Optimization of Headspace Solid-Phase Microextraction Conditions for the Identification of *Phytophthora cinnamomi* Rands

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Abstract

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A robust technique was developed to identify *Phytophthora cinnamomi* using headspace solid-phase microextraction (HS-SPME) combined with gas chromatography (GC) coupled to a flame ionization detector (FID) for analyzing volatile organic compounds (VOCs). Six fiber types were evaluated and results indicated that the three-phase fiber 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) had the highest extraction efficiency for both polar and nonpolar GC columns. The maximum extraction efficiency (equilibrium absorption) was achieved 16 h after fiber exposure in the HS. Absorbed compounds on the fiber were completely desorbed in the GC injector after 5 min at 250°C. Compared with the nonpolar column, the polar column showed optimum separation of VOCs released from *P. cinnamomi*. Under the optimized HS-SPME and GC/FID conditions, lower detection limits for the four external standards was found to be between 1.57 to 27.36 ng/liter. Relative standard deviations <9.01% showed that the method is precise and reliable. The method also

showed good linearity for the concentration range that was analyzed using four standards, with regression coefficients between 0.989 and 0.995, and the sensitivity of the method was 10^4 times greater than that of the conventional HS method. In this study, the VOC profiles of six *Phytophthora* spp. and one *Pythium* sp. were characterized by the optimized HS-SPME-GC method. The combination of the VOCs creates a unique pattern for each pathogen; the chromatograms of different isolates of *P. cinnamomi* were the same and the specific VOC pattern of *P. cinnamomi* remained consistently independent of the growth medium used. The chromatograms and morphological studies showed that *P. cinnamomi* released specific VOCs at different stages of colony development. Using the optimized HS-SPME GC method, identification of *P. cinnamomi* from 15 in vivo diseased soil samples was as high as 100%. Results from this study demonstrate the feasibility of this method for identifying *P. cinnamomi* and the potential use of this method for physiological studies on *P. cinnamomi*.

Phytophthora cinnamomi is a microscopic soilborne plant pathogen that can invade and destroy the root systems of susceptible native and introduced plant species. It is the most widely distributed *Phytophthora* sp., with over 3,000 host species (15). The most significant crop losses due to *P. cinnamomi* root rot occur in avocado and chestnut. *P. cinnamomi* has been associated with the widespread mortality of oak trees and is the cause of one of the most extensive epidemics in natural ecosystems in the southwest area of Western Australia (4,26). A range of fauna are affected by *P. cinnamomi*-induced changes to vegetation, including small mammals, reptiles, birds, and invertebrates (12,32). *P. cinnamomi*, with its enormous host range spanning many genera of rare and endemic plants, has been named by the Australian Government as one of the 15 “Key Threatening Processes” endangering ecosystems and ecological processes in Australia (9).

Developing robust, timely, and sensitive diagnostic methods for *P. cinnamomi* infection is very important to prevent its introduction and spread. Traditionally, identification of *Phytophthora* spp. start

with isolation, and the main method to isolate *Phytophthora* spp. from symptomatic plants is by plating necrotic material onto *Phytophthora*-selective agar or baiting diseased tissues and rhizosphere soil with fresh young leaf material as “baits”; necrotic lesions from the baits are then plated onto selective agar (10). This process, though effective, is slow and requires experienced personnel to identify the pathogen based on morphological characteristics. Electrophoretic patterns of isozymes and serological methods based on use of antibodies are only useful for *P. cinnamomi* identification from pure cultures and these methods are slow and expensive, have limited usefulness, are not necessarily species-specific, and need to be confirmed by morphological or molecular methods (6). More recently, a variety of molecular techniques have been used for the identification of *P. cinnamomi* and other *Phytophthora* spp. Conventional polymerase chain reaction (PCR) identification is based on amplification and pyrosequencing (30); fingerprinting techniques that include restriction fragment length polymorphism of the internal transcribed spacer region (5,21), random amplified polymorphic DNAs (20), and amplified fragment length polymorphisms (8) with nested PCR are all based on conventional PCR and are sensitive and simple but require an agarose or acrylamide gel electrophoresis to visualize the amplified bands, and increase the time required for sample analysis. Real-time PCR eliminates the requirement for post-amplification amplicon detection procedures, including gel electrophoresis, and avoids the problem of cross contamination of reactions that is inherent in nested PCR (11). The technique can also be implemented in the field by using portable real-time PCR machines. Also, without the addition of ethidium

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bromide, health risks for operators and environmental contamination are reduced (29,31). However, it is restricted by the number of fluorescent probes and, 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 (18,28). Therefore, there is an important need to develop other robust, rapid, highly specific, and cost effective diagnostic methods for the identification of *Phytophthora* spp. from plant and soil material.

A novel nondestructive and noninvasive extraction method (no preprocessing of the sample before separation is required, ensuring sample integrity) for the detection of specific microbial-emitted volatile organic compounds (VOCs) has been developed to discriminate microbes in soil, food, feeds, and grains (17,23,25), and a number of studies have shown that VOCs released by microbes change with the different stages of their growth (13,22,34). Although several marker volatiles of *P. infestans* have been identified (7,19) and applied to detect *P. infestans* infection, specific volatiles have not been discriminated for the identification of *P. cinnamomi*. Solid-phase microextraction (SPME) is a new sample enrichment and solvent-free extraction technique which integrates sampling, extraction, concentration, and sample introduction in a single step. Since its development in 1989 by Arthur and Pawliszyn (2), its applications have dramatically increased. The SPME in combination with headspace (HS) analysis by gas chromatography (GC; a very common laboratory analytical instrument) is a convenient alternative method for the analysis of VOCs.

The objectives of this article were to prove the feasibility of using VOCs together with HS-SPME as an identification tool and to establish optimal HS-SPME and GC conditions for the identification of *P. cinnamomi*.

Materials and Methods

Reagents. BBL agar, grade A (reference 212304), used for preparation of V8 juice agar (V8A), and Difco potato dextrose agar (PDA; reference 213400) were purchased from Becton, Dickinson and Company. Organic solvents, including methanol, ethanol, and ethyl formate, were purchased from MERK (high-performance liquid chromatography [HPLC] grade); hexane was purchased from Fisher Scientific (catalog number H306, HPLC grade).

Equipment and apparatus. Erlenmeyer flasks (100 ml; Quickfit, catalog number QFY-372-P) were used for preparation of samples. Each flask was fitted with an adapter (Quickfit, part number AQST53/13) equipped with a septum (Grace, catalog number 6518). Glass bottles of 250 ml (Alltech, catalog number 9535), each fitted with a Mininert valve and septum (Alltech, catalog number 95326) were used for preparation of gas standards. An Olympus BX51 microscope (serial number 3M08876) was used to study the morphology of *P. cinnamomi*.

Six different types of SPME fiber were compared to evaluate their effectiveness on the extraction of VOCs produced by *P. cinnamomi*. The tested SPME fibers were 7 µm polydimethylsiloxane (PDMS) fiber (Sigma-Aldrich Australia, catalog number 57307), 100 µm PDMS fiber (Sigma-Aldrich Australia, catalog number 57300-U), 50/30 µm divinylbenzene/carboxen (DVB/CAR)/PDMS fiber (Sigma-Aldrich Australia, catalog number 57348-U), 65 µm PDMS/DVB fiber (Sigma-Aldrich Australia, catalog number 57310-U), 85 µm polyacrylate (PA) fiber (Sigma-Aldrich Australia, catalog number 57307), and 85 µm CAR/PDMS fiber (Sigma-Aldrich Australia, catalog number 57334-U). Prior to use, the fibers were conditioned according to the manufacturers' recommen-

dations, and cleaned between each extraction by exposing the fibers into the GC injection port for 15 min at 250°C for the 100 µm PDMS and PDMS/DVB fibers, and 15 min at 260°C for the other four fibers.

Analysis of VOCs was performed on an Agilent 6890 series (serial number US00021731) gas chromatograph coupled to a flame ionization detector (FID). Injector and detector temperatures were 250°C, except when the injector temperature was varied to assess its effect on desorption of VOCs from the fiber. In order to get the optimal blueprint of *P. cinnamomi*, both polar and nonpolar columns were used for the separation of VOCs. Separation was achieved on a ZB-WAX plus capillary polar column (30 m by 0.25 mm ID, film thickness 0.25 µm; Zebron, part number 7HG-G013-11) and an Rxi-5ms nonpolar column (30 m by 0.25 mm, film thickness 0.25 µm; Crossbond 5% diphenyl and 95% dimethyl polydioxane; RESTEK, catalog number 13423) separately. Hydrogen was used as carrier gas at a flow rate of 1.0 ml min⁻¹. The GC column temperature was programmed to hold temperature at 45°C for 5 min, then increased by 5°C min⁻¹ to 250°C and held for 5 min, and the GC-FID instrument was operated under the splitless mode.

Data were collected with GC online software and analyzed by ChemStation instrument offline software and Microsoft Excel 2007.

***Phytophthora* and *Pythium* spp., *P. cinnamomi* isolates, VOC detection, and microscopic observation.** Six *Phytophthora* spp., including five isolates of *P. cinnamomi* (Table 1), *P. niederhauserii* (PAB 13-29), *P. elongata* (VHS 13784), *P. humicola* (VHS 25241), *P. inundata* (VHS 25170), and *P. multivora* (VHS 14926); and one *Pythium* sp., *Pythium dissotocum* (SA370), were obtained from the Centre for Phytophthora Science and Management (CPSM), Murdoch University, Australia.

Phytophthora and *Pythium* spp. were grown on 10% V8A in petri dishes in the dark at 24 ± 1°C and subcultured every 10 days via mycelial mass transfer. A single 4-mm-diameter plug from the edge of the 5-day-old pathogen cultures was used as inoculum and transferred to a 100-ml Erlenmeyer flask containing 50 ml of V8A. Inocula of the five *Phytophthora cinnamomi* isolates were also transferred to 50 ml of PDA. All the cultures were incubated at 24 ± 1°C in the dark for 6 days and VOC detection was conducted.

A single 4-mm-diameter agar disc colonized by a 5-day-old *P. cinnamomi* isolate (MP94.48) colony grown on V8A was transferred to a 100-ml Erlenmeyer flask containing 50 ml of V8A, the culture was incubated for 15 days at 24 ± 1°C in the dark, and morphological observation and VOC detection were conducted daily. Each treatment combination was replicated three times and each experiment was repeated more than three times.

Inoculation. Seedlings of *Lupinus angustifolius* 'Danja', the commonly used bait plant for *P. cinnamomi*, were used as the susceptible host plant for this study. Seed of *L. angustifolius* Danja 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 seed were germinated on sterilized filter paper, which was premoistened with sterilized distilled water at 24 ± 1°C in the dark for 2 days.

***Lupin* sample inoculation.** Three uniform 2-day-old lupin seedlings were transferred to a 100-ml Erlenmeyer flask with 1 ml of sterilized distilled water and grown for a further day. A single 4-mm plug from the edge of the 5-day-old *P. cinnamomi* MP 94.48

Table 1. *Phytophthora cinnamomi* isolates used in this study^a

Isolate code	Type	GenBank number	Country	Host	Year
MP 94.48	A2	JX113294	Australia	<i>Eucalyptus marginata</i>	1994
W4	A2	JX113312	Australia	<i>Chamaecilla corymbosa</i>	2011
W15	A2	JX113308	Australia	<i>Stylidium diuroides</i>	2011
MP75	A1	NA	Australia	<i>Eucalyptus marginata</i>	ND
DCE 25	A1	JX454790	Australia	<i>Hypocalymma angustifolia</i>	1969

^a NA = not applicable, and ND = no data.

culture was used as inoculum, placed on the tip of the root, and kept in dark at $24 \pm 1^\circ\text{C}$ for 3 days to allow lesions to develop.

Soil sample inoculation. Washed white river sand was purchased from Sand and Soil. To ensure that the seedlings were grown at "container capacity" conditions for optimum growth, 13.6 ml of 10% V8 broth was added to a 100-ml Erlenmeyer flask containing 100 g of sand and sterilized. Three lupin seedlings were transferred to the sterilized sand and grown for a further 2 days. Six 4-mm-diameter agar plugs colonized with 5-day-old *P. cinnamomi* MP94.48 were transferred aseptically to the middle of the sand layer in each flask; controls consisted of six similar-sized V8A plugs that had not been colonized by the pathogen. All the treatments were then incubated for 6 days at $24 \pm 1^\circ\text{C}$ in the dark, at which time VOC detection was conducted.

Optimization of HS-SPME. According to Ristic et al. (24), a typical SPME method can be optimized for many aspects and, based on the objectives of the current study, the following SPME parameters were evaluated and optimized: fiber coating, extraction time, and desorption conditions. While optimizing one condition, all other conditions were kept constant. Some parameters such as the VOC collection chamber (100-ml Erlenmeyer flask with an adapter and a septum), 250°C GC detector temperature, and the fiber extraction temperature of $24 \pm 1^\circ\text{C}$ were kept constant throughout the experiment.

The sensitivity of an HS-SPME in combination with GC relies on optimized conditions. The first step followed in this study was to optimize the HS-SPME method to maximize the range and amount of VOCs extracted. An optimized chromatographic separa-

tion begins with the column: a nonpolar column is better for analyses of nonpolar compounds while polar columns most effectively separate polar compounds. In order to get the most reliable information, both polar and nonpolar columns and polar and nonpolar fibers were used in this study.

Selection of fiber. The six fiber types were exposed in the HS of a 100-ml Erlenmeyer flask over the 5-day-old *P. cinnamomi* MP 94.48 colony grown on V8A and V8A alone (as control) for 3 h of exposure and 5 min of desorption in the GC injector.

Determination of exposure time of fiber extraction. The selected fiber was exposed to the HS of the 100-ml flasks containing a 5-day-old *P. cinnamomi* MP 94.48 colony grown on 50 ml of V8A and V8A alone for a time periods of 3 to 24 h for both the polar column and nonpolar column. After exposure, the fiber was retrieved and injected into the heated GC injection port (250°C) and desorbed for 5 min. For each treatment and time combination, there were three replicate flasks and three replicate extractions per flask.

Determination of desorption temperature and time. The selected fiber (50/30 μm DVB/CAR/PDMS) and the optimized fiber extraction time (16 h) were used to optimize the GC injector temperature and desorption time. Determination of the optimal injector temperature was conducted on both the polar and nonpolar columns. The fiber was desorbed in the GC injection port at different injector temperatures (from 200 to 270°C ; 270°C is the highest temperature the fiber can withstand) for 5 min. The fiber was desorbed for different desorption times (1, 3, 5, and 7 min) at the selected optimal injector temperature to determine the optimal desorption time.

Table 2. Soil samples used to detect the presence of *Phytophthora cinnamomi* in this study

Sample code	Host ^a	Location
716	<i>Banksia attenuata</i>	30°38'02.6"S, 115°25'45.3"E
717	<i>Banksia menziesii</i>	30°38'02.3"S, 115°25'45.5"E
722	<i>Trachymene</i> sp.; Ericaceae (unid.)	30°37'52.8"S, 115°25'58.3"E
T2+4	<i>Hibbertia</i> sp.	30°38'02.7"S, 115°25'48.2"E
T2+6	<i>Banksia attenuata</i> ; Myrtaceae (unid.)	...
T3+4	<i>Hibbertia</i> sp.; Melaleuca sp.	30°37'59.2"S, 115°25'47.8"E
T3+6	<i>Banksia attenuata</i> , <i>Conostephium</i> sp., Ericaceae (unid.)	...
T3+8	Ericaceae (unid.); <i>Banksia</i> sp. (unid.) roots	...
T6-4	Ericaceae (unid.)	30°38'02.7"S, 115°25'45.6"E
MU22-1	<i>Calytrix</i> sp.; <i>Banksia</i> sp. (unid.) roots	32°04'39.4"S, 115°49'56.4"E
MU22-2	<i>Hibbertia</i> sp.; <i>Banksia</i> (unid.) roots	...
MU22-3	<i>Brachyloma preissii</i>	...
MU22-4	<i>Banksia</i> sp. (unid.) roots and soil	...
MU22-5	<i>Brachyloma preissii</i>	...
MU04	<i>Stirlingia latifolia</i>	32°04'28.5"S, 115°50'32.4"E

^a Unid. = unidentified family or genus.

Fig. 1. Peaks produced by *Phytophthora cinnamomi* growing on V8 juice agar and V8 juice agar alone extracted by six different fiber types that were separated by a polar column (ZB-WAX plus) and a nonpolar column (Rxi-5ms).

Selection of GC column. The separation efficiency of the polar and nonpolar columns was evaluated with the optimized three-phase fiber at the optimal time of extraction and time and temperature of desorption.

Determination of the limit of detection for the HS-SPME coupled to GC-FID method. The limit of detection (LOD) was evaluated with hexane, methanol, ethanol, and ethyl formate as external standards. For preparation of the standards, the appropriate volumes of each standard were added into a sealed 250-ml bottle. After 5 min of extraction with the fiber 50/30 μ m DVB/CAR/PDMS at room temperature ($24 \pm 1^\circ\text{C}$), the fiber was injected into the gas chromatograph at the injector temperature of 250°C .

All samples were duplicated and each sample were analyzed as triplicate injections.

Identification of *P. cinnamomi* from forest soil samples using the optimized HS-SPME GC method. Fifteen soil samples naturally infected by *P. cinnamomi* were collected from diseased, susceptible plant rhizosphere from Murdoch University campus and sites south of Perth (Table 2). The youngest fully expanded oak (*Quercus suber*) leaves (Kings Park, WA) were used as the baits for *P. cinnamomi*. All the soil samples were separated into two groups; one group was used for HS-SPME GC analysis and the other group used for baiting.

Soil samples for baiting were transferred into 1-liter takeaway containers and filled with deionized water. The fresh oak leaves were then floated on the water surface. These baits were checked

every 24 h for 10 days, and any leaves that developed water-soaked lesions were removed with forceps and placed into a 100-ml Erlenmeyer flask in preparation for HS-SPME extraction. In addition to the above, some of the soaked baits were dried on paper towels; then, small sections approximately 5 mm square were excised with a sterile scalpel and plated onto *Phytophthora* selective medium (NARPH; 16) and the plates were incubated at $24 \pm 1^\circ\text{C}$ in the dark. The plates were checked daily under the compound microscope to determine whether *P. cinnamomi* had been successfully isolated from the soil. Baiting water (50 ml) for each soil sample was filled into a 100-ml Erlenmeyer flask in preparation for HS-SPME extraction also.

In addition to baiting, 50 g of each soil sample was placed directly into a 100-ml Erlenmeyer flask for HS-SPME extraction.

The optimized HS-SPME GC method was used for analyzing the VOCs from fresh soil, baiting water, and baits.

Statistical analysis. The variations (standard errors of the mean) of VOCs and external standards concentrations, the triplicate samples, and the triplicate injections in comparison with average readings were analyzed by Microsoft Excel 2007.

Results

Selection of fiber and determination of extraction time. The extraction efficiency of the six different commercially available SPME fibers (7 and 100 μ m PDMS, DVB/CAR/PDMS, PDMS/DVB, PA, and CAR/PDMS) was evaluated by comparing

Fig. 2. A, Effects of extraction time with different fibers on the extraction efficacy of the total compounds identified from *Phytophthora cinnamomi* + V8 juice agar at 3, 6, 9, 12, 16, and 20 h for the nonpolar column (Rxi-5ms); **B,** effects of extraction time with the 50/30 μ m divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber on the extraction efficiency of seven analytes of interest (>85% of total identified volatile organic compounds) at 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h for the polar column (ZB-WAX plus). Bars represent standard errors of mean ($n = 3$).

the peak numbers of the compounds from *P. cinnamomi* + V8A and V8A alone treatments under the same extraction, desorption, and GC conditions.

After separation of VOCs desorbed from different fibers on both polar and nonpolar columns, the results showed that the three-phase fiber (50/30 μ m DVB/CAR/PDMS fiber) had the highest extraction efficiency (Fig. 1). The GC response of fiber 50/30 μ m DVB/CAR/PDMS after 16 h of extraction was more than two times that of fiber 85 μ m PA and four times that of fiber 85 μ m CAR/PDMS when separated on the nonpolar column (Fig. 2A), and trapped a wider range of VOCs that separated on both the polar and nonpolar columns (Fig. 1). There were no VOCs extracted with the 7 μ m PDMS fibers because no compounds separated from both polar and nonpolar columns (Figs. 1 and 2A).

Therefore, the three-phase fiber was selected as the appropriate fiber to optimize the other parameters. For determination of extraction time, the selected three-phase fiber was exposed in the HS of the flask for different times (3 to 24 h). The levels of desorbed compounds increased with increasing extraction time until 16 h on the nonpolar column (Fig. 2A) and polar column (Fig. 2B), when equilibrium was achieved.

Evaluation of GC injector (fiber desorption) temperature and desorption time. The effect of the GC injector temperature

was evaluated with the selected 50/30 μ m DVB/CAR/PDMS fiber at GC inlet temperatures of 200 to 270°C under the HS fiber extraction time of 16 h over *P. cinnamomi* + V8A and V8A alone. The results indicated that the total area of desorbed compounds detected increased with increasing injector temperature for both the polar and nonpolar columns, and the maximum desorption from the fiber was obtained at 250°C (Fig. 3A). The total desorbed compounds produced decreased when the temperature was greater than 250°C.

The various desorption times had a significant effect on GC response of *P. cinnamomi* + V8A and V8A alone. The total area of desorbed VOCs increased with an extension of desorption time from 1 to 5 min and then reached equilibrium at 5 min; the GC response remained constant after a desorption time of 5 min, which was determined as the optimum (Fig. 3B).

Selection of column. The three-phase fiber was exposed in the HS over a 6-day-old *P. cinnamomi* + V8A colony for 16 h and separated on both polar and nonpolar columns under the optimized HS-SPME GC conditions. The VOCs of the samples had better separation and were more sensitive for most separated VOCs on the polar column than the nonpolar column, and a broader range of compounds was captured on the polar column (Fig. 4). Therefore, the polar column was selected for the subsequent studies.

Fig. 3. A, Effects of gas chromatography (GC) injector temperature on desorption of volatile organic compounds (VOCs) of *Phytophthora cinnamomi* + V8 juice agar and V8 juice agar alone from the 50/30 μ m divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber after 16 h of extraction; **B,** effects of desorption time on desorption of VOCs of *P. cinnamomi* + V8 juice agar and V8 juice agar alone from the 50/30 μ m DVB/CAR/PDMS fiber. Bars represent standard errors of mean ($n = 3$).

Analysis of six *Phytophthora* spp. and one *Pythium* sp. The optimized HS-SPME and GC conditions were applied to detect the VOCs of the six *Phytophthora* spp. and one *Pythium* sp. The GC chromatograms were different between the six *Phytophthora* spp., although one or two of the same retention time peaks (a, b, c, d, f, and g) were present in each species. *Phytophthora* spp. belonging to the same clade (*P. niederhauserii* and *P. cinnamomi*, clade 7; *P. multivora* and *P. elongata*, clade 2; *P. inundata* and *P. humicola*, clade 6) had different chromatograph patterns. The GC chromatogram patterns were also different between *Pythium dissotocum* and the six *Phytophthora* spp. (Fig. 5), with peak e only present in *P. dissotocum* and, except for peak b, no further specific peaks common to *P. dissotocum* were observed.

The optimized HS-SPME and GC conditions were also used to detect the VOCs from the five *Phytophthora cinnamomi* isolates. The results showed that all five *P. cinnamomi* isolates had the same GC chromatogram patterns when grown on V8A or PDA (Fig. 6). The VOCs from *P. cinnamomi* MP94.48 had the same GC chroma-

togram patterns when it was grown on different substrates, except for two additional peaks (a and b), when *P. cinnamomi* was inoculated into lupin seedlings growing in sand or in the absence of sand (Fig. 7).

LOD. The LODs of the four external standards were evaluated under the optimal experimental conditions in a range of concentrations from 0 (control) to 300 ng/liter (0 to 176.4 parts per billion) that separated on the polar column. The GC response for the four standards increased with concomitant increase in the concentrations of the standards and there were significant (regression coefficient > 0.989) linear regressions for all four standards (Fig. 8; Table 2). The LODs were calculated by comparing the signal-to-noise ratio (S/N) of the lowest concentration to S/N = 3. The method allows detection of the external standards at concentrations lower than 43.08 ng/liter, and the four standards had good separation on the polar column (Table 3).

In order to fully validate the HS-SPME-based procedure, its performance was compared with that of the conventional HS syringe

Fig. 4. Gas chromatography (GC) of a 6-day old *Phytophthora cinnamomi* + V8 juice agar that was separated on A, Rxi-5ms nonpolar column and B, ZB-WAX plus polar column.

Fig. 5. Gas chromatography (GC) chromatograms of five different *Phytophthora* spp. and one *Pythium* sp. grow on V8 juice agar.

procedure. Ethyl formate can be detected at less than 475 parts per trillion with the HS-SPME method, whereas concentrations at 3 ppm were not detected by conventional HS chromatography, which is 0.63×10^4 times less sensitive than the concentrations detected by the HS-SPME method.

The precision of the method was obtained by analyzing the VOCs from five replicate flasks each of *P. cinnamomi* + V8A and V8A. The retention time fluctuation of all the VOCs of interest with relative standard deviation values were between 0.852 and 9.010% for *P. cinnamomi* + V8A and between 0.671 and 1.912% for V8A.

The GC chromatograms and morphology observation. The VOCs from *P. cinnamomi* that were grown on V8A were detected under the optimized HS-SPME and GC conditions: in comparison with the control (V8A alone), compounds 1, 2, 3, 4, 7, and 9 were lost; compound 8 was dramatically reduced; and compound 6 was increased. However, compounds 5, 11, and 12 were present during all growth stages of *P. cinnamomi*, whereas compound 13 was observed only when hyphae were present and was lost when chlamydospores were produced, and compound 10 was only produced

with the formation of chlamydospores (Fig. 9). According to the separation principle that the longer the retention time, the larger the molecular weight, we can conclude that the VOCs of *P. cinnamomi* were large molecular weight compounds.

Detection of *P. cinnamomi* from naturally infested soil samples. The HS-SPME GC method resulted in 100% identification from the 15 naturally infested forest soil samples (Table 4); 3 and 13 samples using the HS-SPME GC directly from the soil and the baits, respectively, were *P. cinnamomi* positive and none of the baiting water samples were *P. cinnamomi* positive. When the baits were plated onto the selective agar, 13 soil samples were positive.

Discussion

The bipolar three-phase fiber 50/30 μ m DVB/CAR/PDMS had the highest efficiency as an absorbent to extract VOCs in the HS over an actively growing colony of *P. cinnamomi* under separation with both polar and nonpolar GC columns. This efficiency was followed by the CAR/PDMS, PDMS/DVB, and PA fibers, and no compounds were detected with the PDMS fibers (except for two compounds detected by the 100 μ m PDMS fiber when separated on

Fig. 6. Gas chromatography (GC) chromatograms of five *Phytophthora cinnamomi* isolates grown on A, V8 juice agar and B, potato dextrose agar.

a nonpolar column). This result indicates that the volatile metabolites produced by *P. cinnamomi* are mainly polar compounds. These are consistent with the specified characteristics of the fibers; for example, the CAR/PDMS coated fiber is the best for extracting small analytes, the PDMS/DVB is suitable for more volatile polar molecules, the PA fiber is suitable for the extraction of very polar and large-sized analytes, and the three-phase bipolar fiber is suitable for a broad range of volatile and semivolatile analytes of C₂ to C₂₀. Therefore, we can conclude that the VOCs released by *P. cinnamomi* are polar volatile compounds.

Increasing the sample temperature can increase the HS capacity or analyte partitioning coefficient between air and solid phases, which leads to an increase in the total mass extraction or the rate of mass transfer onto the fiber coating, shortened equilibration times, and a faster overall SPME procedure (24). However, raising the sample temperature further can decrease the analyte distribution constant between the sample matrix and the fiber coating and decrease the sensitivity and recovery of the method (22). The optimal temperature for *P. cinnamomi* growth is 24 to 28°C; lower or higher temperatures will reduce the activity of *P. cinnamomi*

(14,27) and, in turn, could limit the release of VOCs. Consequently, in this study, we only used the optimal growth temperature 24 ± 1°C. Also, in this study, optimal fiber extraction was 16 h, which is quite a long time in comparison with Barbieri et al. (3), who reported 10 min as the extraction time necessary to reach equilibrium for compounds produced by *Staphylococcus pasteurii*. This is because the HS-SPME technique is a dynamic equilibrium process. The extraction time depends on the distribution constant, chemical nature of the desired compounds, and fiber polymeric phase, and analytes with bigger molecular mass (e.g., sterols and polyunsaturated fatty acids) are expected to need longer equilibrium times due to their lower partitioning and diffusion coefficient. Moreover, a 5-min desorption time was needed to completely remove the absorbed VOCs from the fiber, which indicated that some VOCs are bigger molecular volatility compounds and agrees with Grant's findings that the metabolic compounds of *Phytophthora* spp. are characterized by large unsaturated compounds (13).

Desorption is an endothermic process and is disfavored at low temperatures; theoretically, desorption temperature should be high enough to completely release absorbed volatiles from the fiber.

Fig. 7. Gas chromatography (GC) chromatograms of *Phytophthora cinnamomi* grown on different substrates.

Fig. 8. Linearity of four external standards separated on the polar column; right Y-axis is for solid lines and the left Y-axis is for dashed lines.

However, two factors should be considered: (i) in the SPME technique, only those injector temperatures that are within the temperature stability range of a particular fiber coating should be tested (e.g. for the three-phase fiber, 270°C is recommended as the limit of temperature tolerance) and (ii) the higher desorption temperature can reduce GC sensitivity; this could be due to denaturation, destruction, or decomposition of the chemicals at higher temperature. Also, the application of very high temperatures can shorten the fiber's life and result in loss of fiber polymer. Because the optimum desorption efficiency was achieved at 250°C, this temperature was selected for the subsequent experiments.

Using the optimized HS-SPME method developed in this study, we found that the GC chromatogram of *P. cinnamomi* differed from the other *Phytophthora* spp. and *Pythium dissotocum*, and even the chromatogram patterns of species in the same clade were different. The closer the genetic distance of *Phytophthora cinnamomi* to other *Phytophthora* spp., the more similar were the GC chromatogram patterns. The chromatograms of different isolates of *P. cinnamomi* were the same. Using different growth media did not confound the VOCs that were released by *P. cinnamomi*, although two additional peaks were evident when *P. cinnamomi* infected and colonized lupin seedlings (grown in sand or in the absence of sand); this may be due to more adequate nutrition from these substrates resulting in larger concentrations of VOCs. The concentration of these two VOCs was below the detection limit when *P. cinnamomi* was grown on V8A and PDA. However, the majority of the peaks were consistent across the different substrates on which *P. cinnamomi* was grown. The results clearly demonstrated that the HS-SPME combined with GC coupled to FID can be reliably used to analyze VOCs for the specific identification of *P. cinnamomi* and that other *Phytophthora* spp. produced different VOCs, although

the methods could not differentiate between the different isolates of *P. cinnamomi*. This indicates that the method appears to be *P. cinnamomi* specific. The quantity of *P. cinnamomi* in soil is very small (33), and the soil samples used in this study were collected in November, when the weather is very dry and hot, conditions that are not generally conducive for *P. cinnamomi* activity. Conse-

Table 4. Gas chromatography coupled to a flame ionization detector (GC-FID) detection of *Phytophthora cinnamomi* from fresh soil samples and baits

Soil sample	GC-FID detection ^a			Baits plating (%) ^b
	Soil	Water	Baits	
716	–	–	+	71
717	–	–	+	66
722	–	–	+	80
T2+4	–	–	+	97
T2+6	–	–	+	89
T3+4	–	–	+	100
T3+6	–	–	+	100
T3+8	–	–	+	100
T6+4	–	–	+	77
MU22-1	–	–	+	47
MU22-2	+	–	+	44
MU22-3	–	–	+	71
MU22-4	+	–	–	0
MU22-5	–	–	+	68
MU04	+	–	–	0

^a Soil = fresh soil sample, Water = baiting water, + indicates positive detection, and – indicates not detected.

^b Percent baiting efficiency (%) = (baits yielding *P. cinnamomi*/total baits exposed) × 100.

Table 3. Analyzed external standards, limits of detection (LOD), and linearity data in gas chromatography coupled to a flame ionization detector by using 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane fiber

Standards	RT (min) ^a	Linearity (r^2) ^b	LOD (ng/liter)	LOD (ppb) ^c	Molecular number ^d
Methanol	5.929	0.993	27.36	19.15	1.287×10^{14}
Ethanol	6.766	0.989	5.29	2.58	1.731×10^{13}
Ethyl formate	4.734	0.995	1.57	0.47	3.191×10^{12}
Hexane	1.642	0.988	43.08	11.20	7.526×10^{13}

^a RT = retention time.

^b Regression coefficient.

^c In parts per billion (ppb).

^d For all standards, 1 mol molecular number = 6.022×10^{23} .

Fig. 9. Chromatograms of A, V8 juice agar alone and *Phytophthora cinnamomi* at different stages of growth on V8 juice agar: B, chlamydospores and C, hyphae.

quently, under these conditions, *P. cinnamomi* will likely be dormant in the soil, resulting in limited detection of VOCs due to a lower metabolic rate of *P. cinnamomi*. After flooding, *P. cinnamomi* will produce sporangia and release zoospores which, in turn, infect and colonize the baits, resulting in the release of concentrated specific VOCs, beneficial for HS-SPME GC analysis. These life cycle traits could be the reason for low identification efficiency from the fresh but dry soil samples, and for the high identification efficiency from the baits by HS-SPME GC analysis. Although *P. cinnamomi* was isolated by directly plating root material from the naturally infested soil samples MU22-4 and MU04, the baiting results were negative but HS-SPME GC results were positive. The sensitivity of baits to different *P. cinnamomi* isolates has been shown to differ (1); consequently, this could be a potential reason for the negative result from the baits; alternatively, these soils contained microorganisms that suppressed sporangial production or zoospore release. All these results proved the feasibility and sensitivity of HS-SPME GC method for the detection of *P. cinnamomi* from different substrates.

The efficiency and low background interference of GC/FID and its nondestructive nature allows the SPME fiber to be used repeatedly. In addition, with the method developed in this study, VOCs can be detected at levels lower than 19.15 ppb, which demonstrates that this method can be used to identify and determine *P. cinnamomi*-specific VOCs at very low levels.

The loss or decrease of smaller compounds from V8A and the massive increase or emergence of larger compounds from *P. cinnamomi* samples may have resulted from an increase in the metabolism of *P. cinnamomi* between the mycelial and sporangial stages. Also, different VOCs were released from *P. cinnamomi* during its different growth stages; these results are in accordance with other researchers' opinions on physiological metabolism (13,34). We believe that study of metabolite profiles at the various stages of development of *P. cinnamomi* may provide important clues as to the major metabolic pathways that operate during its different growth stages. Therefore, the optimized method developed in this study can be used either as a detection tool or as a new qualitative methodology for physiological studies of *P. cinnamomi* and other oomycetes.

SPME is a simple preparation technique that can be used both in laboratory and on-site (35). HS-SPME can shorten the time of extraction significantly because of the faster diffusion rate of the molecules in the gaseous phase. Because the fiber is suspended in the HS above the sample layer, there is no contamination of the fiber by the sample. From our studies, it is observed that the fibers can be reused more than 200 times, thus reducing the cost of the materials. The HS-SPME GC technique does not require a great deal of skill and can be readily used after a short period of training.

To our knowledge, this is the first use of VOCs as an indicator of *P. cinnamomi* colonization and infection and it is the first time the HS-SPME technique has been used to identify VOCs of *P. cinnamomi*. This study had shown that HS-SPME GC is a robust and cost-effective system for the diagnosis of *P. cinnamomi* based on the production of specific VOCs, and studies are in progress to characterize and identify the VOCs that are released by *P. cinnamomi* to eventually develop an efficient control method for *P. cinnamomi*. Currently, we are using this method to characterize the VOC's from *P. cinnamomi* and other *Phytophthora* spp. by GC/MS.

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