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Volatile-organic compound changes in rose twigs consequent to infection with rose powdery mildew

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ABSTRACT

The chemical mechanisms involved in indirect plant-mediated interactions between insects and phytopathogenic fungi on the host plant are poorly understood. Fungus-induced changes in the volatile organic compound (VOC) contents of plants need to be elucidated to address this. Here, changes in VOCs in rose (*Rosa chinensis* Jacq.) leaves infected with rose powdery mildew (*Podosphaera pannosa* [Wallr.: Fr.] de Bary) were studied. VOCs were collected from undamaged live leaves of healthy and infected intact rose plants by dynamic headspace adsorption and identified by GC-MS. VOCs were extracted using *n*-hexane, and 38 chemicals were found to be produced by *P. pannosa*. A total of 71 VOCs not produced by *P. pannosa* were produced to different degrees by infected and healthy plants, and 18 of these were produced only by infected plants. Principal component analysis of chromatographic data gave VOC profiles distinguishing between infected and healthy plants. Hexadecanol, octadecanol, tetradecanol, *n*-butyl hexadecanoate, and *n*-butyl stearate dominated the VOCs produced by infected plants. These chemicals can be used as markers for detecting mildew-infected rose plants even 4-7 d after infection before symptoms appear. Clear temporal changes in the concentrations of these five chemicals were found. The results improve our understanding of the chemical mechanisms involved in interactions between insects and phytopathogenic fungi.

Key words: Biomarker, GC-MS, hexadecanol, induced plant defense, *Podosphaera pannosa*, *Rosa chinensis*, semiochemicals, volatile organic compound.

INTRODUCTION

Plants produce and emit many volatile organic compounds (VOCs), which play important roles in host plant selection by herbivorous insects. The VOCs produced by a host plant can change (in terms of VOC species produced and amounts produced) when the plant is infected with phytopathogenic fungi. These changes often influence the preferences of insects. The VOC profile in a plant can change quickly and can be detected by the olfactory receptors of insects. Insect olfaction receptors are particularly good at performing quality assessments based on VOCs emitted by host plants (Beyaert et al., 2010; Beck et al., 2014). VOCs emitted by 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 to identifying valuable resources for their offspring (Gripenberg et al., 2010).

Three-way interactions in ternary inter-organism systems consisting of phytopathogenic fungi, herbivorous insects, and host plants are very common (Karban et al., 1987). Interactions between the fungi and insects in such systems may be direct, plant-mediated, or both. Plant-mediated interactions (PMIs) can be either direct or indirect. PMIs can have local effects (confined to the attacked plant part) or systemic effects (Simon and Hilker, 2005; Franco et al., 2017). For

example, the chemicals produced by fungi can enter a plant and influence insects eating the plant. This is a typical direct PMI. Chemicals produced by the plant only when the plant is infected with a phytopathogen can also influence insects eating the plant. This is an indirect PMI. The olfactory system of an insect allows the insect to detect and identify VOCs and to behave in certain ways in response (De Moraes et al., 1998; Liu et al., 2017). Microorganisms such as fungi can produce VOCs or indirectly induce a plant to produce VOCs, and both types of VOCs can attract or repel insects (Guo et al., 2014). It is important to understand PMIs between fungi and insects to improve integrated pest management programs and to allow new genetic varieties of plants to be developed (Franco et al., 2017).

It is usually difficult to differentiate between indirect and direct PMIs because chemicals produced by the fungi enter the host plant and because fungi tissues (e.g., mycelia and spores) cannot be separated from the plant tissues for analysis. VOCs in a host plant also strongly affect interactions between the fungi and insects on the plant. It is necessary to develop a method to allow the chemical compositions of VOCs in healthy plants, fungus-infected plants, and fungi to be determined.

The rose (*Rosa chinensis* Jacq.) is one of the most important cut flowers, and the compositions of VOCs in roses have been studied extensively. More than 400 compounds in roses have been identified, but most of these have been identified in flowers rather than leaves and stems because previous studies have been focused on the flower scent (Joichi et al., 2005).

In a previous study, we focused on a ternary system consisting of rose powdery mildew (*Podosphaera pannosa* [Wallr.: Fr.] de Bary), rose, and beet armyworm (*Spodoptera exigua* [Hübner]). Rose powdery mildew and beet armyworm (BAW) are the two most important pests affecting cut rose flower production (Yang et al., 2013).

In previous studies, we found that VOCs produced and emitted by rose plants clearly changed when the plants were infected with rose powdery mildew (Yang et al., 2013). BAW preference (e.g., olfactory and ovipositional behavior) and performance (e.g., feeding, growth, and development) were markedly negatively affected when rose plants were infected by rose powdery mildew. Significantly, fewer eggs were found on rose plants infected with rose powdery mildew than on healthy plants, and oviposition by the insects dramatically decreased when healthy plants were treated with an extracted mixture containing fungus-induced VOCs from plants infected with rose powdery mildew. Rose powdery mildew changes the VOCs present in rose plants, and the change in VOCs can be recognized by female BAW moths searching for host plants on which to lay eggs. Identifying the VOCs will improve our understanding of the mechanisms involved in this type of insect inhibition by mildew on the shared host plant. Rose plants cannot be inoculated with rose powdery mildew to control BAWs, but VOCs with the characteristics of those mentioned above could be used to repel BAWs.

Although we have found that VOCs in rose plants change when a plant is infected with rose powdery mildew and that these changes affect insect behavior, the chemical compositions of VOCs in healthy and infected rose plants (leaves and stems) are still not understood. This is because only the inhibitory effects on insect behaviors in response to VOC mixtures extracted from healthy and infected rose plants have been tested. We do not know which new chemicals are synthesized by rose plants because of the induction by infection with rose powdery mildew, for instance, they may be dodecanol or other chemicals. Also, we do not know which of these chemicals act as semiochemicals to allow BAW females to recognize infected rose plants. In this study, we focused on the chemical compositions of VOCs in healthy and infected rose plants. VOC mixtures were collected from rose plants infected with rose powdery mildew using a headspace sampling method. Mycelia and spores could not be completely removed from the rose leaves and stems, so some chemicals produced by the mildew were identified in the VOCs collected from the infected rose plants. We wished to identify plant VOCs induced by the mildew rather than VOCs produced by the mildew itself, so the chemical compositions of VOCs from rose powdery mildew were also analyzed. If a chemical is produced by the mildew, it is not a chemical induced by the mildew.

MATERIALS AND METHODS

Chemicals

Standards for 99 compounds were obtained from J&K Scientific (Beijing, P.R. China) or Sigma-Aldrich (St. Louis, Missouri, USA). Each standard was dissolved in *n*-hexane (HPLC grade; Thermo Fisher Scientific, Waltham, Massachusetts, USA) to give standards with concentrations between 2 and 50 μg mL^{-1} (depending on the gas chromatography [GC] mass spectrometry [MS] response to the compound). Seven chemicals (β-bourbonene, methyl *cis*-9,10-epoxyoctadecanoate, methyl 2-eicosenoate, ethyl tridecanedioate, dihydroagarofurane, γ-eudesmol, and 4b,8-dimethyl-2-isopropyl-

4b,5,6,7,8,8a,9,10-octahydro-phenanthrene) were identified by performing mass spectral matches but were not confirmed using standards.

Plants

Experiments to collect VOCs in rose plants were performed in greenhouses used to produce cut rose flowers in Chenggong and Kunyang counties, Yunnan Province, Southwest China. Healthy plants of the susceptible rose *Rosa chinensis* Jacq. 'Movie Star' were grown in a fungus-free greenhouse compartment at *ca*. 35 °C and 95% RH with a 12:12 h photoperiod. In a separate greenhouse compartment, rose plants were grown under the same conditions but were naturally infected with rose powdery mildew (*Podosphaera pannosa* [Wallr.: Fr.] de Bary). The plants were used in experiments ~ 24 d post infection (dpi).

Rose powdery mildew sample collection and extraction

Fifteen rose powdery mildew samples (including mycelia and spores) were collected from infected rose leaves. The samples were weighed using a BP 121S electronic balance (error ± 0.1 mg; Sartorius, Göttingen, Germany) in the greenhouse. Each sample had a mass of ~ 1 g (range 0.9532-1.0419 g). Each weighed sample was immediately steeped in 5 mL *n*-hexane (HPLC grade; Thermo Fisher Scientific), transported to the laboratory in dry ice, and then stored at -80 °C. VOCs were extracted from each mildew sample by leaving the sample to steep in the *n*-hexane for 4 h at room temperature, then the solution was filtered and concentrated to 250 µL. The VOC concentrations in the solution (on a fresh weight [fw] basis) were then determined by GC-MS.

Collecting VOCs from healthy and infected rose plants

Experiments were performed to analyze VOCs released by healthy rose plants and rose plants infected with rose powdery mildew. VOCs released by healthy and infected rose plants were collected *in situ* from undamaged live twigs still attached to intact plants using the dynamic headspace adsorption method described by Giusto et al. (2010) with some modifications (a different air flux and a different sorbent) (Giusto et al., 2010). VOCs were collected from 15 healthy twigs (each ~ 30 cm long) without any symptoms of rose powdery mildew or other pests (determined by visual inspection) between 6 and 10 August 2015. Each twig was enclosed in a polyethylene terephthalate bag (Nalophan, Kalle, Wiesbaden, Germany) and pure air (cleaned by passing it through a charcoal filter) was drawn into the bag at a flow rate of 600 mL min^{-1} and released through a trap containing 300 mg Tenax TA sorbent in a glass cartridge (Markes, Llantrisant, UK) at flow rate of 500 mL min^{-1}. The difference between inlet and outlet flow rates ensured that leaks in the bag (which could not be completely avoided) were continually purged, so no outside air could enter the system. Another same cartridge containing Tenax TA (the first cartridge) was inserted into the system between the charcoal filter and the bag inlet to ensure no VOCs elsewhere in the circumstance could enter the bag and be collected in the outlet cartridge (the second cartridge). Thus, all of the VOCs trapped in the second cartridge were emitted from rose plants. Multiple samples were collected on each sampling day, and a blank sample (using an empty bag) was simultaneously collected on each sampling day. Each sample was collected for 24 h, then the VOCs trapped in the outlet cartridge were eluted with 250 µL *n*-hexane, and the extract was stored at -80 °C until analysis. VOCs were collected from 15 infected rose plants following the same method at the same time and under the same conditions. The leaves on the selected twigs were completely covered with mildew (determined by visual observation). Once a sample had been collected, the healthy or infected twig was removed from the plant and weighed to allow the VOC contents of leaves to be calculated (ng g^{-1} fw h^{-1}).

GC-MS analysis

The 15 infected rose plant, 15 healthy rose plant, and 15 rose powdery mildew samples were analyzed by GC-MS to allow the individual VOCs to be determined. The GC-MS instrument was an HP 6890 gas chromatograph coupled to an Agilent HP 5973 quadrupole mass selective detector (Agilent Technologies, Santa Clara, California, USA). The GC instrument was equipped with an HP-5 MS capillary column (30 m long, 0.25 mm id, 0.25 µm film thickness; Agilent Technologies). The instrument was controlled and data acquired using ChemStation software (Agilent Technologies). The mobile phase was helium, and the flow rate was 1.0 mL min^{-1}. The inlet pressure was 100 kPa, injector temperature was 250 °C, and split ratio was 2:1 for plant samples and 10:1 for mildew samples. Transfer line temperature was 260 °C, electron energy

was 70 eV, and scan range was m/z 35-500. The oven temperature program started at 40 °C, which was held for 2 min, then increased at 3 °C/min to 80 °C, and then increased at 5 °C/min to 260 °C, which was held for 10 min. A VOC was identified by comparing the mass spectrum for the peak of interest with spectra in the wiley7n.l library and the peak retention time with retention time data from the literature and using Kovats' retention indices (Becker et al., 2014). When possible, the assignment of a peak was confirmed by analyzing the relevant standard using the same analytical conditions. Retention indices for the VOCs identified were calculated using the retention times for a standard C_8-C_{25} alkane mixture (J&K Scientific). The VOCs were quantified after calibrating the GC-MS instrument using standards. If a standard for a VOC of interest could not be purchased, the concentration was calculated using the peak area for the alkane with the nearest retention time.

Temporal effects experiments

Experiments in this section were performed to determine temporal scale of the induction effects by infection with rose powdery mildew. The aim was to measure the changes in amounts of the mildew-induced chemicals over time after infection of rose plants. A total of 50 rose plants were grown in a compartment in a mildew-free greenhouse in the laboratory at ~ 24 °C and 85% RH with a 16 h light, 8 h dark cycle. The plants were inoculated with rose powdery mildew by shaking conidia from mildew-infected leaves (collected from a cut rose flower production site) onto the surfaces of all the leaves. This inoculation method was successfully used in previous studies (Xu, 1999; Yang et al., 2013). At 4 dpi (Yang et al., 2013), the upper surfaces of all the leaves were inspected for rose powdery mildew colonies and conidiophores within the colonies using an adjustable pen-microscope (200X magnification) without damaging leaves or colonies. If 50-60 colonies with conidiophores were found on each leaflet on every leaf on a twig, the VOCs emitted by the twig were collected using the method described above. The VOCs were then analyzed following the method described above. Samples of VOCs were also collected and analyzed 7, 10, 13, and 16 dpi with the same methods. Triplicate samples were collected and analyzed at each time point.

Statistical analyses

The total ion current (TIC) as a function of retention time of volatiles, was recorded every 0.4 s during a 60 min GC-MS run, meaning each GC-MS chromatogram contained > 8000 observations (each corresponding to a point in the chromatogram). Statistical analyses were performed only on useful data (determined by visual inspection) in the retention time range 3.1-50.2 min. Each GC-MS chromatogram that was analyzed therefore had ~ 7051 variables. There were 45 sets of GC data in total. Data processing and statistical analyses were performed using MATLAB v. R2014a (8.3.0532) software (MathWorks, Natick, Massachusetts, USA).

Data processing and statistical analysis of the GC data involved five steps. First, baseline correction was performed using a method described by Baek et al. (2015). Minor retention time drifts could not be avoided, so data alignment was performed before principal component analysis (PCA) was performed (Jackels et al., 2014). The GC data were aligned by correlation-optimized warping (COW), following a procedure described by Kumar (2018). Sample HR13 was used as the reference chromatogram because it had a higher cumulative correlation coefficient of all 45 chromatograms. The parameters used for the alignment (segment 100, slack 20) were determined from the systematic variations found after graphically assessing the aligned chromatograms.

Once the data had been standardized, PCA was performed to classify and compare samples in terms of their entire GC profiles, taking the large number of minor peaks and the peak shapes into account (Jackels et al., 2014). Chemometric PCA was performed using the 'statistics toolbox' in MATLAB v. R2014a (8.3.0532) software. Varimax rotations were applied to the resulting principal component model to allow the sample data to be interpreted in terms of the individual factor loadings (Jackels et al., 2014).

For the temporal effects study data, significant differences between VOC concentrations in samples collected from healthy rose plants and plants after they have been infected were identified using two-sample Student's t-tests using MATLAB software.

RESULTS AND DISCUSSION

We aimed to identify mildew-induced changes in VOCs produced by rose plants infected with *P. pannosa*. VOCs were therefore collected from mildew-infected rose plants. However, mildew was present on the rose plant material VOCs were collected from. It was impossible to completely remove mildew mycelia from the plant tissues, so VOCs produced by the mildew itself were collected and analyzed by GC-MS at the same time. All chemicals produced by the mildew were excluded from the list of chemicals induced to be emitted by rose plants by the mildew. More VOCs were extracted from mildew samples by solvent extraction than by dynamic headspace adsorption. Therefore, more information useful for determining which VOCs produced by the rose plants were affected by the mildew was acquired from solvent extraction samples than dynamic headspace adsorption samples.

Chromatograms

The VOCs produced by healthy and infected rose plants and rose powdery mildew were studied in the first experiment. The VOCs produced by the mildew were extracted with *n*-hexane rather than being collected using the headspace sampling system because it was not possible to collect enough mycelia with spores to provide 15 headspace samples. More VOC species were extracted with *n*-hexane than would have been collected using the headspace method, as stated by Joichi et al. (2005). In the study by Joichi et al. (2005), 16 and 56 VOC species were identified when the same sample of rose flower 'Lady Hillingdon' was subjected to the headspace method and solvent extraction, respectively. The solvent extract of the mildew allowed more VOCs produced by the mildew itself to be identified and excluded from the mildew-infected rose plant sample results. As mentioned above, it was not possible to completely remove the mildew mycelia and spores from the mildew-infected rose plants used in the experiments.

Typical chromatograms of the VOCs emitted by the healthy and mildew-infected rose plants and extracted from the mildew are shown in Figures 1A, 1B, and 1C, respectively. The chromatograms were labeled with the sample names used in the laboratory. For example, HR13 is the 13th sample from a healthy rose (HR) plant (15 samples, HR1-HR15, were collected from HR plants). There were clear differences between the chromatograms for the different sample types. Most peaks, except for peak 29 (nonanal), in the mildew chromatogram were at retention times close to 50 min, making this chromatogram very different from the healthy and infected rose chromatograms. The peaks in Figure 1 are labeled using the numbers shown in Table 1.

Figure 1. Gas chromatograms for a healthy rose plant (sample HR13) (A), a mildew-infected rose plant (sample IR2) (B), and a rose powdery mildew sample (sample PM3) (C). The compounds associated with the peak labels are shown in Table 1.

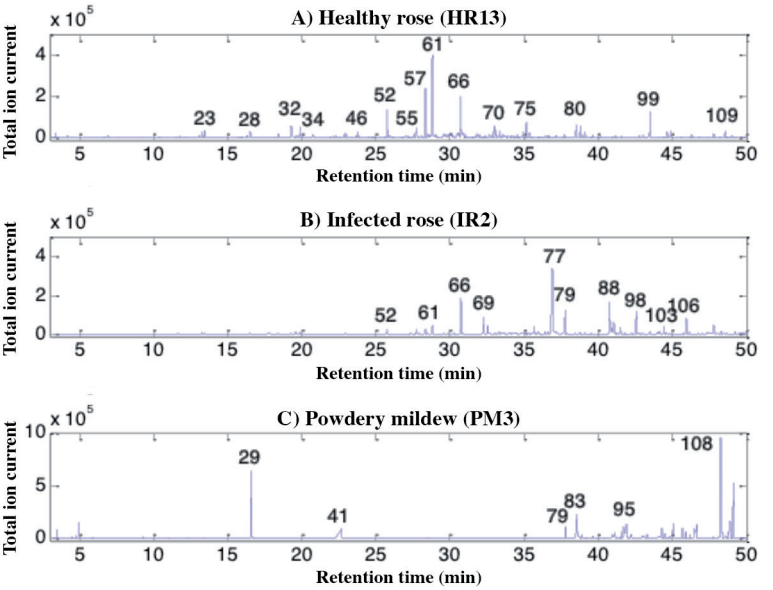


Table 1. Volatile organic compounds found in healthy rose plants (HRP), mildew-infected rose plants (IRP), and rose powdery mildew (RPM).

Label	Retention time (min)	Kovats' RI	Compound	Content (ng g^{-1} fw h^{-1})		
				HRP	IRP	RPM
1	4.563	776	3-Hexanone	2.0 ± 0.7	1.7 ± 0.6	-
2	4.675	792	2-Hexanone	2.0 ± 1.3	2.1 ± 0.8	-
3	4.825	795	3-Hexanol	2.0 ± 0.9	4.0 ± 1.2	-
4	4.953	803	2-Hexanol	1.7 ± 1.1	2.3 ± 1.0	-
5	5.862	837	2-Pentanone, 4-hydroxy-4-methyl-	1.0 ± 0.8	1.7 ± 0.6	-
6	6.273	851	2-Hexenal, (*E*)-	2.3 ± 1.2	3.0 ± 1.1	-
7	6.294	862	3-Hexen-1-ol, (*E*)-	1.3 ± 0.6	2.0 ± 1.4	-
8	8.030	900	*n*-Nonane	-	-	4.3 ± 1.7
9	8.111	905	Heptanal	-	-	3.0 ± 0.8
10	9.200	927	α-Pinene	52.7 ± 5.6	1.0 ± 1.4	7.0 ± 4.2
11	9.564	954	Camphene	6.0 ± 3.5	1.3 ± 0.6	-
12	10.381	962	Benzaldehyde	-	6.3 ± 1.7	-
13	11.054	975	β-Pinene	1.7 ± 0.2	0.7 ± 0.3	5.6 ± 1.9
14	11.540	983	6-Methyl-5-hepten-2-one	3.3 ± 1.2	-	-
15	11.610	989	Hexanoic acid	1.0 ± 0.4	15.3 ± 4.8	-
16	11.663	991	Myrcene	1.7 ± 0.6	3.6 ± 1.3	-
17	12.021	1000	Decane	2.7 ± 1.0	1.8 ± 0.7	-
18	12.090	1005	Octanal	3.3 ± 1.4	0.8 ± 0.1	3.6 ± 0.8
19	12.181	1013	3-Hexen-1-ol, acetate, (*Z*)-	4.3 ± 0.7	5.3 ± 0.7	-
20	12.208	1016	*p*-Cymene	3.3 ± 1.4	0.5 ± 1.1	-
21	13.116	1023	Limonene	95.6 ± 8.3	7.1 ± 0.5	6.0 ± 0.5
22	13.276	1028	Eucalyptol	13.3 ± 5.2	-	-
23	13.447	1031	1-Hexanol, 2-ethyl-	20.2 ± 6.9	4.5 ± 1.1	-
24	14.222	1037	*trans*-β-Ocimene	2.3 ± 1.0	0.3 ± 0.4	-
25	15.023	1066	Acetophenone	3.0 ± 2.0	1.8 ± 0.9	-
26	15.825	1097	Terpinolene	-	0.8 ± 0.2	-
27	16.305	1100	*n*-Undecane	4.0 ± 0.7	1.0 ± 0.5	-
28	16.412	1105	Linalool	28.1 ± 6.1	1.2 ± 0.4	-
29	16.503	1109	Nonanal	-	7.8 ± 0.6	143.1 ± 8.6
30	17.539	1119	Hexanoic acid, 2-ethyl-	-	22.9 ± 1.5	-
31	18.234	1153	Acetic acid, 2-ethylhexyl ester	2.7 ± 0.5	-	-
32	19.308	1179	Naphthalene	32.2 ± 4.9	5.6 ± 0.9	-
33	19.644	1192	α-Terpineol	5.0 ± 1.4	16.6 ± 2.4	-
34	19.874	1200	Dodecane	122.2 ± 9.9	6.6 ± 0.8	-
35	20.098	1207	Decanal	9.3 ± 2.1	2.0 ± 0.7	2.0 ± 0.5
36	20.734	1225	Benzothiazole	10.6 ± 2.5	5.5 ± 1.2	-
37	20.772	1228	Methyl nonanoate	-	-	3.0 ± 0.8
38	21.199	1241	Quinoline	1.7 ± 0.6	-	-
39	21.920	1264	2-Decenal, (*E*)-	-	-	2.0 ± 0.4
40	22.070	1268	Benzene, 1,3-dimethoxy-5-methyl-	23.6 ± 3.9	-	-
41	22.670	1274	Nonanoic acid	7.3 ± 1.4	12.9 ± 1.9	153.2±16.7
42	22.844	1286	Naphthalene, 2-methyl-	7.6 ± 3.2	3.2 ± 1.0	-
43	22.973	1300	*n*-Tridecane	10.9 ± 2.7	3.2 ± 0.4	-
44	23.218	1309	Undecanal	2.0 ± 0.4	-	-
45	23.341	1317	Salicylic acid	2.7 ± 2.6	1.5 ± 0.4	-
46	23.672	1324	*n*-Butyric acid, 2-ethylhexyl ester	4.3 ± 0.8	-	-
47	24.851	1375	Dodecane, 2,6,10-trimethyl-	2.2 ± 1.2	-	-

Continuation Table 1.

Label	Retention time (min)	Kovats' RI	Compound	Content (ng g^{-1} fw h^{-1})		
				HRP	IRP	RPM
48	25.286	1388	Biphenyl	2.7 ± 0.6	-	-
49	25.532	1392	β-Bourbonene[a]	-	-	1.7 ± 1.1
50	25.548	1394	1-Tetradecene	2.7 ± 1.1	-	-
51	25.708	1397	β-Elemene	9.3 ± 1.5	1.8 ± 0.3	2.0 ± 0.5
52	25.761	1400	Tetradecane	151.1 ± 9.7	15.6 ± 0.9	-
53	26.014	1416	9-Oxo-nonanoic acid, methyl ester	3.3 ± 0.7	1.3 ± 0.4	1.7 ± 0.5
54	26.134	1427	β-Caryophyllene	28.6 ± 1.4	0.7 ± 0.3	-
55	27.720	1459	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	7.3 ± 2.2	-	-
56	27.733	1468	1-Dodecanol	-	23.5 ± 3.4	-
57	28.309	1491	Unidentified	32.6 ± 9.2	2.8 ± 0.7	-
58	28.320	1500	*n*-Pentadecane	-	19.1 ± 1.5	-
59	28.368	1506	α-Farnesene	12.1 ± 2.5	3.3 ± 0.6	-
60	28.577	1511	Dihydroagarofurane[a]	-	-	4.6 ± 1.7
61	28.790	1516	2,4-Di-*tert*-butylphenol	177.1 ± 8.8	33.2 ± 4.9	2.7 ± 1.6
62	29.602	1549	Nonanedioic acid, dimethyl ester	-	-	1.7 ± 2.1
63	30.088	1563	2*H*-cyclopropa[a]naphthalen-2-one,1,1a,4,5,6,7,7a, 7b-octahydro-1,1,7,7a-tetramethyl-,(1a*R*,7*R*,7a*R*,7b*S*)-	26.5 ± 3.6	-	-
64	30.457	1581	Fluorene	15.3 ± 6.8	-	-
65	30.580	1591	Caryophyllene oxide	-	-	2.7 ± 0.4
66	30.730	1600	*n*-Hexadecane	83.9 ± 3.0	72.6 ± 2.5	1.7 ± 0.6
67	31.029	1612	Lauryl acetate	-	-	2.0 ± 0.5
68	31.445	1628	γ-Eudesmol[a]	-	-	2.7 ± 0.7
69	32.530	1678	1-Tetradecanol	-	40.0 ± 2.3	-
70	32.995	1700	*n*-Heptadecane	39.8 ± 1.9	4.1 ± 0.6	-
71	33.123	1708	Norphytane	47.1 ± 3.1	-	-
72	33.523	1717	1*H*-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl-	36.1 ± 1.4	-	-
73	33.843	1736	Tetradecanoic acid, methyl ester	-	12.3 ± 0.7	-
74	34.902	1776	Phenanthrene	29.8 ± 2.6	-	-
75	35.142	1800	Octadecane	51.4 ± 3.8	7.5 ± 0.5	-
76	35.335	1812	Phytane	42.4 ± 1.7	-	-
77	36.905	1880	1-Hexadecanol	-	326.1 ± 8.6	-
78	37.195	1900	*n*-Nonadecane	10.9 ± 0.8	-	-
79	37.744	1929	Hexadecanoic acid, methyl ester	1.6 ± 0.2	75.4 ± 9.5	36.1 ± 4.6
80	38.219	1963	Unidentified	37.5 ± 1.3	3.7 ± 1.6	-
81	38.382	1974	1-Eicosene	-	3.0 ± 0.6	-
82	38.502	1984	Unidentified	39.6 ± 1.0	4.5 ± 2.1	-
83	38.577	1991	Palmitic acid	-	5.7 ± 2.3	148.9±17.1
84	39.133	2000	*n*-Eicosane	18.6 ± 0.5	5.5 ± 0.7	-
85	39.624	2012	Tridecanedioic acid, ethyl ester[a]	-	-	8.0 ± 2.7
86	39.651	2019	Phenanthrene, 4b,8-dimethyl-2-isopropyl-4b, 5, 6, 7, 8, 8a, 9, 10-octahydro-[a]	14.9 ± 0.9	-	-
87	39.662	2022	Heptadecanoic acid, methyl ester	-	1.7 ± 0.4	-
88	40.773	2082	1-Octadecanol	-	128.7 ± 7.6	-
89	40.944	2095	Linoleic acid, methyl ester	1.7 ± 0.2	26.7 ± 1.4	10.6 ± 0.8
90	40.982	2100	Heneicosane	4.3 ± 1.7	-	-
91	41.056	2108	Methyl *cis*-12-octadecenoate	1.0 ± 0.6	10.6 ± 2.0	15.3 ± 2.8
92	41.110	2114	Methyl linolenate	9.3 ± 0.5	42.3 ± 3.7	23.9 ± 2.5
93	41.358	2129	Methyl stearate	3.3 ± 0.2	7.8 ± 1.8	23.2 ± 1.8
94	41.729	2142	Linoleic acid	1.3 ± 0.7	1.0 ± 1.2	69.0 ± 5.8

Continuation Table 1.

Label	Retention time (min)	Kovats' RI	Compound	Content (ng g^{-1} fw h^{-1})		
				HRP	IRP	RPM
95	41.890	2156	Linolenic acid	2.7 ± 1.3	1.8 ± 1.1	89.9 ± 7.2
96	42.071	2161	Linoleic acid, ethyl ester	-	1.8 ± 0.7	-
97	42.242	2182	Octadecanoic acid	-	-	19.9 ± 3.9
98	42.584	2189	*n*-Butyl hexadecanoate	-	72.8 ± 4.6	-
99	43.487	2245	2,6-Diphenylphenol	57.0 ± 3.2	9.9 ± 2.1	-
100	43.962	2269	Tributyl acetylcitrate	-	5.0 ± 3.4	-
101	44.026	2273	1-Eicosanol	-	3.2 ± 0.6	26.5 ± 1.9
102	44.091	2282	(*E*,*Z*)-2,13-Octadecadienyl acetate	-	8.3 ± 0.9	-
103	44.443	2308	*cis*-9, 10-Epoxyoctadecanoic acid, methyl ester[a]	-	25.9 ± 1.5	-
104	45.704	2374	Eicosanoic acid	-	1.9 ± 0.7	51.7 ± 4.7
105	45.934	2382	Methyl 2-eicosenoate[a]	-	1.3 ± 0.6	21.2 ± 3.1
106	45.971	2389	Butyl stearate	-	48.4 ± 10.0	-
107	46.163	2396	Hexanedioic acid, dioctyl ester	-	7.8 ± 1.1	-
108	48.290	2531	Docosanoic acid, methyl ester	-	3.8 ± 0.4	156.5 ± 7.6
109	48.599	2550	Unidentified	8.3 ± 3.9	0.9 ± 0.7	2.6 ± 0.5
110	49.000	2567	Docosanoic acid	-	2.6 ± 0.5	123.3 ± 9.8

[a]Identified by mass spectra matching but not confirmed using a standard.
Kovats' RI: Kovats' retention index.

The VOCs assigned to the main peaks are shown in Table 1. A total of 73 VOCs were found in the healthy rose samples. The main components were 2,4-di-*tert*-butylphenol (11.58% of the total VOC concentration), tetradecane (9.88%), linalool (8.38%), dodecane (7.99%), limonene (6.25%), *n*-hexadecane (5.49%), 2,6-diphenylphenol (3.73%), and α-pinene (3.45%). A total of 78 VOCs were found in the mildew-infected rose samples. The main components were 1-hexadecanol (26.49%), 1-octadecanol (10.46%), methyl hexadecanoate (6.13%), *n*-butyl hexadecanoate (5.91%), *n*-hexadecane (5.90%), *n*-butyl stearate (3.93%), methyl *cis*-12-octadecenoate (3.44%), and 1-tetradecanol (3.25%). A total of 37 VOCs (in several chemical classes) were found in the rose powdery mildew samples. The main components were methyl docosanoate (13.26%), nonanoic acid (12.98%), palmitic acid (12.62%), nonanal (12.12%), docosanoic acid (10.45%), linolenic acid (7.62%), linoleic acid (5.85%), and eicosanoic acid (4.38%), which together contributed 79.27% of the total VOC concentrations.

To the best of our knowledge, this is the first study of VOC profiles in rose powdery mildew and rose plants infected with rose powdery mildew. But for healthy roses, VOCs from flowers (not from twigs) have been studied extensively, and the main components have been found to be *cis*-3-hexenyl acetate, *cis*-3-hexenol, hexyl acetate, 2-phenylethyl alcohol, 1,3-dimethoxy-5-methylbenzene, linalool, dihydro-β-ionol, and other compounds with similar chemical structures. 1,3-Dimethoxy-5-methylbenzene is mainly responsible for the scent of fresh modern rose flowers. In our present study, *cis*-3-hexenyl acetate, *cis*-3-hexenol, 1,3-dimethoxy-5-methylbenzene, and linalool were found to be emitted from the rose twigs (Table 1) rather than flowers. The VOCs emitted by rose leaves and rose flowers were obviously different.

PCA of the chromatograms

The PCA results are presented in Figures 2 and 3. The scores biplot (PC1 *vs*. PC2) from the 45 data objects for three types of samples (healthy and infected rose plants and rose powdery mildew) are plotted in Figure 2. Varimax rotation was applied to align the clusters well on the PC1 axis. Three clearly separated clusters can be seen in Figure 2. These clusters corresponded to the healthy rose samples, infected rose samples, and rose powdery mildew samples. The rose powdery mildew samples were clearly different from the other samples, as was found when visually inspecting the chromatograms. The differences were more related to the ranges of VOCs present rather than the amounts of VOCs present.

The healthy and infected rose PC1 and PC2 scores (not the rose powdery mildew scores) are plotted against each other in Figure 3. The healthy and infected rose samples formed two different clusters, indicating that mildew infection obviously changed the VOCs emitted by the rose plants. The infected rose (IR) samples and healthy rose (HR) samples

Figure 2. Principal component (PC) analysis score plot (after varimax rotation had been performed) for healthy rose samples (HR), mildew-infected rose samples (IR), and rose powdery mildew samples (PM).

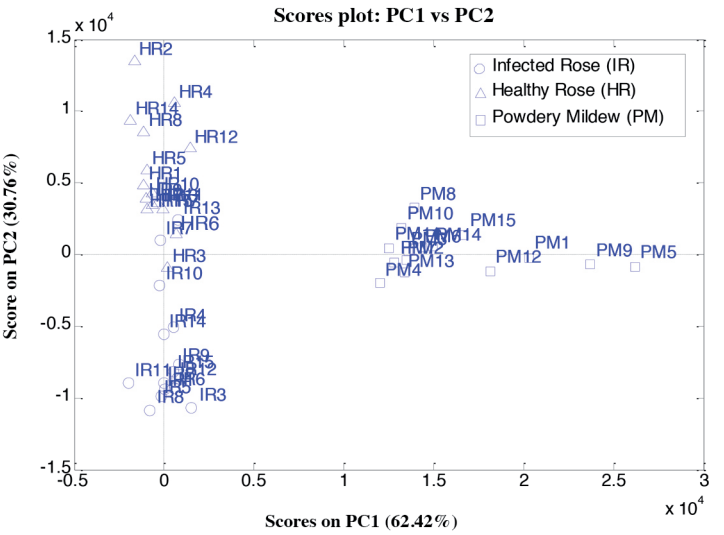
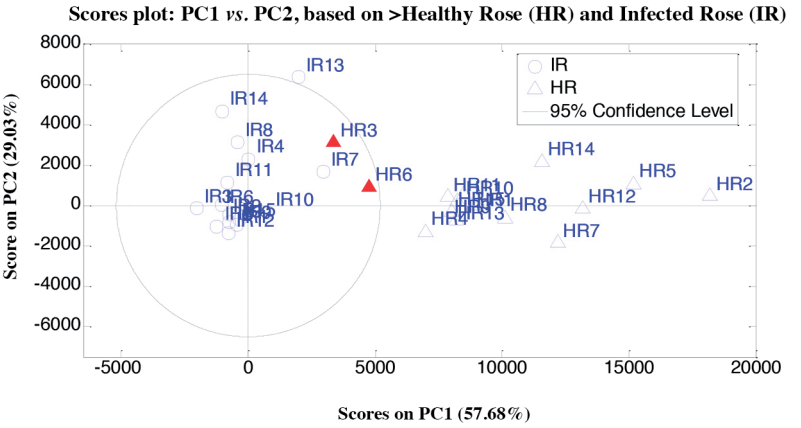


Figure 3. Principal component (PC) analysis score plot (after varimax rotation has been performed) for healthy rose samples (HR) and mildew-infected rose samples (IR).



formed well-separated clusters. The IR cluster was close to the negative PC1 axis (on the left-hand side) in the scores biplot, indicating that the loadings could be used as the characteristic of the VOC profile that classified these points as IR and separated them from the other samples. The loadings (on the left of the graph) indicated that, in general, total VOC contents of the infected rose samples were lower than total VOC contents of the healthy rose samples, consistent with the results shown in Table 1. Samples HR3 and HR6 were classed as healthy rose twigs by visual observation, but the data points for these samples were clearly within the infected rose cluster (within the 95% confidence interval), strongly suggesting that these twigs had been infected with mildew for days, although any symptom of infection on the twigs could not be found by visual inspection. This indicated that for these symptom-free plants, it was necessary to diagnose whether they were infected before they were used in experiments. The 13 remaining healthy rose data points were spread along a small part of the PC1 axis (agreeing with the conclusions drawn from the healthy rose sample chromatograms), so samples HR3 and HR6 were placed in the infected rose group for further analysis. The VOCs identified in the samples supported this decision.

The infected rose cluster was close to the negative PC1 axis in Figure 3, indicating that the concentrations of most of the VOCs emitted by the infected rose samples were lower than the concentrations of the VOCs emitted by the healthy rose samples (Table 1). The results shown in Figures 1-3 and Table 1 indicated that: 1-hexadecanol (labeled 77 in Table 1),

octadecanol (88), and *n*-butyl hexadecanoate (98) concentrations were much higher in infected rose than healthy rose samples; *n*-butyl stearate (106) and methyl *cis*-12-octadecenoate (91) concentrations were somewhat higher in infected rose than healthy rose samples; 1-tetradecanol (69), hexanoic acid (15), and methyl hexadecanoate (79) concentrations were higher in the infected rose than healthy rose samples; and 2,4-di-*tert*-butylphenol (61), 2,6,10,14-tetramethylpentadecane (71), linalool (28), and β-caryophyllene (54) concentrations were lower in infected rose than healthy rose samples. The most important features of the infected rose profiles were the higher hexadecanol (77) concentrations and lower 2,4-di-*tert*-butylphenol (61) concentrations than in the healthy rose profiles. The 2,4-di-*tert*-butylphenol (61) concentration was clearly affected by mildew infection, being 177.1 ± 8.8 and 33.2 ± 4.9 ng g^{-1} fw h^{-1} in the healthy and infected rose samples, respectively. The chemicals mentioned above may have strong antioxidant activities in plants and can be used by plants to protect themselves from injury by phytopathogens (Dangles, 2012). The salicylic acid (45) concentration was not affected by mildew infection.

Mildew infection of the rose plants could have caused these results. The mildew completely infected the rose plants, causing some plant defense pathways (e.g., salicylic-acid-mediated defense pathway) to be inhibited, meaning some defensive chemicals (e.g., phenolic chemicals) could not be synthesized. Mildew infection decreased the 2,4-di-*tert*-butylphenol concentration, as mentioned above, but it also decreased the 2,6-diphenylphenol (99) concentration (which was 57.0 ± 3.2 and 9.9 ± 2.1 ng g^{-1} fw h^{-1} in the healthy and infected rose samples, respectively, as shown in Table 1. Methyl *cis*-9,10-epoxyoctadecanoate (103), a fatty-acid-derived oxylipin, was found at a concentration of 25.9 ± 1.5 ng g^{-1} fw h^{-1} in the infected rose samples but was not found in the healthy rose plant samples. Oxylipins in plants are mainly derived from linole(n)ic acid through the lipoxygenase pathway, and some oxylipins (e.g., methyl *cis*-9,10-epoxyoctadecanoate) are involved in defenses against pathogenic fungi and insect pests (Blée, 1998). The methyl *cis*-9,10-epoxyoctadecanoate concentrations in the infected rose samples were low, so this chemical could not inhibit mildew growth.

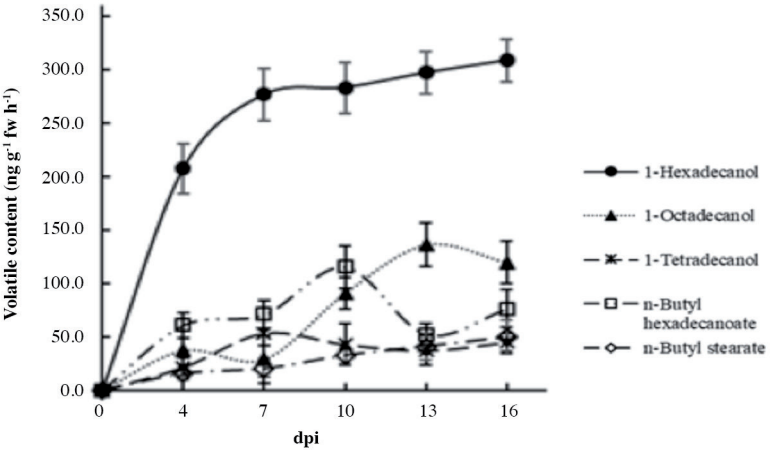
Infection marker VOCs

Some of the identified VOCs were considered to be markers of infection. These VOCs were not emitted by the mildew but were found at markedly different concentrations in healthy and infected rose plants. A total of 71 VOCs could be used as infection markers (Table 1). These were released at markedly higher (10 VOCs) or lower (43 VOCs) concentrations by infected than healthy plants or were emitted only by infected plants (18 VOCs). None of these 71 VOCs was found in the mildew samples. The concentrations of another 18 VOCs were affected by mildew infection, but these VOCs could not be used as markers because they were also found in the mildew samples. The main VOC infection markers (11 VOCs) were defined as VOCs with concentrations ≥ 40.0 ng g^{-1} fw h^{-1} (Table 1) because VOCs at such concentrations could easily and accurately be quantified by GC-MS. The 11 infection markers were identified using authentic standards, MS fragmentation patterns, and Kovats' retention indices. The infection markers were C_{12}-C_{22} VOCs. Three were alcohols (1-hexadecanol [labeled 77 in Table 1], 1-octadecanol [88], and 1-tetradecanol [69]), three were alkanes (tetradecane [52], dodecane [34], and octadecane [75]), two were esters (*n*-butyl hexadecanoate [98] and *n*-butyl stearate [106]), one was a phenolic compound (2,6-diphenylphenol [99]), one was a terpenoid (phytane [76]), and one was a norterpenoid (norphytane [71]) (Table 1).

The VOC infection biomarkers are promising indicators that could allow the early detection of fungal infection before symptoms appear and could be used to discriminate between healthy and infected rose plants. Rose plants should be assessed using the GC-MS method described here before being used in experiments to ensure they are not infected with rose powdery mildew. For example, nonanal has previously been found in healthy roses but we found that it is one of the main VOCs produced by rose powdery mildew (number 29 in Table 1). The material used in the previous study could have been infected with rose powdery mildew without symptoms being visible, or there could have been dead mildew on the rose plant surfaces.

The second experiment was performed to determine how quickly the VOC infection markers indicated infection after plants had been inoculated with the mildew. The results are shown in Figure 4. The concentrations of five VOC infection markers (1-hexadecanol [labeled 77, in Table 1], 1-octadecanol [88], 1-tetradecanol [69], *n*-butyl hexadecanoate [98], and *n*-butyl stearate [106]) were determined using the GC-MS method because these VOCs were not found in the healthy plant samples and the concentrations of these VOCs increased markedly after plants were infected with mildew. Samples were collected every 3 d between 4 and 16 dpi. The first sampling time point (4 dpi) was selected because no symptoms were visible on the rose leaves or stems. The five VOC infection markers were already able to be detected 4 and 7 dpi. Mildew infection clearly strongly induced production of the five VOC infection markers. The concentrations of the VOC

Figure 4. Temporal effect of induction of five volatile organic compound markers emitted by rose plants infected with rose powdery mildew (*Podosphaera pannosa*) at different times after the plants became infected. The points are the mean values and the error bars are the standard errors (n = 3), and dpi means days post inoculation with the mildew.



infection markers increased with time and reached maxima between 13 and 16 dpi. The ability to detect the VOC infection markers very soon after infection means measuring these VOCs could be a nondestructive way of detecting early mildew infections in rose plants in the field, to distinguish between healthy and infected plants and therefore allow informed decisions to be made about applying fungicides.

Temporal effects

The results shown in Figure 4 indicated that the temporal effect existed in the induction of the changes of secondary metabolites in rose plants. The concentrations of all five VOC infection markers changed markedly with time after infection.

In a previous study, we found that BAW ovipositional behavior on rose plants was clearly affected by changes in VOCs induced by rose powdery mildew infection (Yang et al., 2013). In a two-choice bioassay, BAW moths preferred to oviposit on healthy rose twigs sprayed with distilled water (controls) rather than on healthy twigs sprayed with a mixture of VOCs extracted from mildew-infected rose plants. It was concluded that mildew infection therefore inhibited BAW oviposition (Yang et al., 2013). However, it was not clear which chemicals played key roles in inhibiting BAW ovipositional behavior. We therefore needed to investigate the constituents of mildew-induced VOCs. In general, a female moth ready to oviposit on a plant can easily find and recognize a suitable (healthy rather than fungus-infected) host plant from the VOCs released by the host plant if the VOC concentrations in the host plant and in the circumstance are high (Costa et al., 2009; Hashemi and Safavi, 2012; Ponzio et al., 2013; Wyckhuys et al., 2017; Beck et al., 2018). The five VOCs shown in Figure 4 were present at high concentrations in infected rose plants and were not produced by the mildew or by healthy rose plants under normal circumstance. Production of these VOCs by the plants was induced by the mildew infection (Figure 4). In particular, 1-hexadecanol was found at a very early stage of mildew infection (4 dpi), and was found at a higher concentration than all the other VOCs emitted by the mildew-infected rose plants (Figure 4). In future, we will investigate the inhibitory effects of all mildew-induced VOCs identified in this study on the ovipositional behavior of BAW moths.

In previous studies, hexadecanol and octadecanol were found in the pheromone glands of the cotton boll worm *Helicoverpa armigera* and were described as being part of the insect pheromone blend (Bober and Rafaeli, 2010; Zhang et al., 2012). However, the activities of these chemicals in BAW have not yet been determined.

Temporal effects have also been found in other fungus-plant systems (Rostás et al., 2003). For example, consumption of the willow hybrid *Salix* × *cuspidata* by the beetle *Plagiodera versicolora* decreased with time (at 8, 12, and 16 dpi) after the willow was infected with the rust fungus *Melampsora allii-fragilis* (Simon and Hilker, 2005). However, the chemical mechanism involved in this inhibition of feeding activity remains unclear.

Understanding changes in host plant metabolites after infection is key to understanding the chemical mechanisms involved in indirect interactions between phytopathogenic fungi and herbivorous insects mediated by their shared host plant (Beck et al., 2018). Total phenolic compound concentrations in cucumber (*Cucumis sativus*) leaves were

significantly higher 3 dpi of the leaves with the fungus *Cladosporium cucumerinum* relative to healthy controls, but the total phenolic compound concentrations then gradually decreased (at 4, 5, and 6 dpi) (Moran, 1998). The infection was clearly beneficial to the spotted cucumber beetle (*Diabrotica undecimpunctata howardi*) and harmful to the aphid *Aphis gossypii*, suggesting that infections of host plants have complex effects on insects (Moran, 1998). These effects on insects typically involve indirectly plant-mediated interactions between the fungus and the two herbivorous insects. However, the chemical mechanisms involved in such interactions still need to be investigated. Different effects of phytopathogen-induced metabolic changes in plants have been found for different herbivorous insect species, and range from increased susceptibility of the plant (beneficial to the insect) to increased resistance of the plant (detrimental to the insect). It can therefore be concluded that different and complex chemical mechanisms are involved in different interactions (Franco et al., 2017; Hung and Wang, 2018).

The ovipositional behavior of *Epiphyas postvittana* moths have been found to be negatively affected by *Botrytis cinerea* infection of *Vitis vinifera* berries, and this was attributed to changes in VOC concentrations induced by the presence of *B. cinerea*. Ovipositional assays indicated that ethanol and 3-methyl-1-butanol played key roles in regulating the olfactory behavior of *E. postvittana*, and wind-tunnel assays indicated that 2-hexene-1-ol, 2-hexenal, 1-hexanol, 3-octanone, and 1-octen-3-ol may be used by *E. postvittana* as signals when selecting oviposition sites (Rizvi and Raman, 2016; 2017). Interestingly, these VOCs (2-hexene-1-ol, 2-hexenal, 1-hexanol, 3-octanone, and 1-octen-3-ol) were released by grape plants infected with *B. cinerea* but not by healthy plants, and the concentrations of the VOCs in infected plants were very high. These VOCs could therefore be used as semiochemicals by *E. postvittana* females, to allow infected grape plants to be recognized. That conclusion was similar to the conclusion drawn from the results of our present study. In our study, changes in VOC emissions by rose plants were induced by rose powdery mildew infection, and the results improve our understanding of the chemical mechanisms involved in interactions between the two pest organisms and the shared host plant. In future, we aim to investigate the activities of mildew-induced VOCs against BAW, and we will analyze the induction of non-volatile-compound production by rose plants by mildew infection.

CONCLUSIONS

We found that volatile organic compound (VOC) emissions from rose plants were strongly affected by rose powdery mildew infection. Chemometric and bioinformatic analyses were performed. We identified 18 VOCs produced by rose plants infected with *Podosphaera pannosa* that may be used as semiochemicals by beet armyworm moths when searching for a host plant on which to oviposit. We also identified five VOCs that could be used as biomarkers for detecting mildew infections in rose plants before symptoms become visible. VOCs found in rose powdery mildew samples, healthy rose plants (not flowers), and rose plants infected with rose powdery mildew are reported here for the first time. The results improve our understanding of the chemical mechanisms involved in interactions between insects and phytopathogenic fungi on host plants.

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