Headspace Solid-Phase Microextraction and Gas Chromatography-Mass Spectrometry for Analysis of VOCs Produced by *Phytophthora cinnamomi*

Rui Qiu, College of Natural Resources and Environment, Northwest A&F University, Yangling, Shaanxi 712100, China; School of Veterinary and Life Sciences, Murdoch University, Murdoch, WA, 6150 Australia; Cooperative Research Centre for National Plant Biosecurity, Bruce, ACT 2617; **Dong Qu**, College of Natural Resources and Environment, Northwest A&F University, Yangling; **Robert Trengove**, Separation Science & Metabolomics Laboratory, Murdoch University, Murdoch, WA, 6150, Australia; WA Node of Metabolomics Australia; **Manjree Agarwal**, School of Veterinary and Life Sciences, Murdoch University, Murdoch; Cooperative Research Centre for National Plant Biosecurity, Bruce, ACT 2617; **Giles E. St. J. Hardy**, Centre for *Phytophthora* Science and Management (CPSM), School of Veterinary and Life Sciences, Murdoch University, South Street, Murdoch; Cooperative Research Centre for National Plant Biosecurity, Bruce, ACT 2617; and **Yonglin Ren**, School of Veterinary and Life Sciences, Murdoch University, Murdoch; Department of Agriculture and Food, Western Australia, 3 Baron-Hay Court South Perth, WA 6151; Cooperative Research Centre for National Plant Biosecurity, Bruce, ACT 2617

Abstract

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Volatile organic compounds (VOCs) from *Phytophthora cinnamomi*-infected lupin seedlings were collected by headspace solid-phase microextraction (HS-SPME). The sampling was done 28 to 44, 52 to 68, and 76 to 92 h after inoculation (HAI). The HS-SPME samples were analyzed by gas chromatography-flame ionization detector (GC-FID) to assess the differences in volatile compounds between the *P. cinnamomi*-infected lupin seedlings and the control. Three specific peaks were identified after 52 to 68 h with the infected lupin seedlings, at which time there were no visible aboveground symptoms of infection. Subsequently, the VOCs of five different substrates (V8A, PDA, lupin seedlings, soil, and soil + lupin seedlings) infected with *P. cinnamomi* and the corresponding controls were analyzed by gas chromatography-mass spectrometry (GC/MS). A total of 87 VOCs were identified. Of these, the five most abundant that were unique to all five inoculated substrates included: 4-ethyl-2-methoxyphenol, 4-ethylphenol, butyrolactone, phenylethyl alcohol, and 3-hydroxy-2-butanone. Therefore, these metabolites can be used as markers for the identifica-

tion of *P. cinnamomi* in different growing environments. Some VOCs were specific to a particular substrate; for example, 2,4,6-trimethylheptanes, dl-6-methyl-5-hepten-2-ol, dimethyl trisulfide, 6,10-dimethyl-5,9-undecadien-2-ol, and 2-methoxy-4-vinylphenol were specific to *P. cinnamomi* + V8A; heptanes and 5-methyl-3-heptanone were specific to *P. cinnamomi* + PDA; 3-methyl-1-butanol, ethyl acetate, 2-methyl-propanoic acid, ethyl ester, and ethyl ester 2-methyl-butanoic acid were specific to *P. cinnamomi*-inoculated lupin seedlings; and benzyl alcohol and 4-ethyl-1, 2-dimethoxybenzene were only detected in the headspace of inoculated soil + lupin seedlings. Results from this investigation have multiple impacts as the volatile organic profiles produced by the pathogen can be utilized as an early warning system to detect the pathogen from contaminated field soil samples. Data from this investigation have also illuminated potential metabolic pathways utilized by the oomycete during infection which may serve as potential targets for the development of specific control strategies.

Phytophthora cinnamomi is a soilborne oomycete filamentous plant pathogen that causes destructive diseases in agricultural, horticultural, and forestry settings (7,43,45). The damage caused by *P. cinnamomi* is mainly below ground level and frequently not investigated in detail; it is also often neglected or confused with other fungi. Control of the pathogen is difficult, and currently there is no available method to eradicate *P. cinnamomi* without destroying plants in the process (12). Therefore, current management strategies are designed around hygiene and quarantine measures to stop the spread of the pathogen into noninfested areas, and through integrated methods such as chemical and biocontrol, mapping the incidence of *P. cinnamomi* and the disease it causes, together with detection and disease forecasting systems (3,17,18,32,47). To detect the pathogen, microbiological and molecular tests such as methods based on conventional polymerase chain reaction (PCR) and real-time PCR have been developed (13,42). However, methods based on conventional PCR require running samples on an agarose/acrylamide gel to visualize the amplified band, which in-

creases the time required for sample analysis, together with the time-consuming and high throughput sample processing. While real-time PCR is restricted by the number of fluorescent probes, in some cases the primers in multiplex reactions interfere with each other, resulting in a lower sensitivity of detection or the generation of additional amplicons. Therefore, robust, rapid, highly specific, and cost effective diagnostic methods for the identification of *P. cinnamomi* are urgently required.

Microbial indicators are frequently utilized to determine the presence of specific microorganisms in a substrate. For example, volatile organic compounds (VOCs) as intermediate gaseous metabolites can be used to detect microbial activity, abundance, and community composition through distinct chromatographic profiles, and can be measured in a nondestructive manner (15,27,52). For instance, 2-ethylhexanol and methyl 2-ethylhexanoate indicates an enhanced degradation of substrates by most fungi (41). Volatiles that are emitted by three mold species (*Aspergillus versicolor*, *Penicillium chrysogenum*, and *Stachybotrys chartarum*) have been used for the preliminary indication of mold infestation (16). de Lacy Costello et al. (11) studied VOCs from *P. infestans* or *Fusarium coeruleum* inoculated potato tubers and found benzothiazole and 2-ethyl-1-hexanol as the most abundant VOCs produced by both pathogens. Laothawornkitkul et al. (24) studied the VOCs of *P. infestans* infected potato plants and found three marker volatiles including: (E)-2-hexenal, 5-ethyl-2(5H)-furanone, and benzene-ethanol. To date, volatile metabolites have not been identified for *P. cinnamomi* and may be useful in detecting this oomycete.

Corresponding authors: Yonglin Ren, E-mail: y.ren@murdoch.edu.au, and Giles E. St. J. Hardy, E-mail: G.Hardy@murdoch.edu.au

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Qiu et al. (33) developed and evaluated the use of the HS-SPME and GC-FID method for analysis of *P. cinnamomi*. Their study proved the feasibility of using VOCs together with HS-SPME as an identification tool and established the optimal HS-SPME and GC conditions for the identification of *P. cinnamomi* from infected samples.

This study reports the optimized manual sampling (HS-SPME) method followed by (GC-MS) analysis to profile the specific VOCs from *P. cinnamomi*, and to compare the in vitro production of VOCs by *P. cinnamomi* grown on different substrates. The aims of this study allow a determination of which VOCs are typically produced on a specific substrate. Secondly, *P. cinnamomi* may be able to produce specific compounds independently of the substrate used. If such metabolites are found, they can then be used as specific markers for *P. cinnamomi* identification. These VOCs will not only be important for establishing a *P. cinnamomi* online identification system, but can also contribute to the understanding of the pathogens' metabolic pathways, which may lead to the development of efficient *P. cinnamomi* control strategies.

Materials and Methods

Reagents. BBL agar, grade A (Ref. 212304, NJ USA), which was used for preparing the V8 juice agar (V8A) and Difco Potato Dextrose Agar (Ref. 213400, HJ USA), were purchased from Becton, Dickinson and Company (BD). C₁₅ was purchased from CHEM SERVICE (Lot: 377-114A, PA USA), and a custom retention time index standard was purchased from RESTEK (Cat 560295, PA USA).

Equipment and apparatus. Erlenmeyer flasks 100 ml (Quickfit, Australia; Cat QFY-372-P) were used for growing the oomycete samples. Each flask was fitted with an adapter (Quickfit, Australia; Part AQST53/13) equipped with a septum (Grace, Australia; Cat 6518). An autoclave was used for sterilization and a laminar flow hood was used for sterile operation.

The 50/30 µm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fiber (Sigma-Aldrich Australia, Cat. No. 57348-U) was used for collecting of volatile organic compounds (VOCs) from the headspace of the samples.

***P. cinnamomi* isolate.** *P. cinnamomi* (isolate MP 94.48) was obtained from the Centre for Phytophthora Science and Management (CPSM), Murdoch University, Australia. The isolate was maintained on 10% V8 juice agar (V8A) in petri dishes at 24 ± 1°C in the dark; a single 4-mm plug from the margin of the pathogen cultures was transferred to a new V8A petri dish every 10 days.

Substrate and water holding capacity of the container substrate. Washed white river sand purchased from Sand and Soil, WA, was used as the soil substrate for this study. To determine container capacity for optimum plant growth in washed white river sand, three 500-ml free-draining polyurethane pots were filled with 100 g of dry soil. Water was added until each pot was saturated and left overnight, then excess water was allowed to drain. The sand was then weighed when water no longer dripped from the container. The soil was oven dried at 35°C for 2 days and then

weighed. The mass difference between the two weights was considered the container capacity, which was determined as 13.6% for the washed white river sand.

Inoculation. *Lupinus angustifolius* 'Danja' seedlings, a commonly used bait plant for *P. cinnamomi*, were used as a susceptible host for this study. Seeds were surface sterilized by soaking them with 70% ethanol for 2 min followed by immersion in 50% bleach solution (6.25% available chlorine) for 5 min, and then rinsed five times in sterile distilled water. The sterilized seeds were germinated on sterilized filter paper premoistened with sterilized distilled water at 24 ± 1°C in the dark for 2 days. Lupin seedlings and sand inoculation were conducted according to Qiu et al. (33). Briefly, seedling inoculation involved transferring 2-day-old lupins into 100-ml Erlenmeyer flasks with 1 ml sterilized distilled water. After 24 h, a single 4-mm plug from the edge of the 5-day-old *P. cinnamomi* MP 94.48 culture was used as inoculum, placed at the tip of the root and kept in dark at 24 ± 1°C for 3 days to allow lesions to develop. The sand was inoculated by placing six 4-mm-diameter agar plugs colonized with 5-day-old *P. cinnamomi* MP94.48 into a 100-ml Erlenmeyer flask containing 100 g of sterile sand at container capacity and three 3-day-old lupin seedlings. Controls for both methods were inoculated with V8A plugs that had not been colonized by the pathogen. All the treatments were incubated for 6 days at 24 ± 1°C in the dark, at which time VOCs detection was conducted.

Disease progression and headspace volatile compound collection. Lesion lengths were measured at 12, 24, 48, 72, 96, and 120 h after lupin seedlings were inoculated (HAI). Three replicates per treatment, each with three lupin seedlings, were used per sampling period. Based on the progression of symptoms in *P. cinnamomi*-inoculated lupin seedlings, we chose time points 28 to 44, 56 to 72, 104 to 120, 128 to 144, 152 to 168, and 176 to 192 HAI for VOCs collection from the noninoculated and inoculated lupin seedling groups. The 50/30 µm DVB/CAR/PDMS fiber was exposed to the headspace of each sample for 16 h, then the fiber was retrieved and manually injected into the heated GC injection port for sample desorption and analysis (33). The VOCs from the inoculated sterilized washed river sand group and the corresponding controls were collected 6 days after inoculation, with the DVB/CAR/PDMS fiber.

Analysis and identification of VOCs. Gas chromatography-flame ionization detector (GC-FID) was used to detect differences in volatile compounds and to estimate the need to continue with gas chromatography-mass spectrometry (GC-MS). The system was based on an Agilent series 6890 gas chromatograph (serial US00021731, USA) with a 30 m × 0.25 mm internal diameter, Stabilwax capillary polar column with 0.25-µm film thickness (RESTEK, America; Cat 10623). Hydrogen was used as carrier gas at a flow rate of 1.0 ml min⁻¹. The GC column temperature was programmed as: injection at 250°C, temperature held at 45°C for 5 min and then increased by 5°C min⁻¹ to 250°C and held at 250°C for 5 min. All samples were duplicated, and each sample was run in triplicate injections.

Fig. 1. The progressions of *Phytophthora cinnamomi* symptoms on inoculated and control lupin seedlings over time 120 h after inoculation [HAI].

A total of five group samples (three replicates for each treatment and two samples for each replicate) were analyzed by the GC-MS system. This system was based on an Agilent 6890 gas chromatograph (serial US00035629, USA) equipped with the same column that the GC-FID used that was coupled to an Agilent 5973 Network mass selective detector (MSD) with an Agilent ChemStation (serial US02480181, USA). The column temperature program was set the same as the GC-FID. The injection port (splitless mode), interface, and MS source temperatures were 250°C, 250°C, and 230°C, respectively. The helium inlet pressure was controlled at a constant flow of 1.0 ml min⁻¹. The ionization potential was set at 70 eV, and scanning was performed from 35 to 500 atomic mass units at a rate of 3.15 scan/s. However, GC-MS has its limitations for distinguishing between isomers and usually gives very similar mass spectra. Because a favorable match factor between mass spectra is not sufficient for the proper identification (50), the retentions of Kovats-index (22) were used to identify the VOCs by comparison of the mass spectrum with the NIST08 mass spectra library together with retention indices, for the proper identification.

Statistical analysis. The standard deviation of VOCs, the triplicate samples, and the triplicate injections in comparison with aver-

age readings were analyzed using statistical procedures in Microsoft Excel 2007.

Results

Disease progression on inoculated lupin seedlings. No symptoms were observed at 24 HAI; however, the visible symptoms of *P. cinnamomi* MP 94.48 infection appeared as a white mycelial growth from the surface of the root tip at 48 HAI and 24 ± 1°C in dark (Fig. 1). By 72 HAI, the symptoms of *P. cinnamomi* infection were evident, as the roots of the seedlings became necrotic (brown) with a dark brown liquid exuded from the tissues and the absence of lateral root growth. No symptoms were observed on the lupin shoots. However, unlike inoculated samples, control seedlings inoculated with V8A showed a slight browning at the root tip, and the seedlings remained intact throughout the 120-h experimental period (Fig. 1).

Volatile fingerprints of GC-FID chromatograms. The VOCs from GC-FID chromatograms of the different substrates inoculated with *P. cinnamomi* had apparent differences when compared to the controls, and there were similar chromatogram patterns among the infected samples (Fig. 2). Three peaks, RT: 27.31, 29.70, and 32.32 min, with ±0.02 min shifting, were separated in all inoculated sam-

Fig. 2. The chromatograms of different substrates inoculated with *Phytophthora cinnamomi* (A), and their corresponding controls (B).

ples and had consistent chromatogram patterns; two more peaks, RT: 26.29 ± 0.01 and 26.68 ± 0.01 min were found in *P. cinnamomi*-inoculated lupin seedling, soil, and soil + lupin seedling samples (Fig. 2A). Two significant peaks, RT: 17.49 ± 0.01 and 18.86 ± 0.01 min, were found in PDA, V8A, and sand control samples, that disappeared in the corresponding inoculated samples (Fig. 2B). A small quantity of compound RT 21.27 ± 0.02 min was found in all inoculated samples.

The two major GC-FID peaks at 29.70 ± 0.02 min and 32.32 ± 0.02 min indicated volatile metabolites from inoculated lupin seedlings at different sampling periods (Table 1). The volatile metabolites from the inoculated seedlings were not significantly ($P > 0.1$) different from the noninoculated control seedling group in the first

sampling period. However, lupin seedlings inoculated with *P. cinnamomi* isolate MP 94.48 were significantly ($P < 0.05$) greater than noninoculated control seedlings (e.g., $P = 5.94 \times 10^{-8}$ and 3.33×10^{-6} for RT = 29.701 and RT = 32.424 min, respectively, at 76 h), although both specific peaks were detected from the second sampling period, the peak 27.31 ± 0.02 min was only trapped 68 HAI from the inoculated samples.

GC-MS identification of volatile fingerprints. The GC-MS data analysis was conducted using five sample groups: *P. cinnamomi* inoculated PDA, V8A, lupin seedlings, soil, soil + lupin seedlings, and the corresponding noninoculated controls (Table 2). A total of 87 VOCs were identified by GC-MS, and all samples produced well-defined spectra. Sixty-one of the VOCs were found in the

Table 1. The three selected important gas chromatography-flame ionization detector (GC-FID) response volatile organic compounds detected from lupin seedlings inoculated with *Phytophthora cinnamomi* isolate MP 94.48 and noninoculated control seedlings ($n = 3$)^a

Sampling period	Compounds produced by lupin control (Area/GC readings)			Compounds produced by inoculated seedlings (Area/GC readings)		
	RT = 27.2 min	RT = 29.7 min	RT = 32.3 min	RT = 27.2 min	RT = 29.7 min	RT = 32.3 min
First (28-44 HAI)	0	5.93 ± 0.2	3.96 ± 0.1	0	5.83 ± 0.6	3.94 ± 0.4
Second (52-68 HAI)	0	5.94 ± 0.1	3.91 ± 0.5	0	20.11 ± 0.9	17.47 ± 0.7
Third (76-92 HAI)	0	5.92 ± 0.3	3.95 ± 0.1	21.25 ± 0.4	3,614.68 ± 1.2	442.33 ± 1.0

^a HAI = hours after inoculation; RT = retention time.

Table 2. Volatile organic compounds (VOCs) collected from *Phytophthora cinnamomi* (Pc) inoculated samples and the corresponding noninoculated controls identified from gas chromatography-mass spectrometry (GC-MS) analysis and Kovats values calculation

RT ^a (min)	VOCs	Kovats indices	Match quality (%)	Rep ^b	Pc + V8A	V8A alone	Pc + PDA	PDA alone	Lupin control	Pc inoc. lupin	Soil sample	Pc inoc. soil	Pc inoc. soil + lupin	Soil + lupin
1.35	Hexane	824	79.5		+++	+++	+++		+	+				
1.58	Dimethyl sulfide	843	77	2	++	+								
1.80	Acetone	862	79.7	44	++++	++++	++++	+		+++				
1.95	Cyclotrisiloxane, hexamethyl-	875	91.8		++	++	+++		++	++				
2.19	Ethyl acetate	895	85	23						+				
2.25	<i>n</i> -Nonane (STD) ^c													
2.29	2-Butanone	905	71.3		+		++			+				
2.39	Butanol, 2-methyl-	912	77.6			+		+						
2.43	Butanol, 3-methyl-	916	87.2			++		+++						
2.95	Propanoic acid, 2-methyl-, ethyl ester	960	71.9	2						+				
3.13	2-Pentanone	975	80.1		+					+				
3.15	2,3-Butanedione	977	81			+++								
3.40	Decane, 5-methyl-	999	81.7		+	+	+			+				
3.42	<i>n</i> -Decane (STD) ^c													
4.50	Butanoic acid, 2-methyl-, ethyl ester	1050	89							+++				
4.98	Disulfide, dimethyl	1072	97.7		+++		+++					++		
5.28	Hexanal	1087	71.7	14		++++		+++						
5.58	<i>n</i> -Undecane (STD) ^c													
8.96	<i>n</i> -Dodecane (STD) ^c													
9.49	1-Butanol, 3-methyl-	1216	77.6	28						+++				
9.86	Heptane, 2,4,6-trimethyl-	1228	94.3		+++									
10.48	3-Heptanone, 5-methyl-	1247	93.4				++							
11.66	Octanal	1284				++								
11.78	2-Butanone, 3-hydroxy-	1288	76.6		+++++		+++			+++		+++	+++	+++
12.16	<i>n</i> -Tridecane (STD) ^c													
13.26	5-Hepten-2-one, 6-methyl-	1338	75.9		+++	+++++					+++++	++	+++	+++++
14.12	Dimethyl trisulfide	1364	84.4	2	+									
14.48	Cyclohexasiloxane, dodecamethyl-	1380	94		+++	++	+++		++	+				
14.73	Nonanal	1389	86.5	14	+	++++								
14.96	2,6-Octadiene, 2,6-dimethyl-	1396	91			+++								
15.07	<i>n</i> -Tetradecane (STD) ^c													
15.71	2-Octenal, (E)-	1424	96.1			+								
16.42	1-Octen-3-ol	1451	92.5	2, 28	+		++							
16.77	dl-6-methyl-5-hepten-2-ol	1464	88.2	28	+++									

(continued on next page)

^a RT, retention time; VOCs, volatile organic compounds; Trace level (tr), <1%; +, 1-5%; ++, 6-10%; +++, 11-20%; +++++, 21-30%; ++++++, 31-50%; ++++++, 51-100%; ++++++, >100%; blank spaces indicate the compound was not detected. Percentages were calculated using the total ion current (TIC) for each compound relative to the TIC of phenol, 4-ethyl-2-methoxy- that recorded (Phenol, 4-ethyl-2-methoxy- =100%).

^b Reported in other microbe.

^c Compounds in italics (labeled STD) are Kovats standards injected into the GC/MS system.

headspace of the control samples. The corresponding *P. cinnamomi*-inoculated samples had 57 of the VOCs that were observed above trace levels in the headspace of the samples, with 21 volatiles found to be specific to the inoculated substrates only (Table 2).

The largest peak in the spectra of inoculated samples was 4-ethyl-2-methoxy phenol. For comparative purposes, the data in each column of Table 2 have been scaled relative to 4-ethyl-2-methoxy phenol. In addition, a number of minor peaks were found, but could not be identified from the current GC-MS library. Therefore, the retentions of Kovats-index were used for identification of VOCs and the results are shown in Table 2.

Most volatiles were found to be common from the five inoculated samples; however, some VOCs were specific to an inoculated substrate. Dimethyl trisulfide, 6,10-dimethyl-5,9-undecadien-2-ol, and 2-methoxy-4-vinylphenol were detected at low levels (1 to 5%), and heptanes, 2,4,6-trimethyl- and dl-6-methyl-5-hepten-2-ol were detected at the level of 11 to 20% of the reference peak in the headspace of *P. cinnamomi* + V8A. Heptane and 5-methyl-3-heptanone were present at the level of 11 to 20% and 6 to 10% only found in the headspace of *P. cinnamomi* + PDA. While 3-methyl-1-butanol, ethyl acetate, 2-methyl-propanoic acid, ethyl ester, and 2-methyl-butanolic acid, ethyl ester were only found in the headspace of *P. cinnamomi*-inoculated lupin seedlings, benzyl alcohol and 4-ethyl-1, 2-dimethoxybenzene were only detected in the headspace

of inoculated living substrates (lupin seedlings and soil + lupin seedling).

The five specific volatile compounds, 3-hydroxy-2-butanone, butyrolactone, phenylethyl alcohol, 4-ethyl-2-methoxyphenol, and 4-ethylphenol, were found at concentrations significantly ($P < 0.01$ when compared with other peaks) higher in all the infected substrates but not found in the controls, with 4-ethyl-2-methoxy phenol the most concentrated, followed by 4-ethyl-phenol, with butyrolactone and phenylethyl alcohol occurring at 6 to 10% and 6 to 20% of the reference peaks, respectively. 2-Pentanone was detected in the headspace of *P. cinnamomi* + V8A and *P. cinnamomi*-inoculated lupin seedlings; 1-octen-3-ol, dimethyl disulfide, 2-undecanol, and phenylethyl alcohol were found in the headspace of *P. cinnamomi* + V8A and *P. cinnamomi* + PDA. Furfural and benzaldehyde were at the highest and second highest concentrations in the headspace of the controls, but were not present in the inoculated samples. Six additional VOCs occurred at >10% of the reference peaks in the headspace of the controls; these were benzeneacetaldehyde, (E)-2,7-dimethyl-2,6-octadienal, (Z)-3,7-dimethyl-2,6-octadienal, 2,3-butanedione, 2,6-dimethyl-2,6-octadiene, and hexanal.

Discussion

The five marker VOCs, 4-ethyl-2-methoxy phenol, 4-ethyl phenol, butyrolactone, phenylethyl alcohol, and 3-hydroxy-2-buta-

Table 2. (continued from preceding page)

RT ^a (min)	VOCs	Kovats indices	Match quality (%)	Rep ^b	Pc + V8A	V8A alone	Pc + PDA	PDA alone	Lupin control	Pc inoc. lupin	Soil sample	Pc inoc. soil	Pc inoc. soil + lupin	Soil + lupin
16.79	Furfural	1464	97			+++++		+++++						
17.42	1-Hexanol, 2-ethyl-	1488	81.5	11	++	+	+++	+		+				
17.55	Decanal	1498	90.1	26		+								
17.76	<i>n</i> -Pentadecane (STD) ^b													
18.17	Benzaldehyde	1517	94.6	26		+++++		+++++						
19.54	2-Furancarboxaldehyde, 5-methyl-	1574	85.1			+								
19.80	2-Cyclopentene-1,4-dione	1585	79.7			++		+						
20.18	<i>n</i> -Hexadecane (STD) ^c													
20.77	Butyrolactone	1626	79.2		++		++			++		++	++	
21.11	Benzeneacetaldehyde	1641	75.1			+++		+++++						
21.66	2-Furanmethanol	1664	70.1			+	+	+						
22.00	2,6-Octadienal, 2,7-dimethyl-, (E)-	1679	75.5			+++								
22.49	<i>n</i> -Heptadecane (STD) ^c													
22.95	2-Undecanol	1720	77.1		+		+							
23.18	2,6-Octadienal, 3,7-dimethyl-, (E)-	1730	90.6			+++++								
24.69	<i>n</i> -Octadecane (STD) ^c													
25.18	2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexandien-1-yl)-, (E)-	1809	88		++		++++							
25.83	5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	1844	80.4			++								
26.31	Benzyl alcohol	1865	99	6						+++		++++	+++	
26.41	Benzene, 4-ethyl-1,2-dimethoxy-	1869	98							+++		+++	+++	
26.80	<i>n</i> -Nonadecane (STD) ^c													
27.21	Phenylethyl alcohol	1895	95.7	2, 28	++		++			++++		+++	+++	
27.94	5,9-Undecadien-2-ol, 6,10-dimethyl-	1935	74.3		+									
29.18	Bicyclo[3.2.0]heptan-2-one, 6-hydroxyl-5-methyl-6-vinyl	1988	86.1			+								
29.41	Phenol, 4-ethyl-2-methoxy-	1998	92.8		+++++		++++			+++++		+++++	+++++	
31.22	2-Pentadecanone, 6,10,14-trimethyl-	2076	75					+						
31.38	3,5,9-Undecatrien-2-one, 6,10-dimethyl-, (E,E)-	2083	76.4			+								
31.77	2,6-Diisopropyl-naphthalene	2100	81			+								
32.20	Phenol, 4-ethyl-	2118	97.1		+++++		+++			+++++		++	+++++	
32.54	2-Methoxy-4-vinylphenol	2133	80.1		+									
34.25	2-Ethylhexyl salicylate	2207	75.3					+						
35.12	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,13,13,15,15-hexadecamethyl-	2245	76.6		++	+	++	+						

none, were found for the first time by GC-MS during the *P. cinnamomi*–lupin interaction. None of these compounds have been reported previously for *P. cinnamomi* or other *Phytophthora* species (24,36). Using GC-FID analysis, only two specific volatiles (4-ethyl-2-methoxy phenol and 4-ethyl phenol) were identified in the second sampling period. However, three more specific volatiles were found using GC-MS analysis during the second sampling period. Therefore, the GC-MS can provide a higher detection power compared with the GC-FID. This result is consistent with Qiu et al. (33).

4-Ethylphenol is a volatile phenol with an unpleasant odor characteristic of a 'wet horse' or a 'phenolic' compound. It has been reported to occur in various substrates, including olive products (8,9), as a component of the volatile fraction of cigarette smoke (10), the smoky taste of cocoa powder (4), and the bad smell of piggeries (37). It is naturally produced by microbes (e.g., the yeast *Brettanomyces*) and is useful for monitoring its presence, and is an important component of the character of *Brettanomyces*-based wine and beer (19,29,31). In Clark and Bunch's study (10), the HS-SPME GC/MS method has been applied to detect 4-ethylphenol, but our HS-SPME GC/MS detection system was more sensitive, as VOCs were detected at levels lower than 19.15 parts per billion (33).

The other three common compounds found only in the inoculated samples and not in the controls were 3-hydroxy-2-butanone, butyrolactone, and phenylethyl alcohol, where they were relatively minor components (20 to 30%, 1 to 5%, and 5 to 20%, respectively) of the spectrum. No reports of these three compounds from *P. cinnamomi*-inoculated substrates have been reported previously. All these compounds are common solvents and reagents that are widely used for various purposes (1,40,48), and may be produced from 2-methyl-butanol, 3-methyl-butanol, and furfural, which were only found in the headspace of the control substrates in this study. Together with 4-ethyl-2-methoxy phenol and 4-ethyl-phenol, they are potentially useful markers that could be used to identify the presence of *P. cinnamomi* in various substrates. Most VOCs evolved from the different substrates that were inoculated with *P. cinnamomi* were similar, which indicated that *P. cinnamomi* utilized the same metabolic pathway(s) for all substrates, and produced the same metabolites.

Five specific VOCs (4-ethyl-2-methoxy phenol, 4-ethyl phenol, butyrolactone, phenylethyl alcohol, and 3-hydroxy-2-butanone) were detected in the headspace of *P. cinnamomi* + V8A and were more concentrated in the *P. cinnamomi* + PDA substrate. Six additional volatiles, 2,4,6-trimethyl-heptane, dimethyl trisulfide, dl-6-methyl-5-hepten-2-ol, 6,10-dimethyl-5, 9-undecadien-2-ol, and 2-methoxy-4-vinylphenol, were specifically produced by *P. cinnamomi* inoculated onto V8A. However, none of these volatiles were detected in the headspace of *P. cinnamomi* + PDA, while two more compounds (heptane and 5-methyl-3-heptanone) were found only in the headspace of the *P. cinnamomi*-inoculated PDA. This is most likely due to V8A containing different nutrients than PDA, allowing *P. cinnamomi* to grow more abundantly. In contrast, when *P. cinnamomi* was inoculated onto a living substrate, in this case lupin seedlings or soil + lupin seedlings, two additional specific VOCs (benzyl alcohol and 4-ethyl-1,2-dimethoxybenzene) were observed. Overall, less VOCs were detected in the inoculated living substrates compared to V8A or PDA. This might be due to the soil or lupin seedlings adsorbing or consuming most of the VOCs, or it is more likely *P. cinnamomi* utilized a different metabolic pathway(s) when it infected and colonized living substrate. It may also be possible that these two compounds were produced by either the plant or the pathogen during their interaction as a defense signal (30,34).

It is well known that terpenes can protect plants from pathogens and are oxidized to form alcohols and ketones to aid in their excretion by pathogens. Ethyl acetate and 3-methyl-1-butanol were the derivatives of oxygenated monoterpene and were identified only in the headspace of *P. cinnamomi*-inoculated lupin seedling samples, which indicates that different substrates infected by *P. cinnamomi* resulted in different VOCs.

Schnürer et al. (36) found 2-ethyl-1-hexanol in significant amounts in the headspace of *P. infestans*-infected samples, and considered this compound as a useful marker volatile for this pathogen; while Laothawornkitkul et al. (24) found three marker volatiles (5-ethyl-2(5H)-furanone, (E)-2-hexenal, and benzene-ethanol) that were used as chemical signals for *P. infestans*. In the present study, none of these *P. infestans* marker volatile compounds were found in *P. cinnamomi*-inoculated samples, except 2-ethyl-1-hexanol, which was found at a relatively low level in both the inoculated and control samples. None of the *P. cinnamomi* marker VOCs obtained in this study were reported as *P. infestans* or other *Phytophthora* species marker volatiles, indicating the existence of *Phytophthora* species specific VOCs.

Most of the VOCs evolved from the V8A and PDA controls were the same except that a few volatiles present in V8A or PDA were profiled in the control lupin seedlings, soil, and soil + lupin seedling. This might be due to soil adsorption and lupin seedling assimilation. The two most concentrated compounds that were only profiled in the V8A and PDA controls were furfural and benzaldehyde, followed by benzeneacetaldehyde, none of which were detected in any of the *P. cinnamomi*-infected samples.

Furfural and benzaldehyde are organic compounds derived from a variety of agricultural by-products and a number of natural sources. Both furfural and benzaldehyde can be produced during autoclaving with their precursor; this should be another reason that they were found only in V8A and PDA (5). Several publications have reported furfural being a fungal metabolite (21,38), and other workers have identified benzaldehyde in the headspace of *P. infestans* and *F. coeruleum* inoculated substrates (11).

However, it has been shown that microbes are capable of utilizing furfural and benzaldehyde as a carbon source (35,46). Kakinuma and Yamatodani (20) were the first to report the isolation of microorganisms capable of degrading furfural. Since then, a few dozen furfural-degrading microorganisms have been found. The majority of them have been isolated using classical enrichment on furfural as a carbon source (25,46,49). However, most of these isolated microorganisms are aerobic Gram-negative bacteria, with only three fungi reported as furfural degraders (23,39,51). Rosche et al. (35) found 14 filamentous fungi that could biotransform benzaldehyde.

From the present work, we can presume that *P. cinnamomi* is another furfural and benzaldehyde degrader which can directly use furfural and benzaldehyde as a natural resource. This could explain why furfural and benzaldehyde were the two most abundant compounds found in the headspace of V8A and PDA controls but were not identified in the corresponding *P. cinnamomi*-inoculated samples. This is the first time an oomycete has been shown to degrade furfural and benzaldehyde.

In conclusion, we found 4-ethyl-2-methoxy phenol, 4-ethyl-phenol, 3-hydroxy-2-butanone, butyrolactone, and phenylethyl alcohol are significant and specific markers for *P. cinnamomi* infection. The knowledge gained from this study could contribute to the development of early warning sensors to detect the presence of *P. cinnamomi* in horticultural and agricultural crops or in natural ecosystems. *P. cinnamomi* could also potentially be used as a natural resource for the production of 4-ethyl-2-methoxy phenol and 4-ethyl-phenol and as furanic aldehyde degraders to remove furfural from contaminated environments, and assisting sugar production from lignocellulosic materials. It is also expected that the characterization of the VOCs specific to *P. cinnamomi*-inoculated samples and the corresponding controls will provide new knowledge on specific metabolic pathways of *P. cinnamomi*.

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