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Optimization of the Headspace Solid-Phase Microextraction Gas Chromatography for Volatile Compounds Determination in *Phytophthora Cinnamomi* Rands

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Abstract**—***Phytophthora cinnamomi* (*P. c*) is a plant pathogenic oomycete that is capable of damaging plants in commercial production systems and natural ecosystems worldwide. The most common methods for the detection and diagnosis of *P. c* infection are expensive, elaborate and time consuming. This study was carried out to examine whether species specific and life cycle specific volatile organic compounds (VOCs) can be absorbed by solid-phase microextraction fibers and detected by gas chromatography that are produced by *P. c* and another oomycete *Pythium dissotocum*. A headspace solid-phase microextraction (HS-SPME) together with gas chromatography (GC) method was developed and optimized for the identification of the VOCs released by *P. c*. The optimized parameters included type of fiber, exposure time, desorption temperature and desorption time. Optimization was achieved with the analytes of *P. c*+V8A and V8A alone. To perform the HS-SPME, six types of fiber were assayed and compared: 7μm Polydimethylsiloxane (PDMS), 100μm Polydimethylsiloxane (PDMS), 50/30μm Divinylbenzene/Carboxen™/Polydimethylsiloxane DVB/CAR/PDMS), 65μm Polydimethylsiloxane/Divinylbenzene (PDMS/DVB), 85μm Polyacrylate (PA) fibre and 85μm Carboxen™/ Polydimethylsiloxane (Carboxen™/PDMS). In a comparison of the efficacy of the fibers, the bipolar fiber DVB/CAR/PDMS had a higher extraction efficiency than the other fibers. An exposure time of 16h with DVB/CAR/PDMS fiber in the sample headspace was enough to reach the maximum extraction efficiency. A desorption time of 3min in the GC injector with the desorption temperature of 250°C was enough for the fiber to desorb the compounds of interest. The chromatograms and morphology study confirmed that the VOCs from *P. c*+V8A had distinct differences from V8A alone, as did different life cycle stages of *P. c* and different taxa such as *Pythium dissotocum*. The study proved that *P. c* has species and life cycle specific VOCs, which in turn demonstrated the feasibility of this method as means of identifying *P. c.*

Keywords**—**Gas chromatography, headspace solid-phase microextraction, optimization, volatile compounds.

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I. Introduction

PHYTOPHTHORA cinnamomi (P. c) is a soil-borne plant pathogen, which has a host range of more than 3000 plant species [1]. It was first described as the causal agent of stripe canker of *Cinnamomum burmannii* in Sumatra [2], but now causes enormous economic losses in agriculture, horticulture and forestry and it has become a major threat to natural ecosystems and biodiversity. The most significant food crop losses due to *P. c* root rot occur in avocado and chestnut, *P. c* has been associated with the widespread mortality of oak trees and is the cause of one of the most extensive epidemics in the natural *Eucalyptus* forest in Western Australia [3], [4].

Preventing the spread of *P. c* requires developing robust, highly specific and sensitive detection techniques. Traditionally, identification of *Phytophthora* species has been based on morphological and cultural criteria [5], but wide variations in morphological characters in different isolates of a single species or under different growth conditions has made this approach unreliable. Other methods include electrophoretic patterns of isozymes and methods based on antibodies which are slow, expensive and known to yield false negatives [6], whilst more recently detection methods have been based on nucleic acid sequences which are more sensitive, simple and rapid than the traditional method [1]. Despite these advances, these methods are still time, labour and money consuming and none of these methods provide a perfect solution to *P. c* detection and discrimination; consequently, a more rapid, sensitive and cheaper method is urgently required.

Recently, volatile organic compounds (VOCs) have been used to identify bacteria in food, feeds and grains [7]. Solid-phase microextraction (SPME) is a simple, rapid, sensitive and solvent-free technique that has been used in a wide range of studies [8]. SPME in combination with

headspace (HP) analysis by Gas Chromatograph (GC) is a convenient alternative method for the analysis of VOCs.

The present study is the first to use VOCs as indicators of *P. c* identification and infection that determined by HS-SPME. The objective of this paper was to prove the feasibility of using HS-SPME as a diagnostic tool and to establish optimal HS-SPME GC conditions for *P. c* identification.

II. Materials and Methods

A. Equipment

Erlenmeyer flasks 100ml (Quickfit, Cat. No. QFY-372-P) were used for the preparation of samples. Each flask was fitted with an adapter (Quickfit, Part No. AQST53/13) equipped with a septum (Grace, Cat. No. 6518).

An autoclave (BMSS Weston, Serial No. DH14626) was used for agar and flasks sterilization; and a laminar flow (CLEMCO, Serial No. 2205/78) was used for *P. c* and *Pythium dissotocum (Py. dissotocum)* subculture and inoculation. An Olympus BX51 microscope (Serial No. 3M08876) was used to study the morphology of *P. c*.

Six different SPME fiber types were used to evaluate their effectiveness on the extraction of VOCs produced by *P. c*. The SPME fibers were 7μm Polydimethylsiloxane (PDMS) fiber (Sigma-Aldrich Australia, Cat. No. 57307), 100μm Polydimethylsiloxane (PDMS) fiber (Sigma-Aldrich Australia, Cat. No. 57300-U), 50/30μm Divinylbenzene/Carboxen™/Polydimethylsiloxane (DVB/CAR/PDMS) fiber (Sigma-Aldrich Australia, Cat. No. 57348-U), 65μm Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) fiber (Sigma-Aldrich Australia, Cat. No. 57310-U), 85μm Polyacrylate (PA) fiber (Sigma-Aldrich Australia, Cat. No. 57307) and 85μm Carboxen™/ Polydimethylsiloxane (Carboxen™/PDMS) fiber (Sigma-Aldrich Australia, Cat. No. 57334-U). All fibers were conditioned prior to use according to the manufacturers' recommendations, and cleaned between analyses by exposing the fibers into the GC injection port 15min at 250ºC for 100μm PDMS and PDMS/DVB, and 15 min at 260ºC for the other four fibers.

VOCs analysis was performed on a Hewlett Packard 6890 series (Agilent, Serial No. US00021731) gas chromatograph coupled to a flame ionization detector (FID). Injector and detector temperatures were 250ºC except when the injector temperature was varied to assess its effect on desorption of VOCs from the fibers. Separation was achieved on a 30m/0.25mm ID/0.25μm film thickness ZB-WAX plus column (Zebron, Part No. 7HG-G013-11), the oven was held at its initial temperature of 45ºC for 5 min, then increased by 5ºC min^{-1} to 250ºC and held at 250ºC for 5 min.

B. Phytophthora and Pythium Strains, Inoculation and Microscopic Observation

P. c (isolate MP 94.48) and *Py. dissotocum* (isolate P370) were obtained from the Centre for Phytophthora Science and Management (CPSM), Murdoch University. The isolates were maintained on 10% V8-juice agar (V8A) at 24ºC in the dark and subcultured every 10 days [9]. A single 4mm 10 day-old V8A disc of *P. c* or *Py. dissotocum* was transferred to a 100ml Erlenmeyer flask containing 50ml V8A and the cultures were incubated at 24ºC in the dark. Morphological observation and VOCs detection were conducted daily for 15 days after inoculation.

C. Basic Methodology

According to Risticevic et al. [10], a typical SPME method could be optimized from many aspects, and based on the objectives of the current study, the following SPME parameters were optimized: fiber coating, extraction time and desorption conditions. While optimizing one condition all other conditions were kept constant, parameters like 100ml Erlenmeyer flask with an adapter and a septum, 250ºC detector temperature, and the extraction temperature of 24ºC were kept constant during the whole experiment.

1. Fiber Coating

Six different types of fibers were exposed to the 4-6 day-old *P. c*+V8A and V8A alone (control) sample headspace, with an exposure time of 3h and a desorption time in the GC injector of 5min. The analytes obtained on the fibers were separated on the GC-FID instrument under the splitless mode. All samples were run in triplicate.

2. Exposure time

The selected fiber was exposed to the HS of the 100ml flasks containing 4-6 day-old *P. c* colonies grown on 50ml V8A and V8A alone for 11 different time periods (4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h). After exposure, the fiber was retrieved and injected into the heated injection port (250ºC) of a GC-FID and desorbed for 5min. Each sample was conducted in triplicate.

3. Desorption temperature and time

The 50/30μm DVB/CAR/PDMS fiber was used to optimize the injector temperature and desorption time. The fiber was inserted into the HS of the samples and exposed for the optimal extraction time. The fiber was then retrieved and placed into the GC injector. To determine the optimal injector temperature, the fiber was desorbed at different injector temperatures 200, 220, 230, 240, 250, 260 and 270ºC (270ºC is the highest temperature the fiber can stand) for 5mins. The fiber was desorbed for different desorption times (20s, 45s, 1min, 2min, 3min, 4min and 5min) at the optimal injector temperature to detect the optimal desorption time. The experiment was conducted in triplicate and the results are presented as mean values.

III. Results and Discussion

A. Selection of SPME Fiber Coating

The extraction efficiency of the six different commercially available SPME fibers (7μm and 100μm PDMS, PA, PDMS/DVB, PDMS/CAR and DVB/CAR/PDMS) were evaluated by comparing the peak areas and peak numbers of the compounds from *P. c*+V8A and V8A alone under the same extraction, desorption and GC conditions. The DVB/CAR/PDMS fiber had a higher extraction efficiency (larger peak areas) for the analytes of interest compared with

the PA, PDMS/DVB and PDMS/CAR fibres and the chromatograms of PA, PDMS/DVB and PDMS/CAR fibers overlapped with the DVB/CAR/PDMS fiber (Fig. 1b). In addition, the DVB/CAR/PDMS fiber traps a wider range of VOCs (more peak numbers) (Fig. 1a), and consequently it should be ideal for the analysis of the whole range of VOCs that are released by *P. c*. The chromatogram patterns between *P. c*+V8A and V8A alone from the DVB/CAR/PDMS fiber had distinct differences (Fig. 1 b), no compounds were extracted by the 7 and 100µm PDMS fibres. Therefore, the DVB/CAR/PDMS fiber was selected for the next steps of the SPME extraction and optimization process.

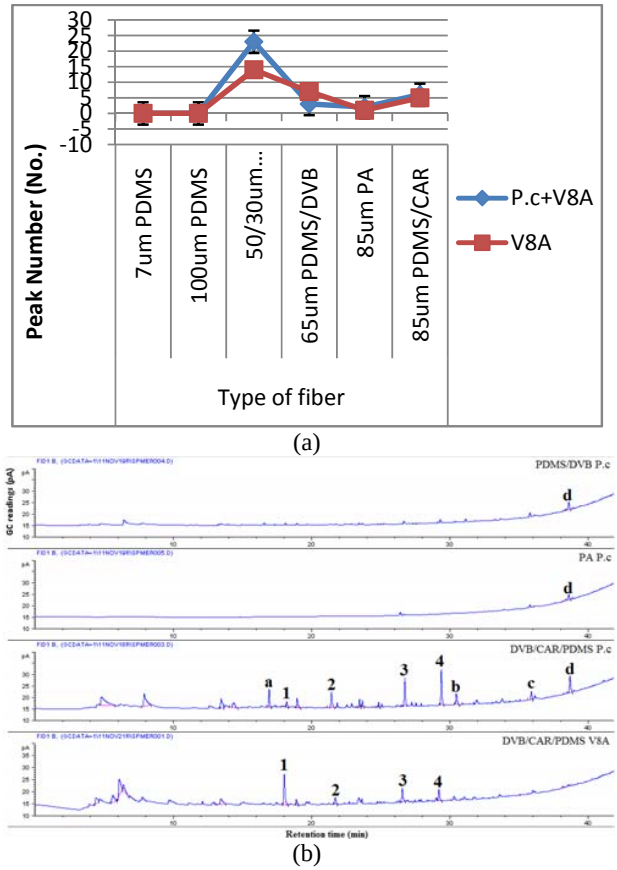

Fig. 1 (a) Number of peaks produced by *P. c* growing on V8A or V8A alone extracted by six different fiber types that were separated by a polar column ZB-WAX plus; (b) VOCs in *P. c*+V8A and V8A alone extracted by SPME fibers, compound d is a common peak from the three fibers, compounds a, b, c and d are distinct peaks from *P. c*+V8A when compared with the peaks from V8A alone, compounds 1, 2, 3 and 4 are common peaks in *P. c*+V8A and V8A alone, but the quantities are obviously different

B. Evaluation of HS-SPME Extraction Time

The determination of the optimum time of extraction is essential to obtain maximum efficiency of the SPME fibers for particular VOCs; and it is recognized as the time-limiting step of the SPME procedure.

The DVB/CAR/PDMS fiber was exposed in the flask HS for different times (4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24h) to determine the phase equilibrium (Fig. 2a). The difference in response depended on the distribution coefficients of the VOCs and the film type of the fiber. The amount of desorbed compounds 1, 2 and 3 increased with increasing extraction time until 16h (Fig. 2). Compound 4 needed a longer exposure time to get a higher response, this may be due to the DVB/CAR/PDMS fiber being an adsorbent fiber, all the physical trap sites or chemically reactive bonds were not saturated with analytes until 20h's of extraction . However, 20h was too long for the HS-SPME procedures and there was no significant difference in sensitivity between the 16h and 20h extraction times, and the response of the analytes decreased after 20h. The GC response for V8A alone had the same trend. Thus, the extraction time of 16h was selected for further analyses.

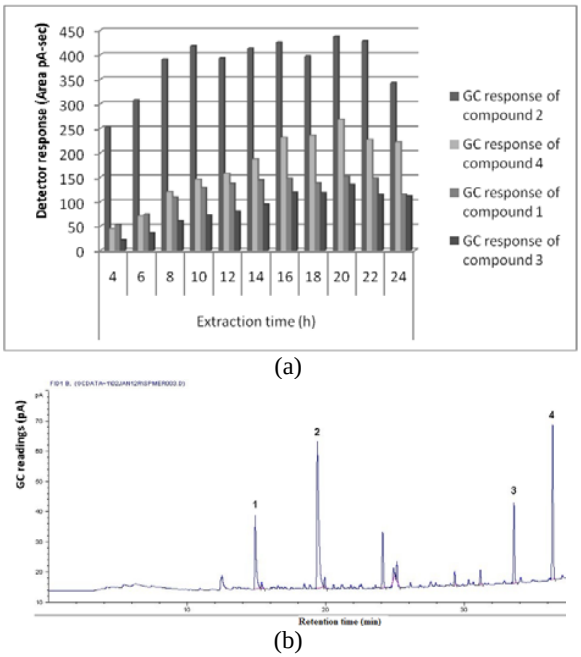

Fig. 2 (a) Effects of extraction time with fiber 50/30µm DVB/CAR/PDMS on the extraction efficiency of analytes of interest at 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 hours; (b) Chromatogram of the compounds of interest with 16h extraction, 5min desorption

C. Evaluation of GC Injector Temperature and Desorption Time

Efficient thermal desorption of an analyte from a SPME fiber depends on the boiling point of the analyte, the type the fiber and the temperature of the injection port. The selection of optimal desorption conditions ensure the maximum transfer of analytes onto the separation (column)/detection system. Injector temperature should be high enough to allow fast and quantitative desorption without decomposing the thermolabile chemicals. Desorption time should be determined at the optimal desorption temperature.

The effects of the GC injector temperature in the desorption yield was evaluated by varying the temperature between 200

and 270ºC with a constant extraction time of 16h for *P. c*+V8A and 230-270ºC for V8A alone (the recommended operating temperature for DVB/CAR/PDMS fiber is 230-270ºC). The desorption temperature profile obtained for *P. c*+V8A and V8A alone using the 50/30µm DVB/CAR/PDMS fiber is shown in Fig. 3. The total area of desorbed compounds increased with increasing injector temperature until it reached a maximum at 250ºC, this is because desorption is an endothermic process and is disfavored at low temperatures. The amount of desorbed compounds decreased when the temperature was higher than 250ºC, this could be due to temperature denaturization, the destruction or decomposition of the chemicals. Thus, the optimum desorption efficiency was achieved at 250ºC and this temperature was selected for the subsequent experiments.

Chromatograms for the DVB/CAR/PDMS fiber showed that compounds from V8A alone and *P. c*+V8A reached the maximum desorption efficiency at 3min (Fig. 4), and the re-injection of the fiber at 3min or less showed that the fiber was completely desorpted of the desired analytes after 3 min of exposure in the injector at 250ºC (Fig. 5). Hence, a desorption time of 3min was selected for complete desorption of the VOCs of interest.

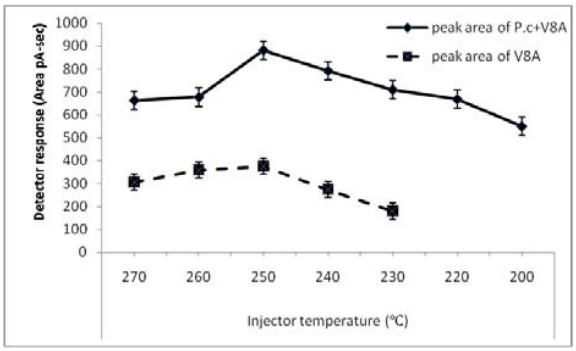

Fig. 3 Effects of GC injector temperature on desorption of VOCs of *P. c*+V8A and V8A alone from the 50/30µm DVB/CAR/PDMS fiber after 16h extraction

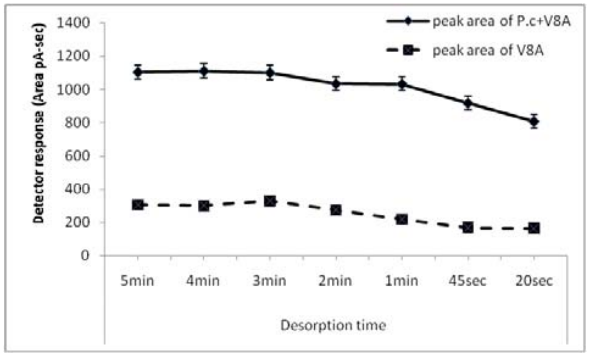

Fig. 4 Time of desorption with a 50/30µm DVB/CAR/PDMS fibre affects the peak areas of *P. c*+V8A and V8A alone

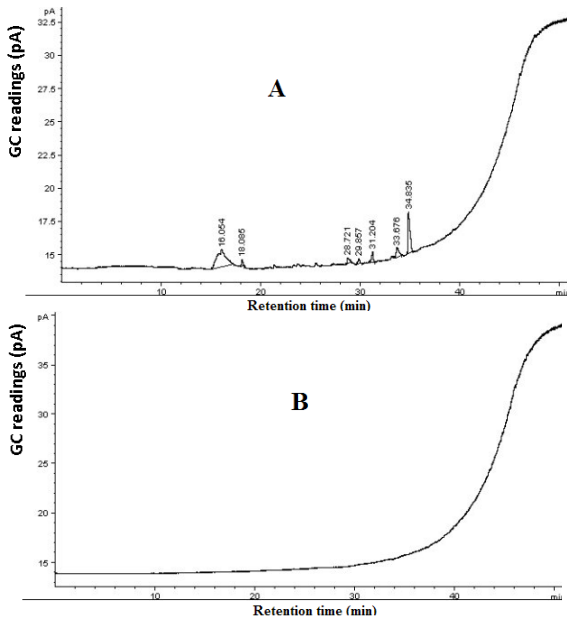

Fig. 5 Reinjection chromatogram of DVB/CAR/PDMS fiber for different desorption times for the extraction of VOCs in *P. c*+V8A after 16h of exposure and desorption times of (A) less than 3 min and (B) 3min and above

D. Chromatograms and Morphology Observation

VOCs of *Py. dissotocum* and *P. c* were detected under the optimized HS-SPME GC conditions, with compounds 1 and 2 specifically belonging to *Py. dissotocum*, and compounds 3, 4 and 5 to *P. c*, compound 5 emerged only when hyphae were present and lost when chlamydospores were produced, whilst compound 3 was only produced with the formation of chlamydospores (Fig. 6). This result corresponds with the findings of Grant et al. [11].

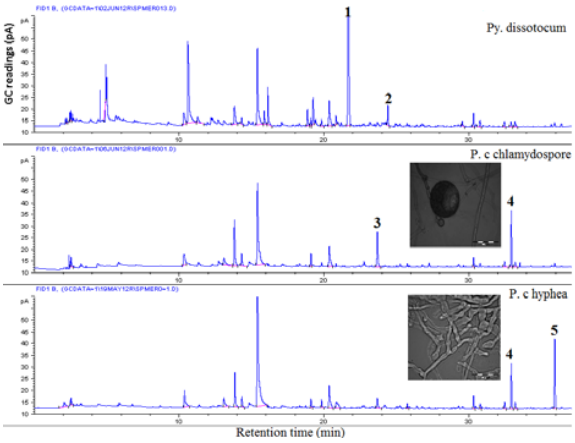

Fig. 6 Chromatograms of *Py. dissotocum*, 6 and 15 day-old hyphae and chlamydospores of *P. c*

IV. Conclusion

The HS-SPME technique is a fast, inexpensive and solvent free technique that has been developed and validated using the analysis of volatile organic compounds produced by *P. c*. The technique allowed the separation of a variety of compounds. The HS-SPME-GC-FID procedure was optimized using a 50/30µm DVB/CAR/PDMS fiber for extraction of the analytes of interest from *P. c*+V8A and V8A alone. It was found that an exposure time of 16h to the sample HS was sufficient to reach the maximum extraction efficiency. A desorption time of 3min in the 250ºC injector of the GC allowed for complete desorption of compounds from the DVB/CAR/PDMS fiber. Using the optimized technique to detect the VOCs from *P. c* and *Py. dissotocum* was possible. In addition, *Py. dissotocum* and *P. c* released different VOCs, and *P. c* released different VOCs at different life cycle stages, indicating that the different microbes have their own species and life cycle specific VOCs. The optimized HS-SPME method has been shown to be a reliable, rapid and precise diagnostic tool for *P. c*.

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