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Phenylacetaldehyde *O*-Methyloxime: A Volatile Compound Produced by Grapefruit Leaves Infected with the Citrus Canker Pathogen, *Xanthomonas axonopodis* pv. *citri*

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An aldehyde oxime *O*-methyl ether, phenylacetaldehyde *O*-methyloxime, was detected using solid-phase microextraction (SPME) and gas chromatography−mass spectrometry (GC-MS) in the headspace above grapefruit leaves infected with *Xanthomonas axonopodis* pv. *citri*, the causal agent of citrus bacterial canker disease (CBCD). This disease is a major phytosanitary concern, and an eradication campaign against it is currently underway in Florida. Phenylacetaldehyde *O*-methyloxime has been reported to be produced by other plants and fragrant flowers, but it was not observed in the headspace above uninfected grapefruit leaves, the pathogenic bacterium *X. axonopodis* pv. *citri* itself, or grapefruit leaves infected with another closely related bacterial pathogen, *X. axonopodis* pv. *citrumelo*, which causes citrus bacterial spot, a disease of no phytosanitary significance. It was also not detected from CBCD infected fruits, including orange, lemon, grapefruit, and lime. We conclude that phenylacetaldehyde *O*-methyloxime may potentially be used to identify CBCD infestations. However, more intensive studies will be required to fully evaluate the potential of phenylacetaldehyde *O*-methyloxime as a diagnostic compound for CBCD. Using SPME and GC-MS to measure phenylacetaldehyde *O*-methyloxime may provide an easy and feasible tool to complement current methods used to detect *X. axonopodis* pv. *citri* in environmental samples.

KEYWORDS: Phenylacetaldehyde *O*-methyloxime; citrus bacterial canker disease; *Xanthomonas axonopodis* pv. *citri*; *Xanthomonas axonopodis* pv. *citrumelo*; solid-phase microextraction

INTRODUCTION

Citrus bacterial canker disease (CBCD) is caused by the plant pathogenic bacterium *Xanthomonas axonopodis* pv. *citri* (*1*). The disease weakens trees by causing cankers on branches and leaves of trees and can be spread rapidly by wind-driven rain (*2*). In addition to being a serious disease, countries or regions where the disease occurs may not export fresh fruit to countries or regions where the disease does not occur, as a result of phytosanitary regulations. Thus, the economic impact of this disease is very large. At least 20% of the $8 billion Florida citrus market ($1.6 billion annually) is at risk because of probable bans on interstate commerce in citrus (*3*). The disease was introduced into Florida early in the 20th century and was eliminated after an eradication campaign that lasted more than 20 years (*4*, *5*). The pathogen was discovered again in Florida in 1986, and since that time eradication programs have been directed against this disease in Florida, where as much as $40 million annually is being spent to eliminate the pathogen from commercial citrus fields (1.56 million commercial trees removed or cut back by 2003) and residential neighborhoods (600 000 dooryard trees removed by 2003) as required by law to prevent reinfection of commercial citrus fields (*3*, *6*). There is also a large eradication campaign currently underway in São Paulo, Brazil, the location of the world's largest citrus industry.

Thus, there are few plant diseases where early and accurate diagnosis is more important. Citrus bacterial spot disease (CBSD) is caused by bacteria very closely related to those that cause CBCD, but neither the pathogen nor the disease is of commercial or quarantine significance (*7*). Currently, identification of citrus canker disease is initially based on visual symptoms (visual differentiation between CBSD and CBCD is easy and each lesion type is quite distinct). Confirmatory tests, including the isolation of the bacterium in vitro, serology, and PCR-based testing for the pathogen are also required (*8*). These tests are accurate but consume both the samples and time. PCR reactions using citrus extracts can be rapidly performed, even in real-time (*9*), but plant extracts often contain natural (*10*) or man-made compounds (*11*) that inhibit the PCR. Regulatory officials, such as U.S. Department of Agriculture (USDA)-Animal and Plant Health Inspection Service (APHIS), routinely employ dogs to search baggage at airports for contraband plant

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material, indicating that plant-produced volatiles are useful for detection purposes. Therefore, we sought to identify volatile compounds produced specifically by citrus plants infected with *X. axonopodis* pv. *citri*. Solid-phase microextraction (SPME) and gas chromatography−mass spectrometry (GC-MS) have been extensively used for headspace analysis (*12*−*16*) and were used in this study to identify characteristic compounds from the headspace above CBCD-infected grapefruit leaves.

MATERIALS AND METHODS

Production of Infected Plant Material. All experiments were carried out under special quarantine arrangements with USDA, APHIS, and the Maryland Department of Agriculture. Fully expanded grapefruit leaves (*Citrus paradisi* MacFadyen Hook) were infiltrated with suspensions of *X. axonopodis* pv. *citri* strain XC62, or *X. axonopodis* pv. *citrumelo* strain F2. These are standard laboratory reference strains for citrus bacterial canker disease and for citrus CBSD, respectively. Because the second pathogen is so closely related to *X. axonopodis* pv. *citri*, it is the ideal strain to use as a control for these experiments. Bacterial suspensions were adjusted turbidimetrically to contain approximately 10^7 CFU/mL, and plants were maintained in a quarantine greenhouse until lesions developed. CBCD infected and healthy fruits of sweet orange, rough lemon, grapefruit, and lime were obtained from the Florida Department of Agriculture and Consumer Services, Citrus Canker Eradication Program. Upon arrival, the fruits were kept in a refrigerator at ∼4 °C until assayed.

Headspace Collection. The pathogen, *X. axonopodis* pv. *citri* on SPA agar plates (*17*), four groups of grapefruit leaves (11, 15, 20, and 26 leaves, 1 leaf from each tree) previously inoculated (∼2 weeks) with *X. axonopodis* pv. *citri*, two groups of grapefruit leaves (20 leaves/each group, 1 leaf from each tree) previously inoculated (∼2 weeks) with *X. axonopodis* pv. *citrumelo*, and four groups of uninoculated healthy grapefruit leaves (11, 15, 20, and 26 leaves, 1 leaf from each tree) were separately placed in 500-mL clear, straight-sided glass jars with Teflon liner caps (Wheaton, Millville, NJ) (*12*). The inoculated leaves contained distinct lesions typical of either CBCD or CBSD. For assays of fruit, one to three (depending on size) healthy and CBCD infected fruits (orange, lemon, grapefruit, and lime) were also separately placed into the glass jars. A 100 mm polydimethyl siloxane coating phase SPME fiber (Supelco Inc., Bellefonte, PA) was conditioned in a GC injector port (250 °C) for 5 min and then passed through the small hole on the cap into the jar. The SPME fiber was exposed for 3−24 h to absorb volatile compounds, and the needle was then pierced into the GC injector port for 2 min to desorb the analytes for GC-MS analysis (*12*). SPME sampling was conducted either in a green house (11 leaves) or in a laboratory (15, 20, 26 leaves, and fruits) at room temperature, and GC-MS analyses from each sample were repeated at least five times.

Besides SPME sampling, the headspace volatiles from healthy and CBCD infected grapefruit leaves were also collected using absorbent in a green house. The CBCD infected and healthy leaves (72 leaves each, 4 leaves from each tree) were introduced separately into two 1-L, four-necked glass containers (*18*). Air was drawn into the container through 6−14 mesh activated charcoal (Fisher Scientific, Pittsburgh, PA) and out of the container through two traps (15 cm × 1.5-cm o.d.) containing 200 mg (each trap) Super Q (Alltech Associates, Inc., Deerfield, IL) by vacuum (∼1 L/min). Aeration was conducted continuously for 4 days at room temperature. The adsorbent traps were changed every 24 h. Adsorbents were eluted with methylene chloride (4 × 0.5 mL); the eluates (2 mL/each sample) were concentrated to ∼50 μL under a nitrogen stream and stored at −30 °C for future analysis.

Chemical Analysis. SPME samplings and volatile extracts were analyzed by gas chromatography−mass spectrometry (GC-MS) in the electron impact (EI) mode. A Hewlett-Packard (HP) 6890 GC was coupled with a HP 5973 mass selective detector. A 60 m × 0.25-mm i.d., 0.25-μm film-thickness DB−WAXetr capillary column (J&W Scientific Inc., Folsom, CA) was used in the splitless mode (50 °C for 2 min, then programmed to 230 °C at 15 °C/min, and held for 15 min). Helium was the carrier gas. Chemical ionization (CI) MS spectra were obtained from a Finnigan 4510 GC-MS spectrometer with ammonia (NH_3) or with deuterioammonia (ND_3) as reagent gases (*19*, *20*) using a 60 m × 0.25-mm i.d., 0.25-μm film-thickness DB-5 column (J&W Scientific Inc.) and the same temperature program as above.

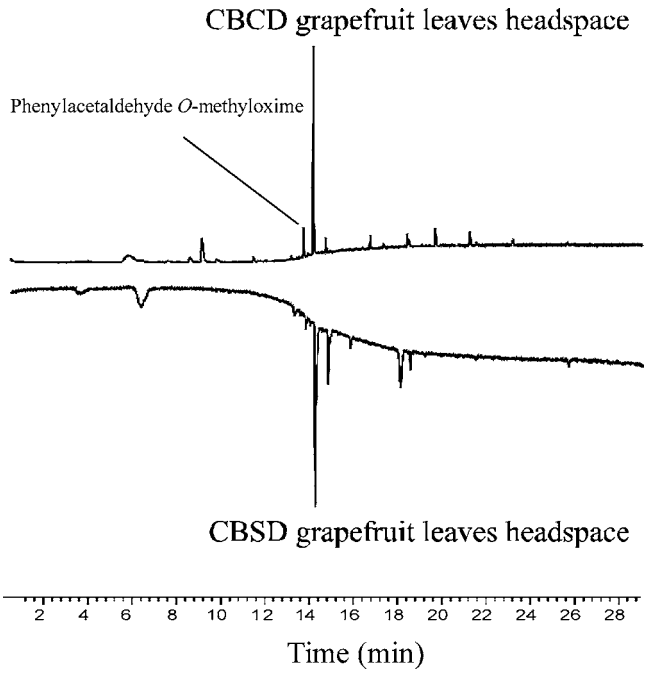

Figure 1. Reconstructed gas chromatograms of volatiles collected by SPME from the headspace above grapefruit leaves with symptoms of CBCD (top) versus CBSD (bottom). The diagnostic compound, phenylacetaldehyde *O*-methyloxime, was marked.

Synthesis of Phenylacetaldehyde *O*-Methyloxime. A solution of phenylacetaldehyde (1.2 g, 10 mmol) in 10 mL of pyridine was cooled with an ice bath. Methoxylamine hydrochloride (1.0 g, 12 mmol) was added slowly with stirring. After the addition was completed, the ice bath was then removed and the solution was allowed to warm to room temperature with stirring for 2 h. Ice-cold water (20 mL) was then added, and the solution was extracted with a 1:1 solution of ether−hexane (3 × 15 mL). The organic phase was washed with water (20 mL), a mixture of ice and 6 N hydrochloric acid (1:1, 2 × 20 mL), water (2 × 20 mL), saturated aqueous sodium bicarbonate, and saturated aqueous sodium chloride solution. After drying over magnesium sulfate, the solvent was evaporated in vacuo, and the residue was purified by flash chromatography (5% ethyl acetate in hexane) to give 1.37 g (92% yield) of phenylacetaldehyde *O*-methyloxime as a colorless oil.

RESULTS AND DISCUSSION

The SPME sampling and GC-MS analysis were repeated at least five times, and the GC traces of the CBCD, CBSD, and healthy leaves were compared. A peak that was present in the GC profile of headspace volatiles from CBCD grapefruit leaves but absent in the GC profile of headspace volatiles from both CBSD and healthy leaves was discovered (**Figure 1**). To confirm this finding, the headspace volatiles from CBCD and healthy grapefruit leaves were collected separately by a traditional aeration method. The same result was obtained (**Figure 2**). The EI mass spectrum of the unknown compound (**Figure 3A**) did not match spectra of known compounds in the Wiley 275 computerized database. The compound contains a single nitrogen atom, and the molecular weight is 149. The assumption that the ion with *m*/*z* 149 was the molecular ion was confirmed by CI mass spectra (*m*/*z* 150 ($[M + 1]^+$, 100) and 167 ($[M + 18]^+$, 25)) with ammonia (NH_3) as the reagent gas. The

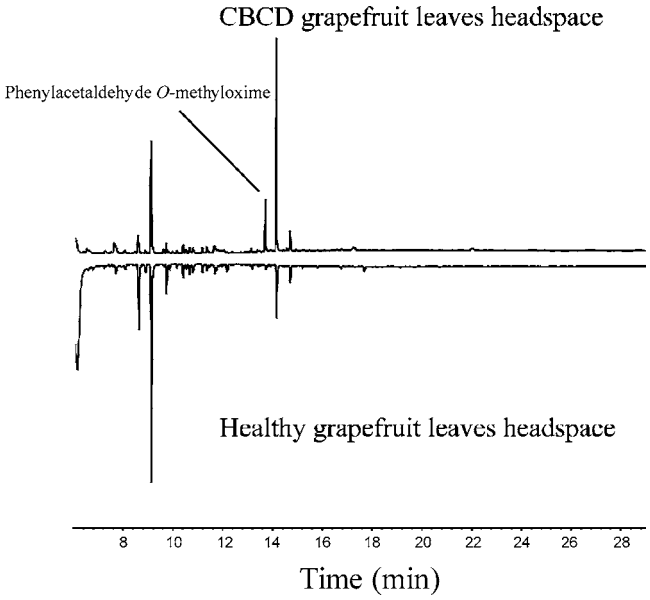

Figure 2. Reconstructed gas chromatograms of volatiles collected by aeration using absorbent from the headspace above grapefruit leaves with symptoms of CBCD (top) versus healthy leaves (bottom). The diagnostic compound, phenylacetaldehyde *O*-methyloxime, was marked.

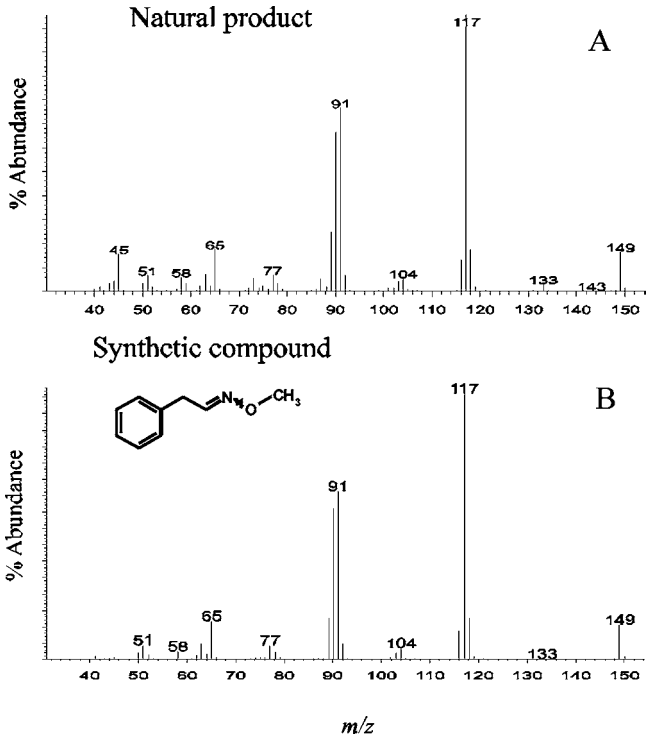

Figure 3. Electron impact (EI) mass spectra of the natural product detected from the headspace above CBCD grapefruit leaves (**A**) and of the synthetic compound (**B**).

corresponding ions, *m*/*z* 151 ($[M + 2]^+$, 70) and 171 ($[M + 22]^+$, 100), were obtained with deuterioammonia (ND_3) indicating that the compound did not contain exchangeable protons. The unknown compound produced fragment ions at *m*/*z* 117 (M − 32) without other significant fragments in the high-mass region between *m*/*z* 149 and 117, (**Figure 3A**), suggesting that a neutral molecule was easily eliminated during fragmentation. It is reasonable to expect that elimination of methanol ($[M - CH_3OH]^{+\bullet}$) from the molecule results in a base peak *m*/*z* 117. The presence of the ion, *m*/*z* 91, at relatively high intensity indicates that the molecule may contain a benzyl group. The mass spectrum of the unknown compound is similar to that of phenylacetonitrile (base peak *m*/*z* 117). These data suggested that the unknown compound was related to phenylacetonitrile but with an extra methoxyl group on either the nitride carbon or on the nitrogen. Experimental data indicate that the latter case is correct. When the compound phenylacetaldehyde *O*-methyloxime was synthesized (*21*), the mass spectrum and GC retention times were identical to those of the natural product (**Figure 3B**) on both polar and nonpolar capillary columns.

Phenylacetaldehyde *O*-methyloxime is not a compound new to science and has been associated previously with flowers (*22*, *23*) and some healthy plants (*24*, *25*) and has been synthesized (*21*, *26*). The same volatile response could also be induced by plants under an environmental stress (i.e., pH, water) or insect damage. For example, phenylacetaldehyde *O*-methyloxime has been tentatively identified from black locust (*Robinia pseudoacacia*) plants infested with spider mites, *Tetranychus urticae*, and from uninfested and infested eggplant (*Solanum melalonga*) (*27*). However, it has not been previously observed with diseased plants. This observation is entirely novel. Our data so far show the compound to be produced by grapefruit leaves infected with *X. axonopodis* pv. *citri* (other varieties of citrus were not tested) and not by the pathogen *X. axonopodis* pv. *citri* itself and also not by grapefruit leaves infected with another member of the same species which also infects citrus but which does not incite CBCD. This second pathogen, *X. axonopodis* pv. *Citrumelo*, causes CBSD, a disease which is not of commercial or phytosanitary significance. Even using the selected ion monitoring method (*m*/*z* 117 and 91), phenylacetaldehyde *O*-methyloxime was not detected in the headspace above grapefruit leaves with CBSD, the nearest bacterial relative of *X. axonopodis.* pv. *citri*, nor above healthy leaves and the pathogen, *X axonopodis* pv. *citri* itself, suggesting that phenylacetaldehyde *O*-methyloxime was produced by interaction between CBCD pathogen, *X axonopodis* pv. *Citri*, and grapefruit leaves. Thus, the ability of our method to distinguish between these two conspecific bacteria may be of practical importance and is evidence of the specificity of the detection method. The grapefruit leaves used in our experiments were inoculated about 2 weeks earlier and significant lesions had developed. Our further research will be conducted to determine the time course of emission of this compound after inoculation of pathogen on susceptible grapefruit leaves, testing leaves of other citrus varieties, and quantitative determination of phenylacetaldehyde *O*-methyloxime produced by CBCD infected citrus leaves.

Although CBCD symptoms are quite distinct, confirmation of the diagnosis of the disease by other methods is required. PCR is an excellent confirmatory test and can also detect the pathogen in the absence of symptoms. Phenylacetaldehyde *O*-methyloxime would be an additional easy, rapid, and methodologically independent test. One attraction of phenylacetaldehyde *O*-methyloxime is that it is a nondestructive assay, since the lesions are not consumed by the assay, as they are whenever samples are collected for isolation of bacteria or for serological or DNA based detection methods. We do not know exactly what the sensitivity would be in terms of number of lesions/headspace volume. Presumably, the speed of detection by phenylacetaldehyde *O*-methyloxime would be proportional to the density of lesions in the sample. Interestingly, phenylacetaldehyde *O*-methyloxime was not detected from any CBCD infected fruits.

We have discovered that phenylacetaldehyde *O*-methyloxime is produced by grapefruit leaves infected by *X. axonopodis* pv. *citri*, the pathogen that causes CBCD. This pathogen/disease is

universally subject to phytosanitary quarantine, and rapid, sensitive, and specific detection methods are urgently needed. At a minimum, this technology will be useful as a completely independent method for the preliminary identification of this pathogen in plant samples. The method is rapid, taking only 30 min to complete once the samples have been collected, and does not require the isolation and culture of the bacterium. However, a more intensive study would be required to evaluate the potential of phenylacetaldehyde *O*-methyloxime as a diagnostic compound and to validate the use of SPME and GC-MS to provide practical tools applicable to CBCD detection in commercial citrus fields.

To our knowledge, our concept of detecting pathogens in plant materials on the basis of the detection and identification of volatile products produced in the course of plant disease is also novel and may be generalized.

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