Baseline and Differential Sensitivity to Two QoI Fungicides Among Isolates of *Phytophthora cactorum* That Cause Leather Rot and Crown Rot on Strawberry

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ABSTRACT

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Sensitivities of 89 isolates of *Phytophthora cactorum*, the causal agent of crown rot and leather rot on strawberry plants, from seven states (Florida, Maine, North Carolina, Ohio, Oregon, South Carolina, and New York) to the QoI fungicide azoxystrobin were determined based on mycelium growth and zoospore germination. Radial growth of mycelia on lima bean agar amended with azoxystrobin at 0.001, 0.01, 0.1, 1.0, 10, and 30 µg/ml and salicylhydroxamic acid (SHAM) at 100 µg/ml was measured after 6 days. Effect on zoospore germination was evaluated in aqueous solutions of azoxystrobin at 0.005, 0.01, 0.05, 0.1, 0.5, and 1.0 µg/ml in 96-well microtiter plates by counting germinated and nongerminated zoospores after 4 h at room temperature. SHAM was not used to evaluate zoospore sensitivity. The effective dose to reduce mycelium growth by 50% (ED₅₀) ranged from 0.16 to 12.52 µg/ml for leather rot isolates and 0.10 to 15 µg/ml for crown rot isolates. The Kolmogorov-Smirnov test showed significant differences ($P < 0.001$) between the two distributions. Zoospores were much more sensitive to azoxystrobin than were mycelia. Differences between sensitivity distributions for zoospores from leather rot and crown rot isolates were significant at $P = 0.05$. Estimated ED₅₀ values ranged from 0.01 to 0.24 µg/ml with a median of 0.04 µg/ml. Experiments with pyraclostrobin, another QoI fungicide, demonstrated that both mycelia and zoospores of *P. cactorum* were more sensitive to pyraclostrobin than to azoxystrobin. Sensitivities to azoxystrobin and pyraclostrobin were moderately but significantly correlated ($r = 0.60$, $P = 0.0001$).

Additional keywords: baseline sensitivity

Phytophthora cactorum (Lebert & Cohn) J. Schröt. (1886) is an oomycete pathogen that causes leather rot and crown rot on strawberry plants (*Fragaria × ananassa* Duchesne). Leather rot affects fruit at all stages of development and can cause significant losses under weather conditions conducive to disease development (25). Crown rot affects the entire plant, causing wilting and, eventually, death (41). Crown rot has been reported in different countries of Europe and the United States (41), and it is becoming a serious problem in some southern states of the United States (e.g., North Carolina, South Carolina, and Georgia) on strawberry plants grown with plasticulture systems (16). Although *P. cactorum* is the causal agent of both diseases, pathogenicity tests and molecular characterization of

leather rot and crown isolates suggest that two different pathotypes are responsible for causing these two diseases (9,41). Currently, management of leather rot is based on integrated use of cultural practices and chemical control. Cultural practices include avoiding saturated soils through proper site selection and improved soil drainage and reducing splash dispersal of the pathogen by the use of straw mulches (10,41). Management of crown rot relies on a similar strategy of avoiding saturated soils and use of chemical control. However, the use of healthy transplants obtained from reliable suppliers also is recommended to minimize losses due to crown rot (16).

Chemical control is an important component of both crown rot and leather rot management programs. Protectant fungicides such as captan and thiram have been used for several years to prevent foliage and fruit diseases of strawberry (M. A. Ellis, *personal communication*). Currently, the most effective fungicides for control of leather rot on strawberry are mefenoxam, fosetyl-aluminum, and several different phosphorous acid-based compounds related to fosetyl-Al. Both classes of fungi-

cides have been used extensively on strawberry and development of resistance is a concern because of their modes of action. Mefenoxam-resistant isolates of *P. cactorum* recovered from symptomatic crowns of strawberry plants recently have been reported in South Carolina (18). To date, no report of insensitivity to fosetyl-al in *P. cactorum* has been published. However, insensitivity to this compound has been reported in other oomycetes, such as *Pseudoperonospora humuli*, which causes downy mildew on hops, in the Pacific Northwest region of the United States (30), and *Bremia lactucae*, which causes downy mildew on lettuce, in California (7). Due to the potential for resistance development, fungicides with different modes of action are needed for use on strawberry plants to control crown rot and leather rot so that further development of fungicide-resistant isolates of *Phytophthora cactorum* is prevented and spread of resistant isolates that already have developed is minimized.

Quinone outside inhibitor (QoI) fungicides (also known as strobilurins) are a relatively new class of fungicides that are used on a broad range of crops against all the major groups of fungus pathogens as well as the oomycetes (5,6). These fungicides inhibit mitochondrial respiration by binding to the Q_o center of cytochrome *b* located at complex III, blocking the electron transport chain, and, therefore, interfering with energy production by halting the formation of ATP (6,14,37,52). Field resistance to these fungicides has been broadly documented in different crop-pathogen systems since their commercial release in 1996 (6,14). In most cases, the G143A mutation at the cytochrome *b* gene has been associated with full resistance in most fungus pathogens (14,17,24). Another mutation, F129L, also has been reported as responsible for field resistance in *Pythium aphanidermatum* and *Alternaria solani*; however, it appears that, with this mutation, pathogens maintain a certain level of sensitivity to fungicides from the same cross-resistance group (6,20,33).

The second mechanism of resistance is associated with the induction of an alternative respiration pathway in the electron transport chain of mitochondria by means of expression of an alternative oxidase (AOX) enzyme (2,51). This alternative

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pathway has been found to be active in mycelia and conidia of fungi in vitro and in planta in the presence of QoI fungicides (2,3,54). However, its role in field resistance is considered of little practical importance (31). Another proposed resistance mechanism is mediated by efflux transporters, such as ATP binding cassette (ABC) transporters, which have been regarded as possible mechanisms of resistance to some strobilurins in fungus and oomycete pathogens (15,19,35).

Two QoI fungicides, azoxystrobin and pyraclostrobin, currently are registered on strawberry plants in the United States for control of anthracnose, powdery mildew, and leaf spot. Although leather rot is not on either label, recent studies have shown that both azoxystrobin and pyraclostrobin are highly effective for control of leather rot (34). In that study, there were no significant differences in efficacy between these fungicides and the currently used fungicides, mefenoxam and phosphorous acid. These results suggest that QoI fungicides could be an effective addition to the fungicides currently registered for leather rot control and may prove useful in preventing or delaying resistance from developing in *P. cactorum* to mefenoxam and phosphorous acid.

Azoxystrobin first was released to the market in 1996 in the United States and countries of Europe and was registered for use on more than 30 crops worldwide (14,21). Although sensitivity to azoxystrobin has been studied in a number of different fungus and oomycete species using both in vitro and in vivo methods (13,20,27,29,36,49,50), information related to the sensitivities of species of *Phytophthora* to azoxystrobin is limited. In vitro studies with mycelia and encysted zoospores to determine sensitivity to azoxystrobin showed that *P. capsici*, *P. citrophthora*, and *P. nicotianae* (= *P. parasitica*) were not highly sensitive to this fungicide (26). Estimated effective dose to reduce mycelium growth by 50% (ED₅₀) values for azoxystrobin were more than 3,000 µg/ml for mycelia and ranged from 256 to 1,000 µg/ml for germinated zoospores. Similar results for the sensitivity of *P. nicotianae* to azoxystrobin, using different methodology, have been reported (22). More recently, Yuan et al. (53) determined the baseline sensitivity of several isolates of *P. infestans* to flumorph and the relationship to azoxystrobin. They reported ED₅₀ values that varied between 0.06 and 0.1 µg/ml.

Azoxystrobin has proven to be a very effective fungicide for controlling leather rot in the field (34), but it has not been fully evaluated for efficacy against crown rot. In addition, sensitivity to QoI fungicides among isolates of *P. cactorum* that cause leather rot and crown rot has not been studied. Defining the sensitivity distribution of isolates of *P. cactorum* from straw-

berry plants to azoxystrobin should be useful for future monitoring of fungicide resistance development once the fungicide becomes broadly used by growers for controlling leather rot and crown rot. Therefore, the main objective of this research was to determine the sensitivities of isolates of *P. cactorum* recovered from fruit and crowns of strawberry plants to azoxystrobin, based on mycelium growth and zoospore germination in vitro. Another objective was to compare the sensitivities of a subset of isolates to a single dose of pyraclostrobin—another QoI fungicide.

MATERIALS AND METHODS

Isolates of *P. cactorum*. Eighty-nine isolates of *P. cactorum* obtained from diverse geographical locations were used in this study (Table 1). Isolates were obtained

from different collections across the country; many of them were collected before QoI fungicides were developed and used commercially. Other isolates were recovered directly from symptomatic strawberry fruit and crowns collected in Ohio fields or from samples sent to our lab between 2003 and 2005 from different locations around the country. Isolates of *P. cactorum* recovered from symptomatic fruit or crowns were isolated on the oomycete-selective medium PBNC, which contained pentachloronitrobenzene, benomyl, neomycin sulfate, and chloramphenicol (40), and PAR-V8, which contained pimarin, ampicillin, and rifamycin (12). Isolates were transferred to lima bean agar (LBA) for use in all fungicide experiments. LBA was prepared by suspending 150 g of frozen lima beans in 500 ml of distilled water and

Table 1. Isolates of *Phytophthora cactorum* used in this study, the type of symptom with which each was found associated in the field, strawberry cultivar from which each initially was isolated, state of origin, the source of each isolate, and isolation date

Isolate no.	Cultivar	Symptom ^a	Origin	Source ^b	Year of isolation
97-35	Sweet Charlie	CR	Florida	UFL	1997
97-37	Sweet Charlie	CR	Florida	UFL	1997
97-38	Sweet Charlie	CR	Florida	UFL	1997
97-40	Sweet Charlie	CR	Florida	UFL	1997
97-41	Sweet Charlie	CR	Florida	UFL	1997
97-42	Sweet Charlie	CR	Florida	UFL	1997
97-54A	Camarosa	LR	Florida	UFL	1997
02-203	Festival	LR	Florida	UFL	2002
02-204	Camarosa	LR	Florida	UFL	2002
04-696	Unknown	CR	North Carolina	NCSU	2004
04-563	Unknown	CR	North Carolina	NCSU	2004
03-3192	Unknown	CR	North Carolina	NCSU	2004
02-2467	Unknown	CR	North Carolina	NCSU	2003
Earlybrite 12-4	Earlybrite	CR	North Carolina	NCSU	2002
Earlybrite 12-7	Earlybrite	CR	North Carolina	NCSU	2004
Northeast H26-2	Noreaster	CR	North Carolina	NCSU	2004
Northeast H26-4	Noreaster	CR	North Carolina	NCSU	2004
Sweet Charlie17-4	Sweet Charlie	CR	North Carolina	NCSU	2004
Sweet Charlie17-5	Sweet Charlie	CR	North Carolina	NCSU	2004
04-NC-CR-1	Sweet Charlie	CR	North Carolina	OSU	2004
04-NC-CR-2	Sweet Charlie	CR	North Carolina	OSU	2004
04-NC-CR-3	Sweet Charlie	CR	North Carolina	OSU	2004
04-NC-CR-4	Sweet Charlie	CR	North Carolina	OSU	2004
04-NC-CR-5	Sweet Charlie	CR	North Carolina	OSU	2004
04-NC-CR-6	Sweet Charlie	CR	North Carolina	OSU	2004
04-NC-CR-7	Sweet Charlie	CR	North Carolina	OSU	2004
04-NC-CR-9	Sweet Charlie	CR	North Carolina	OSU	2004
04-NC-CR-10	Sweet Charlie	CR	North Carolina	OSU	2004
04-NC-CR-11	Sweet Charlie	CR	North Carolina	OSU	2004
04-WLR1	Honeoye	CR	Ohio	OSU	2004
04-WLR2	Honeoye	LR	Ohio	OSU	2004
04-WLR3	Honeoye	LR	Ohio	OSU	2004
04-WLR5	Honeoye	LR	Ohio	OSU	2004
04-WLR6	Honeoye	LR	Ohio	OSU	2004
04-WLR7	Honeoye	LR	Ohio	OSU	2004
04-WLR8	Honeoye	LR	Ohio	OSU	2004
04-WLR9	Honeoye	LR	Ohio	OSU	2004
04-WLR10	Honeoye	LR	Ohio	OSU	2004
04-WLR11	Honeoye	LR	Ohio	OSU	2004
04-WLR12	Honeoye	LR	Ohio	OSU	2004
04-WLR13	Honeoye	LR	Ohio	OSU	2004
04-WLR15	Honeoye	LR	Ohio	OSU	2004

(continued on next page)

^a Isolates were recovered from plants with crown and root rot (CR) or from fruits with leather rot (LR).

^b Isolates were received from collections maintained by J. Mertley at the University of Florida (UFL), F. J. Louws at North Carolina State University (NCSU), or S. N. Jeffers at Clemson University (Clemson), or were recovered from plants by A. Rebollar-Alviter and M. A. Ellis at Ohio State University (OSU).

autoclaving at 120°C and 15 psi for 20 min. The resulting broth was clarified by adding diatomaceous earth and filtering through P8 filter paper (Fisher Scientific Co., Pittsburgh). The clarified broth was mixed with 20 g of agar, adjusted to 1 liter with distilled water, and autoclaved for 20 min. Cultures of all isolates were stored in liquid nitrogen at the Ohio Agricultural Research and Development Center (OARDC), Ohio State University, Wooster.

Isolate identification. Identification of isolates recovered from symptomatic plants was based on standard morphological characteristics produced on both solid and liquid media: presence of caducous sporangia—each with a short pedicel and a conspicuous papilla—produced on sympodial sporangiophores, paragynous antheridia, and aplerotic oospores in single-isolate cultures (11). Isolates obtained from other sources were identified by the provider prior to arrival. However, all of

these isolates were examined in our laboratory for the standard morphological characteristics to confirm identifications.

In addition, a subset of nine isolates (crown rot: 97-35, 04-NC-CR1, 04-NC-CR2, and 04-NC-CR5; leather rot: 04-MLR5B, 04-MLR6, 04-WLR1, 05-STACY1A, and 04-SouthOH4) corresponding to samples from five different locations was identified by amplifying and sequencing the internal transcribed spacer (ITS) region of the 16S ribosomal DNA (rDNA) unit using the polymerase chain reaction (PCR) with ITS1 and ITS4 primers (48). Sequence alignments among the sequenced isolates were performed with the ClustalW algorithm using the default settings (version 1.8; The European Bioinformatics Institute, Cambridge, England). These isolates represented the different regions from which they were recovered. Isolates were grown in dilute lima bean broth (DLB) for 7 days at 23 ± 2°C (room

temperature). DLB was prepared as follows: frozen lima beans were processed as previously described for preparing LBA medium. The resulting broth was clarified by adding diatomaceous earth and filtering through P8 Fisherbrand filter paper. The clarified broth was adjusted to 600 ml with distilled water before autoclaving for 20 min. Mycelia were filtered through P8 filter paper and dried at room temperature for 24 h. Dried mycelia were ground in a mortar using liquid nitrogen, and total genomic DNA was extracted according to the protocol of Lee and Taylor (23). Each 50-μl reaction mixture consisted of 1× buffer, 1.5 mM of MgCl₂, 0.08 mM dNTPs, 0.2 μM each primer, 0.5 μl of go-Taq (Promega Corporation, Madison, WI), and 6 μl of template DNA (16 to 36 ng/μl). PCR was performed in a PTC-100 thermocycler (MJ Research Inc., Waltham, MA) using the following program: 5 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 57°C, and 1 min at 72°C. The final extension step consisted of 5 min at 72°C.

PCR products (10 μl) were separated by horizontal gel electrophoresis in a 1.5% agarose gel (0.5× Tris-borate-EDTA, 100 V for 60 min), and ethidium bromide stained before being visualized using a UV transilluminator, and photographed using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Eastman Kodak Company, New Haven, CT). Prior to sequencing, PCR products were purified with a QIAquick purification kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. PCR products (5-μl aliquots) were sequenced in the forward direction using the ITS1 primer with an ABI Prism 3100 analyzer (Applied Biosystems, Inc., Foster City, CA) at the Molecular and Cellular Imaging Center at OARDC (Wooster). Sequences then were compared to similar sequences in GenBank EMBL, DDBT, and PDB databases by running BLAST (blastn) (1) from the National Center for Biotechnology Information (NCBI).

Single-zoospore isolates. Isolates were cultured on LBA for 5 days at room temperature. Sixteen 5-mm mycelium plugs were taken from the edge of an actively growing colony and were placed in a petri plate with 20 ml of DLB and incubated at 22°C under continuous light in an incubator, and abundant sporangia had developed at 5 to 7 days. DLB was removed and replaced with 15 ml of sterile deionized water. Cultures were incubated for 30 to 40 min at 4°C and then moved to a lab bench at room temperature for 30 min to induce zoospore release. Zoospore suspensions for each isolate were collected, and the density of zoospores in each suspension was determined using a hemacytometer. To produce single-zoospore isolates, zoospore suspensions were adjusted to 10⁵ zoospores/ml, and 5 drops (25 μl/drop) were

Table 1. (continued from preceding page)

Isolate no.	Cultivar	Symptom ^a	Origin	Source ^b	Year of isolation
04-SouthOH1	Unknown	LR	Ohio	OSU	2004
04-SouthOH2	Unknown	LR	Ohio	OSU	2004
04-SouthOH3	Unknown	LR	Ohio	OSU	2004
04-SouthOH4	Unknown	LR	Ohio	OSU	2004
04-40-12	Unknown	LR	Oregon	OSU	2004
04-MLR1B	Chandler	LR	Maine	OSU	2004
04-ML2B	Chandler	LR	Maine	OSU	2004
04-MLR3b	Chandler	LR	Maine	OSU	2004
04-ML5B	Sweet Charlie	LR	Maine	OSU	2004
04-ML6	Chandler	LR	Maine	OSU	2004
04-ML7B	Sweet Charlie	LR	Maine	OSU	2004
04-ML8	Chandler	LR	Maine	OSU	2004
04-MLR10	Unknown	LR	Maine	OSU	2004
SC.99-2554	Chandler	CR	South Carolina	Clemson	1999
SC.99-472	Chandler	CR	South Carolina	Clemson	1999
NY.170	Chandler	LR	New York	Clemson	1982
NCSU.99-47-1	Camarosa or Chandler	CR	North Carolina	Clemson	1999
NCSU.99-1280	Chandler	CR	North Carolina	Clemson	1999
NCSU.99-1277	Chandler	CR	North Carolina	Clemson	1999
NCSU.00-5901	Chandler	CR	North Carolina	Clemson	2000
SC.Ks6	Camarosa	CR	South Carolina	Clemson	2001
SC.Ks7	Camarosa	CR	South Carolina	Clemson	2001
SC.Ks5	Camarosa	CR	South Carolina	Clemson	2001
OSU.194	Unknown	CR	New York	Clemson	1985
OSU.190	Unknown	CR	New York	Clemson	1987
SC.Co1	Unknown	CR	South Carolina	Clemson	2001
SC.Co2	Unknown	CR	South Carolina	Clemson	2001
SC.Co3	Unknown	CR	South Carolina	Clemson	2001
SC.Co4	Unknown	CR	South Carolina	Clemson	2001
FL.97-44	Sweet Charlie	CR	Florida	Clemson	1997
FL.97-42A	Sweet Charlie	CR	Florida	Clemson	1997
SC.Wa1	Unknown	CR	South Carolina	Clemson	2001
SC.Wa4	Unknown	CR	South Carolina	Clemson	2001
SC.NC1-1C	Unknown	CR	North Carolina	Clemson	2001
SC.NC1-2A	Unknown	CR	North Carolina	Clemson	2001
SC.NC1-1E	Unknown	CR	North Carolina	Clemson	2001
SC.NC1-2E	Unknown	CR	North Carolina	Clemson	2001
SC.NC1-2F	Unknown	CR	North Carolina	Clemson	2001
SC.NC1-1D root	Unknown	CR	North Carolina	Clemson	2001
05-Witten 2a	Unknown	LR	Ohio	OSU	2005
05-Waters 1a	Unknown	LR	Ohio	OSU	2005
05-Waters 1b	Unknown	LR	Ohio	OSU	2005
05-Witten 1a	Unknown	LR	Ohio	OSU	2005
05-Witten 2b	Unknown	LR	Ohio	OSU	2005
05-Witten 1b	Unknown	LR	Ohio	OSU	2005
05-Stacy 1a	Unknown	LR	Ohio	OSU	2005
05-Stacy 2a	Unknown	LR	Ohio	OSU	2005

distributed equally on a petri plate containing 1.5% water agar and then slightly tilted to allow drops to run down the plate. Plates were held at room temperature on a lab bench for 5 h. After this period, five germinated zoospores from each isolate were transferred to plates containing PBNC. Single-zoospore colonies were removed and subcultured, and these isolates were stored at 4°C on PBNC in 10-ml glass tubes with screw caps. Isolates were cultured on LBA before use in all experiments.

Fungicides. Technical grade azoxystrobin (96%, Syngenta Crop Protection, Greensboro, NC) and pyraclostrobin (98%; BASF Corporation, Research Triangle Park, NC.) were used in all experiments. Salicylhydroxamic acid (SHAM, 99%; Aldrich Chemical Co., Milwaukee, WI) was added to media amended with fungicide to inhibit the AOX pathway of *P. cactorum* in all mycelium assays.

Effect of SHAM on mycelium growth and zoospore germination. To evaluate the effect of SHAM on mycelium growth and zoospore germination, six representative isolates of *P. cactorum* were tested against the compound at 0, 50, 100, and 150 µg/ml. SHAM was diluted in a 1:1 solution of acetone-methanol and added to molten LBA previously cooled to 50°C. Four plates of each dose were used for each isolate, and the experiment was conducted twice. Control plates were amended with solvent only. An agar plug with mycelium from the edge of a 5-day-old colony was placed at the center of each plate, and plates were held at room temperature for 6 days. After this time, two colony diameters were measured at right angles on each plate, and the two measurements were averaged. A two-way analysis of variance (ANOVA) using Proc Mixed of SAS (version 9.1; SAS Institute, Cary, NC) was conducted on the relative growth of mycelia to compare SHAM concentrations, isolates, and their interactions; replications and trials were considered as random effects. Based on statistical analysis, a single dose of SHAM was selected to be used in fungicide sensitivity experiments with mycelia. The main criterion for selecting the best dose was that mycelium growth was not inhibited more than 30% and provided enough synergism to observe the fungicide effect although, in some isolates, inhibition exceeded this percentage. This level of inhibition was considered effective to detect the effect of fungicide without excessive inhibition of growth by SHAM (C. Avila-Adame, *personal communication*). To confirm that this concentration was sufficient to block the alternative respiration pathway, four isolates were tested using azoxystrobin at 0.001, 0.01, 0.1, 1.0, 10, and 30 µg/ml in the presence and absence of SHAM as previously described. Experiments were conducted at least twice.

To determine if SHAM was required to detect the effect of QoI fungicides on zoospore germination, SHAM at 100 µg/ml was tested alone and in aqueous concentrations of azoxystrobin at 0.005, 0.01, 0.05, 0.1, 0.5, and 1 µg/ml on two isolates of *P. cactorum* using a 96-well microtiter plate assay as described below. The experiment was conducted twice, and the relative germination from the two trials of this experiment was used to fit the log-logistic model to the data (as shown below). The ED₅₀ of zoospore germination, standard errors, and confidence intervals were estimated for zoospore germination at the six concentrations of azoxystrobin in the presence and absence of SHAM.

Effect of azoxystrobin on mycelium growth. Technical grade azoxystrobin was diluted in acetone. LBA was cooled to 50°C and then amended with different amounts of azoxystrobin to obtain final concentrations of 0.001, 0.01, 0.1, 1.0, 10, and 30 µg/ml. For each concentration, SHAM at 100 µg/ml was added to the medium to inhibit the alternative respiration pathway. Fungicide-amended medium (20 ml) was placed in each of five 9-cm-diameter disposable petri plates. Control plates were amended with SHAM only. Final concentration of solvent did not exceed 2 ml/liter, and no effects of solvent on mycelium growth were observed in preliminary experiments (*data not shown*).

Agar plugs (5 mm in diameter) with mycelium were removed from the edge of actively growing 5-day-old cultures of *P. cactorum* growing on LBA and were placed at the center of plates with fungicide-amended LBA. Plates were placed in clear plastic bags, and bags were held on a lab bench at room temperature (23 ± 2°C) for 6 days. Colony diameters were measured as described above, and the average diameter for each colony was used for analysis. Mycelium growth on five replicate plates was averaged and used to calculate the relative growth in relation to the mean mycelium growth on nonamended control plates for each isolate–fungicide concentration combination. Each isolate was tested at least twice and the mean relative growth obtained from each fungicide dose in each trial was used to estimate the ED₅₀ by fitting the log-logistic model to the data as described below.

Effect of azoxystrobin on zoospore germination. A 96-well microtiter plate (Fisher Scientific Co.) assay adapted from Slaweacki (45) was used to evaluate zoospore germination. Zoospores from each isolate were produced as previously described and adjusted with a hemacytometer to a concentration of 10⁵ zoospores/ml. Because zoospores may remain motile for a relatively long period of time, suspensions were vortexed for 12 to 15 s to induce encystment before applying fungicide solutions. Zoospores tend to adhere to the bottom of the plate after encystment and

prior to germination. Therefore, germinated and nongerminated cysts easily can be observed (×160) and counted with an inverted microscope or by inverting the plate and using a standard compound microscope.

Fungicide solutions of 0.01, 0.02, 0.1, 0.2, 1, and 2 µg/ml were prepared in sterile distilled water. An aliquot of 150 µl of each fungicide solution was mixed with an equal volume of zoospore suspension to obtain final fungicide concentrations of 0.005, 0.01, 0.05, 0.1, 0.5, and 1.0 µg/ml in a total volume of 300 µl per well. For each isolate–fungicide concentration combination, two wells (replications) were used to evaluate zoospore germination and values were averaged. Preliminary experiments with SHAM showed that there was no significant difference in estimated ED₅₀ values for zoospore germination among the isolates tested in the presence or absence of SHAM; therefore, SHAM was not used in studies to evaluate zoospore sensitivity. Microtiter plates were placed on a lab bench at room temperature for 4 h. After this time, fungicide solutions were poured gently from plates and the plates were immediately sealed with Parafilm to prevent dehydration of zoospores during counting. For each well, 60 zoospores were observed microscopically and the percentages of germinated and nongerminated zoospores were calculated. A zoospore was considered germinated if its germ tube was at least twice as long as the zoospore diameter and an appressorium was present. Germination percentages from two wells were averaged, and the mean was used to calculate relative germination by comparison with germination in the nontreated control plates. All tests were conducted at least twice. Relative germination obtained for each isolate was used to estimate the ED₅₀ for germination by fitting the log-logistic model (see below).

Estimation of the effective dose. ED₅₀ of relative mycelium growth and zoospore germination was estimated by fitting the log-logistic model with upper asymptote to the data using the NLMIXED procedure of SAS (version 9.1). The model is expressed as:

$$y = \frac{\alpha}{1 + \exp\left[\beta \log\left(\frac{\text{dose}}{\text{ED}_{50}}\right)\right]}$$

where *y* is the relative growth of mycelium or zoospore germination and α , β , and ED₅₀ are parameters estimated from the data; α is the upper asymptote (maximum possible value of *y*), β is the slope of the *y*:dose curve at its steepest point (at the point of inflexion), and ED₅₀ is the dose at which the relative growth or germination is reduced by 50%.

The model is a reparameterization of the log-logistic model in terms of ED₅₀ as indicated by Schabenberger and Pierce (39). Starting values of parameters for

iterations were assigned arbitrarily. Because not all starting values resulted in convergence for all isolates, different sets of starting values were used to reach convergence. Goodness of fit was checked by observing the random pattern of residuals for each of the isolates.

Distribution of sensitivities. Histograms were used to illustrate the distribution of ED_{50} values for mycelium growth and zoospore germination. Moment estimates, basic statistical measures of dispersion and skewness, normality tests, and histograms for distributions were obtained with Proc UNIVARIATE of SAS. Because skewed distributions of ED_{50} values of azoxystrobin were obtained from crown rot and leather rot isolates, even after log-transformation of ED_{50} values, comparisons of ED_{50} distributions were conducted by applying the Kolmogorov-Smirnov two-sample test using the same SAS procedure. In addition, covariance analyses were conducted to compare intercepts and slopes of relative mycelium growth and zoospore germination with azoxystrobin at 10 and 0.05 $\mu\text{g/ml}$ (covariates), respectively. These doses were selected from a set of azoxystrobin doses after graphical exploration and confirmation of linear relationship by determining Pearson and Spearman correlations between relative mycelium growth or zoospore germination and ED_{50} values. After confirmation of a linear relationship between those variables, data were evaluated for normality and for equal variances. Covariance analysis allowed for determination of linear regression equations that could be used for future prediction of ED_{50} values obtained from zoospores and mycelia.

Because some outliers and influential observations were detected during the statistical analysis, they were removed from the data to run the covariance analyses but were kept for most of the other tests. Covariance analysis was conducted using Proc MIXED of SAS (version 9.1). Pearson and Spearman correlations between mycelium growth and zoospore germination also were performed to determine whether there was an association between sensitivities of zoospores and mycelia.

Sensitivities of isolates of *P. cactorum* to pyraclostrobin. Relative growth of mycelia from 69 isolates of *P. cactorum* (30 leather rot and 39 crown rot isolates) was determined using pyraclostrobin at 1 $\mu\text{g/ml}$. This dose was chosen based on previous observations of sensitivity of mycelium growth to azoxystrobin. All procedures were conducted as described previously. For each isolate, three plates of amended medium were used in each trial. An additional set of plates was amended with only SHAM as a control for each isolate. From the edge of a 5-day-old colony, 5-mm plugs were taken and placed at the center of each amended plate, and

plates were held for 6 days at room temperature. Colony diameters were measured and relative growth was calculated as described previously. The experiment was conducted twice, and data from the two trials were combined because there was no significant difference between them. Relative growth was used to construct histograms and box plots for both crown and leather rot isolates to illustrate the distribution of sensitivities to a single dose of pyraclostrobin.

To determine if there were significant differences between crown rot and leather rot isolates and between the two fungicides, ANOVA was conducted using Proc MIXED (SAS, version 9.1) on the relative growth of mycelia from the 69 isolates at a single, common dose of pyraclostrobin and azoxystrobin (1 $\mu\text{g/ml}$). In this analysis, isolates were considered random effects and isolate type (crown rot and leather rot) and fungicide were considered fixed effects.

To determine if the relative mycelium growth resulting from a single dose of pyraclostrobin (1 $\mu\text{g/ml}$) was useful for predicting ED_{50} values, two isolates of each class of the constructed histogram were selected and evaluated against the fungicide at 0.001, 0.01, 0.1, 1.0, 10, and 30 $\mu\text{g/ml}$. In all, 26 isolates were chosen (13 leather rot and 13 crown rot). The assay was conducted twice, using three plates for each fungicide concentration, and mycelium growth was measured as described previously. Mycelium growth from three plates was averaged and the relative growth was calculated. Relative mycelium growth values from the two trials were used to fit the log-logistic model, which was used to estimate the ED_{50} value for each isolate, as described

previously. Estimated ED_{50} values were regressed against the relative growth of mycelia previously obtained from testing the same isolates with pyraclostrobin at 1 $\mu\text{g/ml}$. Normality and goodness-of-fit were confirmed by conducting an Anderson-Darling test and observing a random pattern of residuals, respectively.

Because azoxystrobin and pyraclostrobin belong to the same cross-resistance group of fungicides (6), relative growth of mycelia obtained from using 69 isolates in the presence of pyraclostrobin at 1 $\mu\text{g/ml}$ was correlated with the relative growth obtained with azoxystrobin at the same concentration. The same analysis was conducted with the ED_{50} values obtained for pyraclostrobin and azoxystrobin with 26 isolates. Before the analysis, a normality test was conducted and equality of variances (Levene's test) for the data was tested.

To determine the sensitivity of zoospores to pyraclostrobin, a set of 16 isolates was selected and tested against a range of concentrations (0.01, 0.005, 0.001, 0.0005, 0.0001, and 0.0 $\mu\text{g/ml}$) by using the 96-well microtiter plate assay as described previously. The range of doses was selected based on a preliminary fungicide sensitivity experiment. Comparison of estimated ED_{50} values for the two fungicides was conducted by a *t* test on the $\log_{10}$ of the ED_{50} values. Confidence intervals for the mean ED_{50} value for each fungicide also were estimated.

RESULTS

Identification of isolates. All isolates recovered from symptomatic crowns and fruit and obtained from other locations showed the typical morphological characteristics of *P. cactorum* (11). The isolates

Fig. 1. Relative growth of mycelia of six isolates of *Phytophthora cactorum* with salicylhydroxamic acid (SHAM) at 50, 100, and 150 $\mu\text{g/ml}$, estimated as the ratio of the radial mycelium growth on amended and nonamended media. Isolates were grown on lima bean agar for 6 days at $23 \pm 2^\circ\text{C}$ and then the radial mycelium growth was measured. Vertical bars are means of four replicates; error bars are standard errors of the mean.

selected for amplification and sequencing of the ITS region of the rDNA gene had 100% sequence similarity with the following isolates of *P. cactorum* in the NCBI database of GenBank: DSM 62643 (accession number AY848935), MN10GG (accession number DQ486654), and P25 (accession number AY423290). Amplified sequences corresponded to the ITS1, 5.8s rRNA gene, and ITS2 sequences. Nucleotide sequence alignment between the sequenced crown and leather rot isolates did not show differences.

Effect of SHAM on mycelium growth and zoospore germination. SHAM alone significantly reduced growth of mycelia

for all isolates tested. There was a significant interaction between dose and isolates ($F = 6.98$, $P < 0.0001$) (i.e., isolates responded differently to different concentrations of SHAM; Fig. 1). Although one isolate was very sensitive to SHAM in this test, 100 $\mu\text{g/ml}$ was selected for use in sensitivity tests with azoxystrobin and pyraclostrobin. This concentration provided enough synergism with azoxystrobin and pyraclostrobin to allow observation of a dose-response effect at different concentrations of the fungicides (Fig. 2). In contrast, when SHAM was not included in the medium, there was no response to dose of QoI fungicides, indicating that the oomy-

cete was able to circumvent the effect of the fungicides by the activation of the alternative respiration pathway by means of the AOX. These results confirmed that SHAM at 100 $\mu\text{g/ml}$ was sufficient to effectively reduce interference from the AOX pathway, so that fungicide activity on mycelium growth could be measured.

Including SHAM in fungicide solutions did not significantly change the estimated ED_{50} values for zoospore germination, which indicated that this compound was not needed to observe a response for zoospore germination to doses of azoxystrobin (Fig. 2). Confidence intervals (95%) for ED_{50} values estimated for two isolates in

Fig. 2. Dose-response of two isolates of *Phytophthora cactorum* growing on lima bean agar amended with azoxystrobin alone at 0.001, 0.01, 0.1, 1.0, 10, and 30 $\mu\text{g/ml}$ (filled dots) or combined with salicylhydroxamic acid (SHAM) at 100 $\mu\text{g/ml}$ (empty dots) at $23 \pm 2^\circ\text{C}$ for 6 days: **A**, Crown rot isolate SC.NC1-2F; **B**, leather rot isolate 02-204. Relative mycelium growth was calculated as the ratio of radial growth on fungicide + SHAM amended and nonamended media. Each datum point represents the mean of five replicates from two trials combined. Dose-response curves for germination of zoospores of two isolates of *P. cactorum* at different concentrations of azoxystrobin amended with (empty dots) and without (filled dots) SHAM at 100 $\mu\text{g/ml}$ at $23 \pm 2^\circ\text{C}$ for 4 h: **C**, isolate 97-41 and **D**, isolate 97-35. Each point represents the mean relative germination of zoospores in aqueous suspensions from two trials. In each trial, mean percent zoospore germination from two replications was obtained and relative germination calculated as the ratio of the percent germination in fungicide + SHAM amended and nonamended (control) suspensions.

the presence and absence of SHAM based on the log-logistic model were 0.060 to 0.063 $\mu\text{g/ml}$ with SHAM and 0.05 to 0.1 $\mu\text{g/ml}$ without SHAM for one isolate, and 0.098 to 0.1 $\mu\text{g/ml}$ with SHAM and 0.082 to 0.12 $\mu\text{g/ml}$ without SHAM for the second isolate. Based on these results, sensitivities of zoospores to azoxystrobin and pyraclostrobin were assayed without adding SHAM to fungicide solutions.

Log-logistic model. The log-logistic model with upper asymptote was adequate to describe the relative growth of mycelia and relative germination of zoospores at different fungicide doses (Fig. 3). For each of the isolates evaluated, the effective dose that inhibited 50% of mycelium growth

and zoospore germination could be estimated, and residual plots showed a random pattern (Fig. 3).

Sensitivity distribution of *P. cactorum* isolates to azoxystrobin. Distribution of isolates of *P. cactorum* based on sensitivities of mycelia to azoxystrobin had characteristics of a bimodal distribution, with each peak corresponding to a type of isolate—either crown rot or leather rot (Fig. 4). Therefore, each distribution was analyzed separately. Both distributions of the ED_{50} values were skewed to the right (Table 2; Fig. 4A) and log-transformed values did not normalize the distributions satisfactorily (Fig. 4B). Comparison of ED_{50} distributions for mycelium growth of crown

rot and leather rot isolates by the Kolmogorov-Smirnov two-sample test (which does not depend on normality), showed significant differences ($KSa = 2.65$, $P < 0.0001$). Estimated ED_{50} values for crown rot isolates ranged from 0.1 to 15 $\mu\text{g/ml}$, with a median of 4.83 $\mu\text{g/ml}$. ED_{50} values for leather rot isolates ranged from 0.1 to 12.5 $\mu\text{g/ml}$, with a median of 1.98 $\mu\text{g/ml}$ (Table 2; Fig. 4A). A significant linear correlation between estimated ED_{50} values and relative growth of mycelia at 10 $\mu\text{g/ml}$ ($r = 0.85$, $P < 0.0001$) was observed.

To determine whether 10 $\mu\text{g/ml}$ potentially could discriminate between the two types of isolates and be used as a discriminatory dose, a covariance analysis on the

Fig. 3. Typical dose-response curves for isolates of *Phytophthora cactorum*: **A**, relative mycelium growth of isolate SC.Wa4, (calculated as the ratio of radial growth on fungicide amended and nonamended [control] agar media) and **C**, relative zoospore germination for isolate 04-563 (calculated as the ratio of percentages of zoospore germination in fungicide amended and nonamended [control] aqueous suspensions). Values for the predicted effective dose for 50% reduction and associated residuals for **B**, relative mycelium growth and **D**, relative zoospore germination were estimated and plotted after fitting the log-logistic model to the data.

log-transformed ED_{50} values was conducted, selecting relative mycelium growth at 10 $\mu\text{g/ml}$ as covariate and the $\log_{10}$ of ED_{50} values as the response variable. Isolate type (crown rot and leather rot) was a categorical variable. The results showed that there was a significant interaction ($P = 0.0001$) between the covariate and type of isolate (Fig. 5A), meaning that the change in ED_{50} with change in relative mycelium growth at 10 $\mu\text{g/ml}$ was not the same for the two types of isolates. Because the interaction was significant, models were compared at three different values of the covariate—0.10, 0.35, and 0.50—corresponding to a low, medium, and high value of relative mycelium growth. Results showed that ED_{50} values for both types of isolates were significantly different at relative mycelium growth of 0.1 ($P = 0.002$) and 0.35 ($P = 0.005$) but were not signifi-

cantly different at a relative mycelium growth of 0.5 ($P = 0.90$). These results also suggest that the linear relationship between ED_{50} values for crown rot and leather rot isolates and relative mycelium growth at 10 $\mu\text{g/ml}$ could be useful to predict sensitivity of *P. cactorum* to azoxystrobin (Fig. 5A). The regression equations were: $y = 10^{(-0.13 + 2.05X)}$ for crown rot isolates and $y = 10^{(-0.57 + 2.92X)}$ for leather rot isolates.

Zoospore germination proved to be very sensitive to azoxystrobin. A Kolmogorov-Smirnov two-sample test showed borderline significant differences between ED_{50} distributions for crown rot and leather rot isolates ($KSa = 1.34$, $P = 0.054$). ED_{50} values of isolates ranged from 0.01 to 0.24 $\mu\text{g/ml}$ with a mean of 0.04 $\mu\text{g/ml}$ for crown rot isolates and from 0.01 to 0.07 $\mu\text{g/ml}$ with a median of 0.03 $\mu\text{g/ml}$ for leather rot

isolates (Table 2; Fig. 4C). A significant linear correlation was found between the estimated log ED_{50} values and the relative germination at 0.05 $\mu\text{g/ml}$ ($r = 0.92$, $P < 0.0001$).

Covariance analysis using 0.05 $\mu\text{g/ml}$ as the covariate and isolate type as the categorical variable showed that the common-slope line model was adequate to describe the data (i.e., the slopes of the lines did not differ significantly; $P = 0.35$). Therefore, comparisons between the two types of isolates were conducted based on the intercepts. This analysis confirmed that there was a significant difference between crown rot and leather rot isolates ($P = 0.01$) after adjusting for the mean value of relative zoospore germination at 0.05 $\mu\text{g/ml}$ (covariant), which confirmed the results obtained in the mycelium growth experiment (Fig. 5A). In addition, these results suggest

Fig. 4. Sensitivity distributions for crown rot (CR; black bars, $n = 52$) and leather rot (LR; gray bars, $n = 37$) isolates of *Phytophthora cactorum* to azoxystrobin: **A**, frequency distribution of isolates for each class of estimated effective dose for 50% reduction (ED_{50}) values in sensitivity tests with growth of mycelia on fungicide amended medium; **B**, frequency distribution of isolates for each class of $\log_{10}$ ED_{50} values for mycelium growth; **C**, frequency distribution of isolates for each class of estimated ED_{50} values in sensitivity tests with zoospore germination on aqueous suspensions of fungicide; and **D**, frequency distribution of isolates for each class of $\log_{10}$ ED_{50} values for zoospore germination. Estimation of ED_{50} values was conducted by fitting the log-logistic model to relative mycelium growth and relative zoospore germination data.

that ED_{50} values for zoospore germination potentially could be predicted from a single dose of azoxystrobin at 0.05 $\mu\text{g/ml}$ (Fig. 5B). Linear regression equations were: $y = 10^{(-1.80 + 0.11X)}$ for crown rot isolates and $y = 10^{(-1.90 + 0.11X)}$ for leather rot isolates.

Correlation analysis between ED_{50} values estimated from mycelium growth assays and ED_{50} values estimated from zoospore germination assays (Fig. 6) showed significant but weak correlation ($r = 0.32$, $P = 0.007$). Median ED_{50} values for leather rot and crown rot isolates obtained from mycelium growth assays were 50 and 120 times higher than the median ED_{50} values estimated from zoospore germination assays.

Sensitivities of isolates of *P. cactorum* to pyraclostrobin. Distributions of the relative mycelium growth of crown rot and leather rot isolates in the presence of pyraclostrobin were symmetric (Fig. 7). Means of relative mycelium growth with pyraclostrobin at 1 $\mu\text{g/ml}$ for crown rot and leather rot isolates were significantly different based on a two-sample t test ($P = 0.002$). Relative growth ranged from 0.07 to 0.71, with a mean of 0.38, for leather rot isolates and from 0.15 to 0.84, with a mean of 0.49, for crown rot isolates. In addition, there was an overall significant difference (Type, $P < 0.0001$) between leather rot isolates and crown rot isolates (based on relative mycelium growth) to QoI fungicides (i.e., pyraclostrobin and azoxystrobin combined) at 1 $\mu\text{g/ml}$. There also were significant differences in growth of mycelia (for crown rot and leather rot isolates combined) exposed to pyraclostrobin at 1 $\mu\text{g/ml}$ and azoxystrobin at 1 $\mu\text{g/ml}$ ($P < 0.0001$). Isolates of *P. cactorum* from strawberry plants were more sensitive to pyraclostrobin (mean relative growth = 0.44) than to azoxystrobin (mean relative growth = 0.66). There was no significant interaction between fungicides and type of isolate ($P = 0.36$), indicating that both types of isolates responded similarly to the two QoI fungicides at 1 $\mu\text{g/ml}$. There was significant correlation between fungicides based on relative mycelium growth at 1 $\mu\text{g/ml}$ ($r = 0.6$, $P < 0.0001$).

The difference between estimated ED_{50} values for pyraclostrobin and azoxystrobin, based on mycelium growth for 13 crown rot and 13 leather rot isolates (26 isolates in all), was significant at $P = 0.063$. ED_{50}

Fig. 5. A, Relationship between relative growth of mycelia with azoxystrobin at 10 $\mu\text{g/ml}$ for 86 isolates of *Phytophthora cactorum* and estimated doses of azoxystrobin that caused 50% reduction in mycelium growth (ED_{50} values); solid line and circles correspond to crown rot isolates and dashed line and triangles correspond to leather rot isolates. **B,** Relationship between zoospore germination and estimated ED_{50} values with azoxystrobin at 0.05 $\mu\text{g/ml}$; solid line and circles correspond to crown rot isolates and dashed lines and triangles correspond to leather rot isolates. ED_{50} values were estimated by fitting the log-logistic model to data obtained from mycelium growth and zoospore germination experiments using different doses of azoxystrobin. Regression equations were obtained after covariance analysis using azoxystrobin at 10 and 0.05 $\mu\text{g/ml}$ as covariates and type of isolate (either crown rot or leather rot) as a categorical variable for mycelium growth and zoospore germination, respectively.

Table 2. Moment estimates and basic statistical measures for sensitivity distributions to azoxystrobin of effective dose to reduce mycelium growth by 50% values for two different life stages of isolates of *Phytophthora cactorum* that caused either crown rot (CR) or leather rot (LR)

Stage, symptom ^a	n	Mean	Median	SD ^b	Kurtosis	Skewness	Range	Pr > KS
Mycelia								
CR	51	6.23	4.83	3.72	0.68	-0.089	15.39	<0.0001
LR	37	3.19	1.98	3.06	1.71	1.53	12.47	...
Zoospores								
CR	52	0.055	0.043	0.048	7.31	2.59	0.23	0.054
LR	36	0.036	0.030	0.02	30.83	-1.53	0.06	...

^a Isolate life stage and symptom.

^b Standard deviation.

values for pyraclostrobin for crown rot isolates ranged from 0.09 to 2.56 $\mu\text{g/ml}$, with a mean of 0.92 $\mu\text{g/ml}$, whereas ED_{50} values for leather rot isolates ranged from 0.05 to 1.23 $\mu\text{g/ml}$, with a mean of 0.44 $\mu\text{g/ml}$. The limits of the 95% confidence intervals for the mean ED_{50} for pyraclostrobin were 0.30 and 0.67 $\mu\text{g/ml}$ and for azoxystrobin were 1.67 and 3.78 $\mu\text{g/ml}$. Significant correlation ($r = 0.73$, $P < 0.0001$) between the $\log_{10}$ ED_{50} values for azoxystrobin and pyraclostrobin was observed (Fig. 8). Zoospores also were more sensitive to pyraclostrobin than to azoxystrobin ($t = 0.06$, $P < 0.0001$). Estimated mean ($n = 16$) ED_{50} values for pyraclostrobin was 0.001 (standard error [se] = 0.0008) $\mu\text{g/ml}$ and for azoxystrobin was 0.06 $\mu\text{g/ml}$ (se = 0.05).

DISCUSSION

Sensitivities to azoxystrobin and pyraclostrobin of isolates of *P. cactorum* asso-

ciated with crown rot and leather rot of strawberry plants was determined using mycelium growth and zoospore germination as indicators. To evaluate sensitivities of mycelia, SHAM at 100 $\mu\text{g/ml}$ was added to agar medium. This amount of SHAM was found to be the most appropriate dose to mix with different fungicide concentrations to determine ED_{50} values. Concentrations of SHAM of between 100 and 150 $\mu\text{g/ml}$ have been used in previous sensitivity experiments with QoI fungicides with fungi (4,47). This compound is used to inhibit the alternative respiration pathway, which is dependent on the AOX enzyme in mitochondrion respiration in plants and fungi (46). Alternative respiration is activated in the presence of QoI fungicides and allows fungi and oomycetes to grow in vitro by circumventing the toxic action of the compound (43,51,54).

Prior to these studies, no information was available on the role of the AOX en-

zyme in oomycetes exposed to QoI fungicides. These studies suggest that *P. cactorum* activates the AOX enzyme in the presence of these fungicides. Fungicide doses as high as 30 $\mu\text{g/ml}$ had very little effect on mycelium growth when the medium was not amended with SHAM. In contrast, mycelium growth was greatly reduced when the medium was amended with different doses of fungicide plus SHAM at 100 $\mu\text{g/ml}$, which suggests that the AOX enzyme is expressed in mycelia in the presence of azoxystrobin. SHAM has been used in several in vitro studies to evaluate sensitivities of mycelia and conidia of fungi to different QoI fungicides at doses between 100 and 150 $\mu\text{g/ml}$ (2,32,33,47).

SHAM was not needed to estimate ED_{50} values for the effect of azoxystrobin on zoospore germination because zoospore germination rates in the presence of azoxystrobin with or without SHAM at 100 $\mu\text{g/ml}$ were similar. In these studies, the effects of fungicides were tested on encysted zoospores. To date, no information has been published on the role of the AOX enzyme in zoospore metabolism, but our results indicate that it is not active during germination of zoospores in the presence of QoI fungicides in vitro; however, the AOX enzyme still may be active in motile zoospores or zoospores germinating in situ. In a preliminary experiment, SHAM at 100 $\mu\text{g/ml}$ caused motile zoospores to stop swimming almost immediately and later to lyse (unpublished data). Therefore, molecular studies to determine gene expression in motile and encysted zoospores in the presence of QoI fungicides would be required to determine whether the AOX enzyme is expressed in zoospores. Gene expression studies in zoospores and germinated cysts of *P. nicotianae* have shown that several genes related to energy production are expressed during this stage of development, but the AOX enzyme genes were not reported (42,44).

Sensitivity distributions for isolates of *P. cactorum* to azoxystrobin based on mycelium growth showed a wide variation of ED_{50} values. The wide variation found in the sensitivity tests may be a result of the great genetic diversity of *P. cactorum* isolates found in North America (9,16). Interestingly, the median ED_{50} value for isolates recovered from the crown area (i.e., crown rot isolates) was more than twice as large as the one for isolates recovered from fruit (i.e., leather rot isolates). The reason for this difference in sensitivities between crown rot and leather rot isolates is not known. Previous investigations have suggested that crown rot and leather rot isolates belong to two different pathotypes (9,41). If this is true, then genetic differences with respect to sensitivity to QoI fungicides between these two groups of isolates may also exist.

Very few studies on the effects of QoI fungicides on oomycete pathogens in vivo

Fig. 6. Relationship between effective doses of azoxystrobin to reduce growth of mycelia and germination of zoospores by 50% (ED_{50}) for 84 isolates of *Phytophthora cactorum*. ED_{50} values were transformed to $\log_{10}$ values before plotting and extreme observations were removed; r is the correlation value for this relationship.

Fig. 7. Sensitivity distributions of leather rot (white bars, $n = 30$) and crown rot (black bars, $n = 39$) isolates of *Phytophthora cactorum* to pyraclostrobin based on relative growth of mycelia on lima bean agar amended with 1 $\mu\text{g/ml}$. Relative mycelium growth was calculated as the ratio of radial growth on fungicide-amended and nonamended media and was measured after 6 days at $23 \pm 2^\circ\text{C}$.

or in vitro have been published. Sensitivities to azoxystrobin of mycelia of *P. capsici*, *P. citrophthora*, and *P. parasitica* have been reported with ED₉₀ values >3,000 µg/ml (26) and ED₅₀ values of 56 to 165 µg/ml for *P. nicotianae* (22). These values are much greater than the ones obtained in our experiments. The high ED₅₀ values for mycelia in previous studies may be due to the different species used or may be due to the fact that fungicide-amended media used in these other studies were not amended with SHAM. The higher ED₅₀ values reported in studies conducted by others might suggest the activation of the alternative respiration pathway in the species of *Phytophthora* used in these studies. This may be further evidence for the ability of different species of *Phytophthora* to counteract the toxic effect of QoI fungicides when exposed in vitro.

Although results from our studies produced lower ED₅₀ values than those obtained for other species of *Phytophthora* (22,26), they still are considered high compared with ED₅₀ values published in other pathosystems, such as *Plasmopara viticola* on grape, which causes downy mildew. In this pathosystem, estimated ED₅₀ values for azoxystrobin ranged from 0.04 to 0.94 µg/ml using a leaf disk bioassay (49). In general, published ED₅₀ values estimated from baseline populations of different fungus pathogens based on conidium germination in vitro or in vivo do not exceed 1 µg/ml (3,28,29,32,47,51). Results with mycelia from our study produced median ED₅₀ values of 4.83 and 1.98 µg/ml for crown rot and leather rot isolates, respectively. Because QoI fungicides are more effective in vitro against conidia and zoospores than against mycelia, it is recommended that sensitivity studies be conducted using conidia or zoospores rather than mycelia (6,52). However, for some fungus pathogens, mycelium growth assays have provided useful information to detect variations in sensitivities to these fungicides within pathogen populations (3,29,43).

Compared with mycelium growth, zoospore germination was much more sensitive to azoxystrobin in our study. Similar to results with mycelium growth, zoospores of crown rot and leather rot isolates varied in sensitivity to azoxystrobin. ED₅₀ values for zoospore germination were log-normal distributed, with a median of 0.04 µg/ml for crown rot and 0.03 µg/ml for leather rot isolates. After the removal of outliers and influential observations, covariance analysis allowed a clear separation of the two groups of isolates, as was found with mycelium growth when the two groups of isolates were compared at a single dose of azoxystrobin at 0.05 µg/ml.

Sensitivity studies using zoospores from other species of *Phytophthora* have produced ED₅₀ values of azoxystrobin of 56 to 165 µg/ml (22) and 256 µg/ml (26). These

values clearly contrast with the results from our study. This may be due to the different species of *Phytophthora* tested as well as different methodologies used to conduct the experiments. Our methodology was based on the fact that, when zoospores are induced to encyst, they tend to attach to surfaces and then germinate (11). Inducing zoospore encystment allowed us to target the process of germination more directly, which is the process that appears more sensitive to QoI fungicides (6,38,51,52). Results obtained using our methodology with azoxystrobin are in agreement with those reported for other oomycetes (e.g., *P. viticola*; 50). Similar ED₅₀ values of azoxystrobin have been reported for *Phytophthora infestans* (13,54). The results obtained in our study also are similar to those reported for different fungi exposed to other QoI fungicides (3,8,27–29,31,33,47,49,50). Using a similar methodology, Slawewski et al. (45) evaluated the effect of several fungicides, including azoxystrobin and kresoxim-methyl, on spore germination and adhesion to polystyrene microtiter plates by *Botrytis cinerea* and *Puccinia recondita*. They found that the estimated ED₅₀ value for *B. cinerea* was azoxystrobin at 0.32 µg/ml and kresoxim-methyl at 0.02 µg/ml, and for *P. recondita* was kresoxim-methyl at 0.057 µg/ml. It appears that results obtained in polystyrene microtiter plate assays with fungicides that inhibit respiration (such as strobilurins) are similar to those obtained from in vivo tests in other pathosystems. However, more research is needed to evaluate the effectiveness and accuracy of results obtained from this in vitro methodology, and comparisons with sensitivities in vivo are essential.

Sensitivity of mycelia from a subset of isolates (13 crown rot and 13 leather rot)

of *P. cactorum* to a full range of doses of pyraclostrobin also was evaluated. As with azoxystrobin, leather rot isolates were more sensitive than crown rot isolates to pyraclostrobin, but this difference was not as clear as that with azoxystrobin. This discrepancy between results with the two fungicides may be due to the small sample size used to test sensitivity to pyraclostrobin. However, the results obtained with pyraclostrobin tested at 1 µg/ml appear to confirm that crown rot and leather rot isolates respond differently to QoI fungicides. Whether this difference in sensitivity between groups of isolates has implications from a practical standpoint is not clear, but it may mean that higher rates of QoI fungicides will be needed to manage crown rot compared with those needed for leather rot in the field.

In general, isolates of *P. cactorum* from strawberry plants were more sensitive to pyraclostrobin than to azoxystrobin based on relative growth of mycelia at a single dose of 1 µg/ml and estimated ED₅₀ values. Pyraclostrobin also had more activity than azoxystrobin against zoospore germination. Similar observations in laboratory studies have been reported elsewhere (29,33,50). However, differences in activity between these two fungicides in vitro do not necessarily reflect potential differences in their performance under field conditions. Moderate but highly significant correlation between the effects of pyraclostrobin and azoxystrobin (at 1 µg/ml) on relative growth of mycelia was observed. This correlation was more significant when ED₅₀ values were used. Correlation between the two fungicides was expected because both chemistries share the same mode of action and are classified in the same cross-resistance group (6). Wong and Wilcox (49) found similar correlations

Fig. 8. Relationship between the effective doses of pyraclostrobin and azoxystrobin needed to reduce mycelium growth by 50% (ED₅₀) for 26 selected isolates of *Phytophthora cactorum*. ED₅₀ values were estimated by fitting the log-logistic model to data of relative mycelium growth, calculated as the ratio of radial mycelium growth on media amended with each fungicide tested media at 0.001, 0.01, 0.1, 1, 10, and 30 µg/ml and nonamended media (control); *r* is the correlation value for each relationship.

between the effects of azoxystrobin and pyraclostrobin on *Uncinula necator* on grape.

For *P. cactorum*, sensitivities of mycelia were not strongly correlated with sensitivities of zoospores to these fungicides—at least under the conditions used in this study. This lack of correlation probably is due to the intrinsic physiological and morphological differences between these two life stages (11), and the ability of mycelia of *P. cactorum* to counteract the effect of QoI fungicides through the use of an alternative respiration pathway in vitro. For diseases caused by oomycetes, the propagule most commonly responsible for infection is the zoospore (11). Our results demonstrate that zoospores are more sensitive than mycelia to QoI fungicides and, therefore, may be a better indicator of the sensitivity of *P. cactorum* to these fungicides. In addition, assays with zoospores do not require the use of SHAM to counteract the alternative respiration pathway. Consequently, zoospores should be more effective in evaluating sensitivity to QoI fungicides in future studies and should be useful for monitoring development of resistance in isolates of *P. cactorum* that attack strawberry plants. In fact, the data collected in this study may be used as a measure of baseline sensitivity because many of the isolates evaluated were collected before QoI fungicides were used in commercial fields.

Our results also demonstrate that isolates of *P. cactorum* recovered from different plant organs exhibit a differential response to QoI fungicides (i.e., isolates from roots and crowns tend to tolerate a relatively higher fungicide concentration compared with those recovered from fruit). More importantly, based on previous reports of sensitivities to QoI fungicides in other pathosystems (29,30,31,49), the ED₅₀ values determined in this study indicate that the isolates of *P. cactorum* used here do not show insensitivity to QoI fungicides. Based on our results with regression analysis, it should be possible to evaluate shifts in fungicide sensitivity based on using a discriminatory dose of azoxystrobin at 0.05 µg/ml in a zoospore germination assay.

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