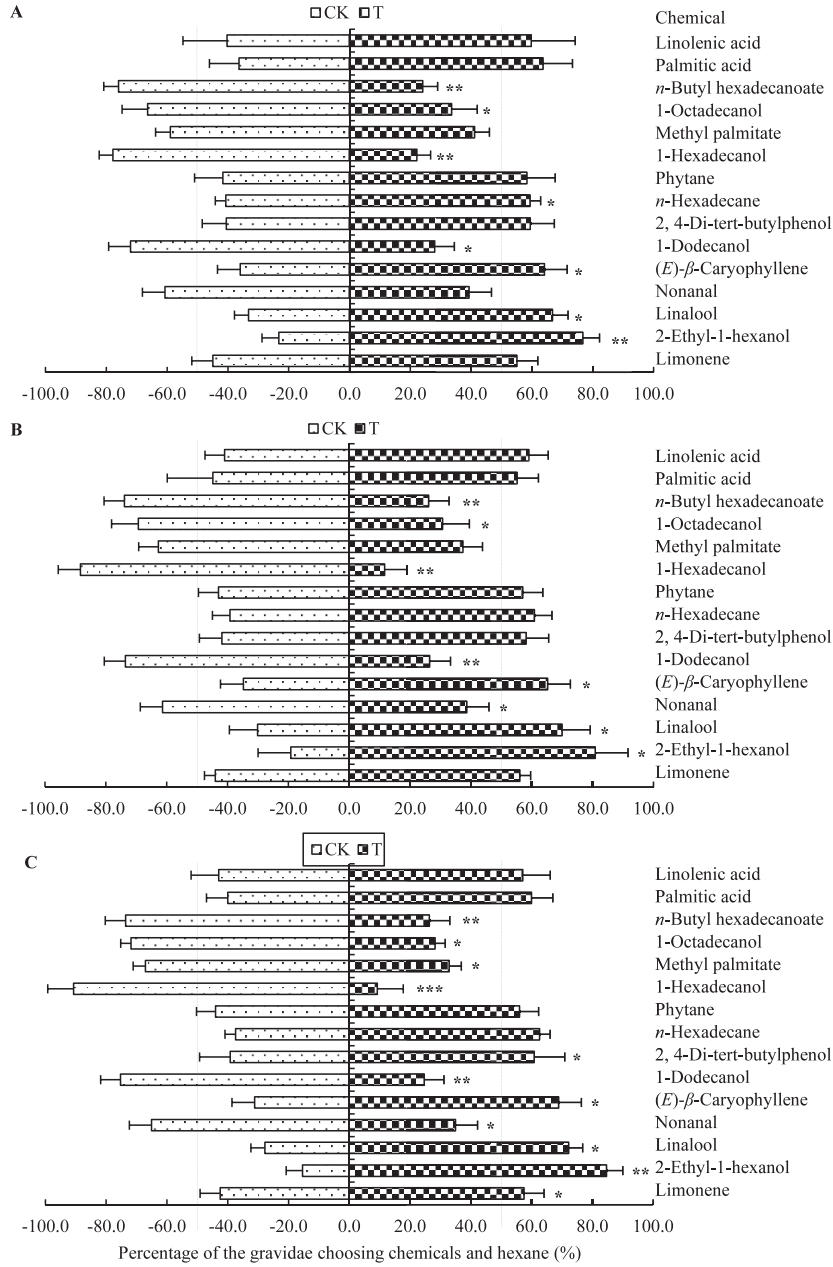

Fig. 5. Behavioral responses of gravid *S. exigua* to the electroantennographic detector (EAD)-active chemicals in olfactometer bioassay. Bars represent the percentages of the moths choosing synthetic chemicals (T) at different concentrations (A, 0.5; B, 5.0; C, 50.0 mg/ml) or hexane (CK). A single asterisk (*) indicates a significant difference at $P < 0.050$, double

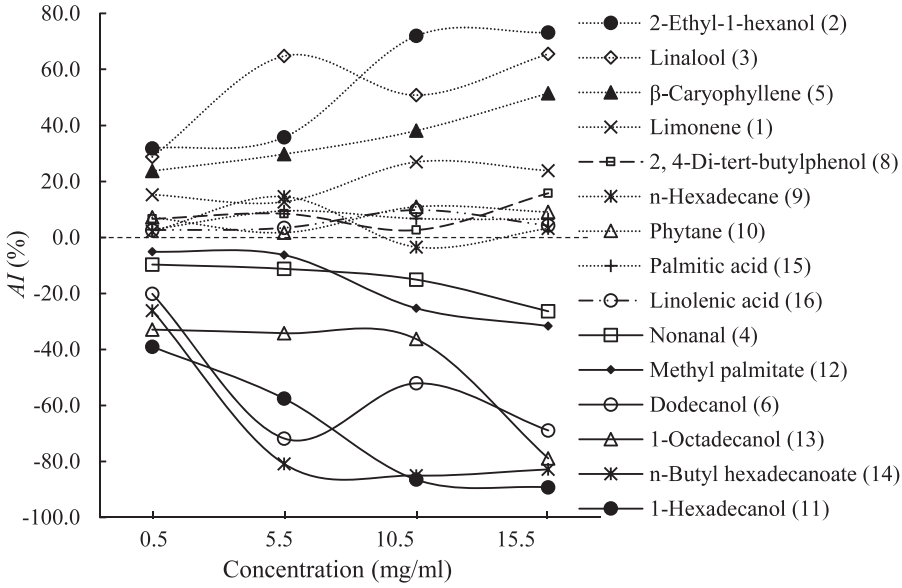

Fig. 6. Effect of the 15 electroantennographic detector (EAD)-active chemicals on ovipositional behavior of gravid *S. exigua*. Activity index (*AI*%) of the chemicals was calculated with the formula, $AI\% = (N_T - N_C) / (N_T + N_C) \times 100\%$. Here, N_T and N_C represent the total numbers of eggs on the rose leaves treated with the chemicals and on the controls in dual-choice oviposition bioassays, respectively. A large *AI* value means strongly attractive or repellent activity of the chemical to the gravid. The chemicals were labeled with the numbers in Table 1.

were found ($P > 0.05$, comparing among different concentrations of each chemical). The chemical, *n*-hexadecane (number 9), had weakly repellent activity to ovipositional behavior of the females only at the concentration of 10.5 mg/ml, but the difference of egg numbers between the treatments and the controls was not significant ($t = 0.287$, df $= 6$, $P = 0.783$).

On the other hand, nonanal (number 4), dodecanol (number 6), 1-hexadecanol (number 11), methyl hexadecanoate (number 12), 1-octadecanol (number 13), and *n*-butyl hexadecanoate (number 14) were repellent to female ovipositional behaviors (Fig. 6). The chemicals 6, 11, 13, and 14 repelled the females strongly, whereas the chemicals 4 and 12 repelled the females moderately. The obvious dose–effect relationships of the three chemicals (numbers 11, 13, and 14) were recorded from the ovipositional results. The activity indices (*AI*%) of the three chemicals decreased significantly with their increasing concentrations, demonstrat-

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asterisks (**) indicate a significant difference at $P < 0.010$, and three asterisks (***) indicate a significant difference at $P < 0.001$ (Pearson's χ^2 test) between T and CK.

ing that their repellent activity against ovipositional behaviors increased significantly. Among all 15 chemicals, 1-hexadecanol possessed the strongest activity to repel oviposition. Its *AI*% could reach −89.3% at the concentration of 15.5 mg/ml ($t = 9.241$, df = 6, $P < 0.0001$).

Discussion

These results (Figs. 1–6; Table 1) demonstrate the chemical mechanism in indirect plant-mediated interaction between *S. exigua* and the rose powdery mildew on their shared host plant, *R. chinensis*, through VOCs. The mildew-induced changes in VOCs from rose plants played a critical role in host plant selection by *S. exigua* moths for oviposition. We previously showed that the mixture of VOCs collected from healthy roses were attractive to ovipositional behavior of *S. exigua* females and that oviposition was inhibited by the mixture of VOCs from mildew-infected roses but not from the mildew alone (Yang et al. 2013). Our results reported herein demonstrated that the ovipositional behavior of the moths could be influenced by the three aspects of VOC changes induced by infection with powdery mildew, including (1) decreases in contents of the chemicals with attractive activity to the moths searching for ovipositional sites; (2) increase in contents of repellent chemicals, and (3) occurrence of the newly discovered chemicals with repellent activity. The new chemicals were produced by the roses only when the roses were infected by the mildew, and not produced by healthy roses. As a consequence, *S. exigua* were negatively affected by infection of rose plants by the mildew through the plant mediation. Therefore, the rose plants had obtained the mildew-induced resistance to *S. exigua*.

From the perspective of the chemical mechanism in searching for healthy roses by the moths as host plants, the most important chemicals were continually released by healthy roses and were 2-ethyl-1-hexanol, linalool, and β-caryophyllene. These were easily recognized by the females so that the roses could be easily located and selected by *S. exigua* as host plants for oviposition. These three chemicals were highly attractive for olfactory and ovipositional behaviors of *S. exigua* females (Figs. 5, 6). Sarles et al. (2017) also reported that the three chemicals had attractive activity. Linalool enhanced attractive activity to *S. exigua* males when it was added to the sex pheromones (Deng et al. 2004). Dickens et al. (1993) also demonstrated that linalool possessed biological activity and elicited a large EAG peak in both sexes of *S. exigua*, results that are similar to ours (Figs. 1, 2, 4, 5, 6). Linalool was a major component in VOCs from the corn, *Zea mays* L, seedling and was attractive to the stink bug *Dichelops furcatus* (F.) (Jacobi et al. 2021). McKibben (2012) illustrated that β-caryophyllene could be used as a synergist in the attractiveness of sex pheromones to male *S. exigua* and *Helicoverpa virescens* (F.). β-caryophyllene also can be a semiochemical for communication between two species of plants (Beck et al. 2018).

The contents of the three chemicals emitted from the roses decreased after the infection of the roses by powdery mildew (Yang et al. 2019a). We, therefore, concluded that one reason for the negative influences of the mildew-infection of rose plants on the ovipositional behavior of *S. exigua* was the mildew-induced decreases of VOCs with attractive activity to *S. exigua* females. Furthermore,

mildew infection induced increases of VOCs with repellent activity. For example, the content of methyl palmitate increased from 1.6 to 75.4 ng per gram fresh weight per hour in rose twigs as a result of mildew infection of roses (Yang et al. 2019a). Figs. 5 and 6 demonstrate the repellent activity of this chemical to *S. exigua* females. In addition, the newly discovered chemicals produced the only roses infected with mildew were repellent. Eighteen new chemicals in roses were induced by infection by mildew with 1-hexadecanol, 1-octadecanol, 1-dodecanol, *n*-butyl hexadecanoate, and *n*-butyl stearate being the predominate ones (Yang et al. 2019a). Our results herein demonstrated that 1-hexadecanol, 1-octadecanol, 1-dodecanol, and *n*-butyl hexadecanoate were EAD-active (Figs. 1–3) and elicited the observed EAG signals (Fig. 4). They clearly repelled *S. exigua* females in the bioassays of olfactory and ovipositional behaviors (Figs. 5, 6), thus, supporting the conclusion that the four new chemicals were responsible for the mildew-induced resistance in roses against *S. exigua*. These chemicals might be used as semiochemicals by the females to recognize the infected roses as unsuitable hosts and to, thus, avoid ovipositing on the infected roses. These chemicals serve as the infection biomarkers for the gravid females to detect the mildew infections in rose plants. VOCs released by host plants can indicate the health statuses of potential host plants to insects and act as semiochemicals allowing plants and insects to communicate. This allows insects to successfully allocate time and energy and, thus, to recognize suitable resources for their offspring (Gripenberg et al. 2010). Therefore, the VOC changes in host plants are responsible for the changes of olfactory and ovipositional behaviors of insects on these host plants.

With the exception of our present study, the attractive or repellent activities of these four chemicals to insects in olfactory or ovipositional bioassays remain unclear to date. In the fruits of *Solena amplexicaulis* (Lam.) Gandhi, 1-hexadecanol, could be produced as a consequence of the induction through feeding damage of the fruits by *Aulacophora foveicollis* Lucas, but the females of *A. foveicollis* did not respond to this compound.

Although it was not a main reason for the chemical mechanism in the mildew-induced resistance of roses against *S. exigua*, the direct interaction between the mildew and the moths also could be found in our ternary system to a slight degree. For instance, nonanal could be released by the mildew itself and be produced by roses as a new chemical resulting from infection with the mildew (Yang et al. 2019a). This chemical showed weakly repellent activity to ovipositional behavior of *S. exigua* females. The females could detect the mildew on the roses by recognizing nonanal and, consequently, exhibit an avoidance response. Microorganisms such as phytopathogenic fungi can produce VOCs or indirectly induce a plant to produce VOCs, and both types of VOCs can repel insects (Guo et al. 2014).

On the whole, the most important reason for the obvious inhibition of infection of rose by *P. pannosa* to *S. exigua* was that the mildew induced the rose plant to produce new chemicals of 1-dodecanol, 1-hexadecanol, 1-octadecanol, and *n*-butyl hexadecanoate. These four chemicals, thereby, repelled the insect. Mildew itself did not obviously attract or repel the females.

Interestingly, when the repellent chemicals were present in the roses, the attractive chemicals coexisting in roses had no effect on *S. exigua* females, thus, females had not been attracted to the roses by these coexisting attractive chemicals (e.g., the headspace VOC blends were collected from *P. pannosa*-infected rose

plants and sprayed on healthy rose plants had no effect as per Yang et al. [2013]). The attractive and repellent chemicals were simultaneously released by these healthy roses sprayed with the blends; however, the females clearly avoided these sprayed roses and preferred to oviposit on healthy roses without any other VOCs in no-choice and dual-choice bioassays of ovipositional behaviors of the females. The females responded only to the repellent chemicals in such situations. Similar results also were reported by Jacobi et al. (2021) in which linalool was a key active chemical emitted by corn seedlings that attracted stink bug of *D. furcatus* to the seedlings. The quantity released by the seedlings significantly increased 2 h after the attack by the stink bug, but the stink bugs clearly avoid these damaged seedlings in response to the repellent chemicals in a blend of VOCs emitted from these seedlings. Here, coexisting linalool in the blend with its larger content no longer affected the behavior of the stink bug.

Perhaps both the attractive and repellent chemicals can competitively bind with the odorant binding proteins (OBPs) or other olfactory proteins in the insects, and the binding abilities of the repellent chemicals is stronger than those of the attractive chemicals so that the attractive molecules cannot be transmitted by OBPs. However, possible explanations for this phenomenon remain hypothetical without intensive insect-physiological analyses. It has been ascertained that such repellent chemicals could be used to protect roses by directly or indirectly applying them to healthy roses to prevent the females from ovipositing on healthy roses. Although roses cannot be inoculated with the rose powdery mildew, *P. pannosa*, for the purpose of improving the resistance of roses to *S. exigua*, repellent chemicals produced by mildew-infected roses could be applied to healthy rose plants to protect the plants from *S. exigua* attack. Hence, our results provide a platform for the development of future semiochemical-based pest management strategies against *S. exigua*.

Future studies should focus on the use of molecular technology to elucidate the physiological mechanisms in host plants responsible for the production of the repellent chemicals by mildew-infected roses. Identification of those host plant genes that control the production of the repellent chemicals could possibly lead to genetic modification of the rose plants to render healthy rose plants that produce the repellent chemicals without infection by the mildew *P. pannosa*.

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