A Rapid Resazurin-Based Microtiter Assay to Evaluate QoI Sensitivity for *Alternaria alternata* Isolates and Their Molecular Characterization

Byron Vega, Citrus Research and Education Center, University of Florida, Lake Alfred; **Daniele Liberti** and **Philip F. Harmon**, Department of Plant Pathology, University of Florida, Gainesville; and **Megan M. Dewdney**, Citrus Research and Education Center, University of Florida, Lake Alfred

Abstract

Vega, B., Liberti, D., Harmon, P. F., and Dewdney, M. M. 2012. A rapid resazurin-based microtiter assay to evaluate QoI sensitivity for *Alternaria alternata* isolates and their molecular characterization. Plant Dis. 96:1262-1270.

Chemical management of *Alternaria* brown spot of citrus is based upon the timely application of site-specific fungicides, many of which are vulnerable to the development of fungicide resistance. A rapid microtiter bioassay based on the colorimetric changes of resazurin (RZ) dye was developed to evaluate the sensitivity of *Alternaria alternata* to quinone outside inhibitor (QoI) fungicides. Four liquid media (complete medium, minimal medium, potato dextrose broth, and yeast peptone dextrose broth), five conidia concentrations (from 10^1 to 10^5 conidia/ml), and five RZ concentrations (10, 20, 30, 40, and 50 μ M) were evaluated. Complete medium at 10^5 conidia/ml and 40 μ M RZ were

identified as optimal for measuring RZ reduction. The effective concentration of two QoI fungicides (azoxystrobin and pyraclostrobin) needed to reduce RZ by 50% (EC_{50}) was calculated and compared with those obtained from conidia germination tests on fungicide-amended media. Concordant EC_{50} values were observed ($R^2 = 0.923$; $P < 0.0001$) from both methods. Resistant phenotypes were further characterized by the partial sequencing of the cytochrome *b* gene. Genetic variability associated with the presence or absence of two introns was observed among isolates. The identified resistant isolates had the amino acid substitution G143A, typical of QoI resistance in other fungi.

Alternaria brown spot (ABS) is one of the most important foliar diseases of tangerine (*Citrus reticulata* Blanco) and tangerine hybrids, affecting yield and fruit quality of high-value fresh fruit cultivars in Florida, such as 'Murcott' tangor, 'Sunburst' tangerine, and 'Minneola' tangelo (42,43). ABS is caused by *Alternaria alternata* (Fr.) Keissl. tangerine pathotype that produces the host-specific ACT toxin (25,42). The fungus infects leaves, twigs, and young fruit, producing small brown to black lesions that become surrounded by a yellow halo (14,60). Fruit are normally susceptible in Florida from petal fall until they are 4 cm in diameter but more susceptible cultivars can be infected up to 5 to 6 cm (11). Fruit abscission occurs with high disease pressure, resulting in reduced yield and diminished marketability (1,60).

The primary tool used to control ABS is fungicide applications. Currently, copper and the quinone outside inhibitor (QoI) or strobilurin fungicides, such as azoxystrobin and pyraclostrobin, are used widely in Florida to control ABS (14). Due to the multi-site activity of copper fungicides, there is no evidence of fungal resistance; however, phytotoxicity caused by applications at higher temperatures limits its use (14). Also, copper is a protectant fungicide that frequently needs to be applied due to a rapid expansion of young fruit and leaf flushes. The QoI fungicides block electron transport by binding to the Qo site of the mitochondrial cytochrome *bc_L* complex. The specific mode of action has an increased risk of resistance development compared with multi-site fungicides (5,6,65). Many cases of QoI resistance have been reported in different plant pathogens (8,40,48,49,58). Recently, *A. alternata* isolates recovered from Florida tangerine groves with a history of

QoI use were found to be insensitive to QoI fungicides, indicating the development of resistant populations (35). A single point mutation of the cytochrome *b* gene, leading to an amino acid substitution that prevents fungicide binding at the target site, has been reported as the primary mechanism of QoI resistance in fungi (6,65). The most common mutation results in the replacement of glycine by alanine at amino acid position 143 (G143A) and confers a high level of resistance. A second, lower-frequency amino acid replacement has been found at position 129 with the substitution of phenylalanine with leucine (F129L), causing a reduction of QoI sensitivity (3,6,26,39,48). A third change from glycine to arginine was identified at amino acid position 137 (G137R). This mutation occurs at a very low frequency and confers a level of resistance similar to the F129L mutation (51).

Traditional techniques for measuring fungicide sensitivity use fungicide-amended agar to characterize inhibition of either mycelial growth or conidia germination (50). These techniques are laborious and time consuming, require considerable space for plates, and the growth inhibition measurements can be subjective. Several automated quantitative methods have been developed for biocidal efficacy in the biomedical field but their implementation has been limited by the requirement of high-cost specialized equipment. These methods include bioluminescence and colorimetric tests using reagents that could be toxic to the user and cells of interest (44,62). In contrast, resazurin (RZ) is a nontoxic redox dye that has been used extensively to test bacteria and fungi in chemical sensitivity assays (31,44,61). RZ is a stable, water-soluble, tetrazolium-based dye where the oxidized, nonfluorescent form (blue) is reduced to a fluorescent form (pink) in response to cell metabolism (16,28,38). Therefore, the fluorescence and color properties of this dye can be measured fluorometrically or spectrophotometrically. RZ reduction takes place in the cytochrome oxidase region, during the final reduction of O_2 and the cytochrome *a₃* complex (23).

Several molecular techniques have also been developed for the rapid detection of QoI-resistant genotypes (52). These techniques include the use of polymerase chain reaction (PCR)-based technologies, such as cleavable amplified polymorphic sequence (CAPS)-PCR, quantitative PCR, or pyrosequencing, among others (17,20,51). The allele-specific PCR or CAPS-PCR techniques are

Corresponding author: M. M. Dewdney, E-mail: mmdewdney@ufl.edu

*The e-Xtra logo stands for "electronic extra" and indicates that three supplementary figures are available online, and that Figures 2 and 3 appear in color online.

Accepted for publication 28 February 2012.

<http://dx.doi.org/10.1094/PDIS-12-11-1037-RE>
© 2012 The American Phytopathological Society

straightforward, versatile, and low cost, and do not require specialized PCR equipment to identify QoI-resistant mutants in a fungal population.

Detection of fungicide resistance based on PCR technology only detects the genotypes that the primers are designed to detect. A major disadvantage to using only a PCR-based technique is the inability to detect other causes of fungicide resistance. For example, it would not be possible to detect the F129L mutation that confers QoI resistance (39) with primers designed for the G143A mutation (29) even though both cause a resistant phenotype. There can also be silent mutations or other DNA features that could interfere with a PCR assay that do not change the phenotype. Thus, although PCR identification is an excellent tool, it is necessary to rule out false negatives. This is especially important when providing management information to producers who will change their practices based on results from an assay. These are the reasons it was chosen to conduct a phenotypic assay that can detect all genotypes and has fewer risks of false-negative results with confirmatory PCR assays to determine what mutations are important in the Florida citrus industry.

Due to the importance of ABS to Florida tangerine production and the need for a rapid, accurate method to detect the QoI sensitivity for a statewide survey of the *A. alternata* population; the purpose of this study was to develop a rapid assay for QoI fungicide sensitivity. The specific aims of this research were to (i) identify an optimal chemically defined medium for the microtiter assay, (ii) standardize both conidia and RZ concentrations, (iii) correlate the microtiter assay with the conidia germination assay, and (iv) identify point mutations conferring the resistant phenotype.

Materials and Methods

Fungal isolates. Twelve monoconidial isolates of *A. alternata* were used in this study, varying in QoI sensitivity from baseline to field sensitive and field resistant (Table 1). Isolates were collected from different citrus orchards in Florida and cultured on potato dextrose agar (PDA; Becton Dickinson, Sparks, MD). Isolates were stored at -20°C on colonized filter paper (41). Conidial production was induced by transferring 3-day-old mycelia on PDA to clarified V8 agar (100 ml of centrifuged V8 juice, 20 g of agar, and 10 g of CaCO_3 /liter). Cultures were incubated at room temperature under white fluorescent light for 8 to 12 days. Conidia were harvested by flooding the plate with sterile distilled water, gently rubbing the surface, and filtering through three layers of sterile cheesecloth.

Medium evaluation at different conidia and RZ concentrations. Six *A. alternata* isolates at five conidial concentrations of 10^1 to 10^5 conidia/ml were evaluated (Table 1). Four liquid media were used: potato dextrose broth (PDB; Becton Dickinson), yeast peptone dextrose broth (YPD; Becton Dickinson), complete medium (CM; 7), and minimal medium (MM; 7). RZ salt (Sigma-Aldrich, St. Louis) was dissolved in distilled water at 5 mM, filter sterilized using 0.22- μm filters (Corning Inc., Lowell, MA), and

stored at 4°C until use. To determine the optimal concentration of RZ, stock solutions of 100, 200, 300, 400, and 500 μM RZ were prepared. The various combinations of liquid media, conidia, and RZ concentrations were evaluated for RZ reduction in the microtiter assay. To obtain a final volume of 200 μl , 100 μl of media were added to each well with 80 μl of a conidia suspension at the concentrations described above, followed by 20 μl of RZ solution to obtain a final RZ concentration of 10, 20, 30, 40, or 50 μM . Each treatment was loaded in triplicate into a 96-well clear, flat-bottom, polystyrene microplate (Corning Inc.). Each plate included wells of media alone and media with RZ as controls. Plates were covered with a plastic lid, placed into a clean plastic humid chamber, and incubated at 28°C for 24 h with shaking at 400 rpm. RZ reduction was calculated based on the absorbance reading at 570 and 600 nm. Absorbance was measured with an Emax precision microplate reader (Molecular Devices Corp., Sunnyvale, CA). The experiment was performed twice.

Effect of salicylhydroxamic acid (SHAM) on *Alternaria alternata* growth in the RZ-based microtiter assay. To determine the effect of SHAM on the growth of *A. alternata*, 12 isolates were evaluated (Table 1). SHAM was dissolved in methanol at 100 mg/ml. All media contained 0.1% (vol/vol) methanol. CM (100 μl) amended with SHAM at either 0, 50, 100, 150, 200, 300, or 400 $\mu\text{g/ml}$ was added to each well, followed by 80 μl of 10^5 conidia/ml and 20 μl of 400 μM RZ. Final SHAM concentration in the wells was 0, 25, 50, 75, 100, 150, or 200 $\mu\text{g/ml}$. Three wells per plate were used for each treatment. Control wells of media and RZ without conidia were included for each SHAM concentration. Incubation conditions as well as absorbance measurement were as described above. The experiment was performed twice.

RZ-based microtiter assay of QoI fungicide sensitivity. A microtiter assay was developed based on percent reduction of RZ in QoI fungicide-amended media. Technical-grade azoxystrobin (Syngenta Crop Protection, Greensboro, NC) and pyraclostrobin (BASF Corp., Research Triangle Park, NC) were dissolved in acetone at 10 mg/ml (active ingredient) and 10-fold serially diluted to create stock solutions, such that the final concentration of acetone in the media was 0.1% (vol/vol). Three wells of the microtiter plate contained 100 μl of CM amended with fungicide at 0, 0.002, 0.02, 0.1, 0.2, 1, 2, or 20 $\mu\text{g/ml}$. Then, 80 μl of 10^5 conidia/ml and 20 μl of 400 μM RZ were added. The final concentrations of the fungicides in the wells were 0, 0.001, 0.01, 0.05, 0.1, 0.5, 1, or 10 $\mu\text{g/ml}$. Incubation conditions as well as absorbance measurements were as described above. Relative percent RZ reduction for two independent experiments was expressed as the ratio of percent RZ reduction in the presence of fungicide to the percent in the absence of fungicide multiplied by 100. Isolate sensitivity expressed in micrograms per milliliter was determined by the effective concentration needed to reduce RZ by 50% (EC_{50}) using an exponential decay function (53):

$$RZ(lc) = ae^{-blc} \quad (1)$$

Table 1. Isolates by host, location, and year of collection

Isolate ^a	Abbreviation	Host ^b	Location	Sensitivity ^c	Year
BL-17-4-3S*	BL	Minneola tangelo	Lake Alfred	S	2008
BLK-17-S-10S*	BLK	Minneola tangelo	Lake Alfred	S	2008
CPI-ORI-2S	CPI	Murcott tangor	Immokalee	BL	1996
EV-3-1S	EV	Minneola tangelo	Lake Alfred	BL	1996
LJ-1-8-2S*	LJ1-8	Minneola tangelo	Oviedo	R	2009
LJ-3-3-2S	LJ3-3	Minneola tangelo	Oviedo	R	2009
LOR-ORI-1-3S	LOR	Sunburst tangerine	Lorida	BL	1997
R3Q26-F9-2S*	R3Q26	Dancy mandarin	Winter Haven	R	2008
R3Q33-1-1S*	R3Q33	Dancy mandarin	Winter Haven	R	2008
VB-RQF-3S*	VB	Grapefruit	Vero Beach	BL	1997
WM-1-2S	WM1-2	Minneola tangelo	Oviedo	R	2009
WM-1-3S	WM1-3	Minneola tangelo	Oviedo	R	2009

^a Asterisk (*) indicates isolates used to determine percent resazurin reduction for different media, conidia and resazurin concentrations.

^b Tangelo = *Citrus paradisi* $\times$ *C. reticulata*, mandarin or tangerine = *C. reticulata*, and grapefruit = *C. paradise*.

^c S = field sensitive, BL = baseline, and R = field resistant.

where $RZ(lc)$ = percent RZ reduction, lc = fungicide concentration, a = constant at initial lc value, and b = slope coefficient.

RZ reduction. Percent RZ reduction was calculated using the equation following the manufacturer's instructions (19,44):

$$\frac{(\epsilon_{OX})\lambda_2 A\lambda_1 - (\epsilon_{OX})\lambda_1 A\lambda_2}{(\epsilon_{RED})\lambda_1 A'\lambda_2 - (\epsilon_{RED})\lambda_2 A'\lambda_1} \times 100 \quad (2)$$

where ϵ_{OX} = molar extinction coefficient of RZ oxidized form (blue; 80,586 and 117,216 for 570 and 600 nm, respectively); ϵ_{RED} = molar extinction coefficient of RZ reduced form (pink; 155,677 and 14,652 for 570 and 600 nm, respectively); A = absorbance of test wells; A' = absorbance of negative control wells; λ_1 = 570 nm; and λ_2 = 600 nm.

Because RZ measures the metabolic activity of fungal growth, the percent RZ reduction corresponds to the fungal respiration rate.

Conidia germination test. The sensitivity of germinating *A. alternata* conidia to QoI fungicides also was determined for the isolates previously described. Stock solutions of azoxystrobin or pyraclostrobin were added to molten water agar (WA) to obtain the final concentrations described above. To suppress the alternative oxidase pathway during spore germination, SHAM (100 µg/ml) was added to WA containing fungicide (29,50). A 100-µl aliquot of 5×10^3 conidia/ml was spread onto WA in each petri dish and incubated in the dark at 27°C overnight. Fifty conidia at each concentration were arbitrarily selected in four replicates. A conidium was considered germinated if the germ tube was equal to or longer than the total length of a conidium, if an appressorium was developed or if multiple germ tubes were observed. The average number of conidia germinated for two independent experiments was converted to percent inhibition. EC_{50} values were determined by the concentration that inhibited conidia germination by 50% using the sigmoidal function (53):

$$I(lc) = \frac{a}{1 + e^{-(lc-c/b)}} \quad (3)$$

where $I(lc)$ = percent germination inhibition, a = percent germination inhibition range, c = fungicide concentration at the maximum rate of change, b = slope coefficient, and lc as defined in equation 1.

DNA extraction and sequence analysis. Mycelia were harvested from 2- to 8-day-old cultures growing on PDA overlaid with cellophane sheets, and ground in liquid nitrogen. Genomic DNA extraction was performed using the DNeasy Plant Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The cytochrome *b* gene of 10 *A. alternata* isolates was partially amplified using the primers DTRcytb2 (5'-CTAGTATGAACTATTGGTAC-3') and DTRcytb2r (5'-GAGCAAAAGATATTCTTT-3') in a total reaction volume of 25 µl (18). Because isolates CPI-ORI-2S and WM-1-2S were not amplified by DTRcytb2/DTRcytb2r, a new set of primers, cytb2f (5'-CTATGGATCTTACAGAGCAC-3') and cytb3r (5'-TGCAGGAGTTTGCATAGGGTTTGC-3'), were designed. The PCR reaction was performed using primers at 0.5 µM final concentration. The REDExtract-N-Amp PCR ready mix (Sigma-Aldrich) was used following the manufacturer's directions. The quantity of extracted fungal DNA template varied from 5 to 50 ng. Cycling parameters for all primer sets used were initial denaturation at 94°C for 5 min; 35 cycles at 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min; with final extension at 72°C for 5 min. The amplification products were visualized with UV light after electrophoresis in a 1.5% agarose gel with 1× Tris-borate-EDTA buffer stained with ethidium bromide.

PCR products were purified using the MiniElute PCR purification kit (Qiagen) and cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI). Plasmids were transformed into *Escherichia coli* high-efficiency competent cells JM109 (Promega Corp.) according to the manufacturer's directions. Both strands of the cloned PCR products were sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida). New primers, cytb2f-walk (5'-AATGAAATATAAATTATTGGTTACA-

3') and cytb2r-walk (5'-GAAGGAGGTATGGATAAATATTC-3'), based on the partial sequence of the cytochrome *b* gene of CPI-ORI-2S and WM-1-2S isolates, were designed to amplify the rest of the sequence. The partial sequence of the cytochrome *b* gene for two representative isolates was submitted to GenBank (accession numbers JQ437357 and JQ446323).

DNA sequences were aligned and manually edited using Sequencher (ver. 4.5; Gene Codes Co., Ann Arbor, MI). Multiple DNA sequence alignments and translation to amino acids were performed using MEGA (ver. 4.1; 59).

Using the DTRcytb2/DTRcytb2r partial sequence of the cytochrome *b* gene, an additional gene-specific reverse primer, DTRcytb2-INTr (5'-GTATGTAACCGTCTCCGTC-3'), was designed and used in combination with the DTRcytb2 forward primer to amplify a 349-bp PCR fragment. PCR conditions were as described above. CAPS analysis of the DTRcytb2/DTRcytb2-INTr PCR products was performed using the *Fnu4HI* (New England Biolabs, Ipswich, MA) restriction enzyme to validate this molecular diagnostic technique and determine whether a simple molecular test could be used to confirm the mutation responsible for phenotypic results (29). Purified PCR (5 µl) product was digested with *Fnu4HI* according to the manufacturer's recommendations, and restriction fragments were separated and visualized as described above.

Statistical analysis. Analyses of the effect of medium and isolate, as well as conidia, SHAM, and RZ concentrations, were performed using PROC GLM (ver. 9.1; SAS Institute, Cary, NC). Calculation of EC_{50} and the linear regression for EC_{50} in conventional and RZ-based microtiter assays were performed with SigmaPlot (ver. 10.0; Systat Software Inc., San Jose, CA). To reduce inequality of variance among treatments, percent reduction of RZ was square root transformed prior to analysis. Standardized residual errors of transformed data plotted against predicted values were graphically analyzed for homogeneity of variance. Univariate analysis was used for testing normality. Homogeneity of variance test using the ratio of variance of two independent experiments was analyzed prior to pooling the experiments. Percent RZ reductions in different media for isolates, conidia, and RZ concentrations were compared by a three-way analysis of variance (ANOVA) with isolate, conidia, and RZ concentrations as variables. The effect of SHAM on percent RZ reduction of different isolates was analyzed by two-way ANOVA with isolate and SHAM concentration as variables. Treatment means were separated using Fisher's protected least significant difference test ($\alpha = 0.05$). EC_{50} values predicted by conidium germination and RZ microtiter tests were obtained by fitting the dose response to a sigmoidal and an exponential decay function, respectively.

Results

Effect of media and conidia concentration. The growth of six *A. alternata* isolates in the microtiter assay at five conidia concentrations was evaluated in CM, MM, PDB, and YPD at five concentrations of RZ dye. Media, isolate, conidia, and RZ concentration significantly affected ($P < 0.0001$) percent RZ reduction 24 h after treatment (*data not shown*). Furthermore, the main effects of isolate, conidia concentration, and RZ concentration as well as the interaction between conidia and RZ concentration were analyzed individually by media. Data presented in Table 2 were not pooled because the RZ doses were not identical between experiments 1 and 2; however, results shown are representative for both experiments. All main factors significantly ($P < 0.001$) affected the percent RZ reduction using CM, MM, and PDB (Table 2), with the exception of conidia concentration in YPD ($P = 0.6395$). The most consistent percent RZ reduction over 24 h was at 40 µM in CM, which ranged from 11.92% at 10^1 conidia/ml to 98.04% at 10^5 conidia/ml (Fig. 1A). Although MM showed the same RZ reduction pattern as CM at 40 µM of RZ (Fig. 1B), the percent reduction was much lower, ranging from 4.75% at 10^1 conidia/ml to 61.83% at 10^5 conidia/ml. Much higher levels of percent RZ reduction were observed in PDB, where the percent reduction increased with

conidia concentration, but the percent change was much smaller (Fig. 1C). In YPD, the lowest percent RZ reduction was 175% and did not change with conidia concentration (Fig. 1D). The highest percent RZ reduction was observed at 10^5 conidia/ml in all media, except YPD. At 10^4 conidia/ml, the percent RZ reduction increase was lower than could be considered accurate for analyses over a 24-h period of time (Fig. 1A–C). Thus, 10^5 conidia/ml and CM were selected for further work.

Effect of SHAM on *A. alternata* growth. To identify the effect of SHAM on the growth of *A. alternata* in our assay, seven SHAM concentrations were evaluated in CM. SHAM concentration, isolate, and the isolate–SHAM concentration interaction significantly ($P < 0.0001$) affected the fungal respiration rate (Fig. 2A). However, whether the isolates were QoI sensitive or resistant did not

significantly ($P = 0.2024$) affect the fungal-respiration response (data not shown). An inverse relationship was found between fungal-respiration rate and SHAM concentration ($R^2 = 0.9861$; $P < 0.0001$). SHAM at 25 $\mu\text{g/ml}$ decreased percent RZ reduction from 96.36 to 91.92% when compared with the nonamended control, whereas, at the highest SHAM concentration, the percent RZ reduction was 52% (Supplementary Figure 1). RZ reduction indicated that *A. alternata* grew but growth was significantly reduced at higher SHAM concentrations in an isolate-dependent manner (Fig. 2B).

Assessment of sensitivity to azoxystrobin and pyraclostrobin. Two methods were used to assess QoI sensitivity: the RZ microtiter assay and the conidia germination assay. Because SHAM affected fungal growth in a dose-dependent response in the RZ microtiter

Table 2. *F* statistics and *P* values for the media effect on the growth of six *Alternaria alternata* isolates measured by percent resazurin reduction under different conidia and resazurin concentrations

Factor ^a	df ^b	Complete medium		Minimal medium		Potato dextrose broth		Yeast peptone dextrose	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Isolate	5	4.33	0.0008	208.72	<0.0001	118	<0.0001	42.05	<0.0001
Conidia	4	1,572.44	<0.0001	14,785.09	<0.0001	559.13	<0.0001	0.63	0.6395
Resazurin	4	77.76	<0.0001	210.46	<0.0001	1,080.73	<0.0001	330.54	<0.0001
Conidia $\times$ Resazurin	16	11.18	<0.0001	4.8	<0.0001	1.68	0.0471	0.33	0.9934

^a Data from the second experiment.

^b Degrees of freedom.

Fig. 1. Resazurin reduction in four liquid media influenced by *Alternaria alternata* conidial concentration and resazurin concentration. **A**, Complete medium; **B**, minimal medium; **C**, potato dextrose broth; and **D**, yeast peptone dextrose. Values are the mean and standard error of six isolates. In C and D, some values exceed 100% resazurin reduction caused by the formation of a colorless compound during the microtiter assay.

assay, it was not included. However, SHAM at 100 µg/ml was used for the conidia germination test to avoid false positive results from respiration by the alternative oxidase pathway (*data not shown*; 33). All of the evaluated isolates were classified into two groups. One group, designated “sensitive”, had EC_{50} values less than 0.80 µg/ml for azoxystrobin and less than 0.02 µg/ml for pyraclostrobin for either method used. The “resistant” isolates had EC_{50} values greater than 10 and 1 µg/ml for azoxystrobin and pyraclostrobin, respectively (Table 3). Highly significant differences ($P < 0.0001$) were found for isolate when either QoI fungicide was evaluated, whereas no significant differences ($P = 0.4724$) were observed for evaluation method and for the interaction between isolate and method ($P = 0.9616$) (Table 4). Sensitive *A. alternata* isolates had EC_{50} values of 0.056 to 0.502 µg/ml for azoxystrobin in the conidia germination test and 0.069 to 0.761 µg/ml in the RZ-based microtiter assay. Resistant isolates had EC_{50} values greater than 10 µg/ml in both methods (Table 3). *A. alternata* isolates were more sensitive to pyraclostrobin than azoxystrobin. For sensitive isolates, the pyraclostrobin EC_{50} values were 0.001 to 0.012 µg/ml and 0.007 to 0.019 µg/ml for the conidia germination test and the RZ microtiter assay, respectively. For resistant isolates, EC_{50} values were 4.006 to 5.228 µg/ml in the conidia germination test and 1.094 to 4.49 µg/ml in the microtiter assay (Table 3). Discrimination between isolate groups could also be visually evaluated by the color change

Fig. 2. A, Effect of salicylhydroxamic acid (SHAM) concentration on *Alternaria alternata* respiration as measured by percent resazurin reduction by isolates. Columns are the mean and error bars are the standard error of three replicates for four representative SHAM concentrations. Effects of SHAM concentration on isolates with the same letter are not significantly different according to the Fisher’s protected least significant difference test at $\alpha = 0.05$. **B,** Colorimetric reaction of resazurin dye showing the effect of SHAM on different isolates of *A. alternata*.

of test wells in the microtiter plate. Isolates that developed a strong pink color in wells with a higher concentration of azoxystrobin (10 µg/ml) were considered resistant whereas isolates in blue-colored wells at 0.5 µg/ml were considered sensitive (Fig. 3). A strong and highly significant relationship ($R^2 = 0.923$; $P < 0.0001$) between EC_{50} values from both methods was observed (Fig. 4).

Cytochrome *b* partial gene sequence analysis. The partial structure of the cytochrome *b* gene in the fragment containing the first “hot spot” region (amino acid positions 120 to 160) was characterized in the 12 isolates. The term hot spot refers to the cytochrome *b* region where amino acid exchange inducing mutations occurs, leading to QoI fungicide resistance (18). A 1,589-bp fragment, encoding for the amino acid residues 112 to 223, was amplified using primers DTRcytb2 and DTRcytb2r. A 2,938-bp fragment, encoding for the amino acid residues 103 to 268, was amplified using primers cytb2f and cytb3r. The length and intron positions of the partial sequence of the cytochrome *b* gene was determined after alignment with the reference cytochrome sequence of *A. alternata* (accession number DQ209283.1). The first intron of 1,187 bp was inserted after the triplet encoding for alanine at amino acid position 126 (A126) and was found only in isolates WM-1-2S (resistant) and CPI-ORI-2S (sensitive). The second intron of 1,252 bp was inserted after the first T of the triplet encoding for phenylalanine (TTC) at position 164 (F164) and was found in all evaluated isolates (Fig. 5). Four silent, transition mutations were found only in isolates carrying both introns at amino acid positions L134, P135, and Y136. An expected cytochrome *b* point mutation was identified in all QoI-resistant isolates. Protein comparisons of the partial cytochrome *b* coding region between isolates identified a glycine (GGT) to alanine (GCT) nonsynonymous point mutation at amino acid position 143 (G143A), which distinguishes sensitive from resistant isolates (Supplementary Figure 2).

CAPS analysis. Using the CAPS technique, it was possible to discriminate between resistant and sensitive isolates with the G143A mutation that carried only one intron and, therefore, could not discriminate among all isolates (Supplementary Figure 3). A 349-bp cytochrome *b* fragment was amplified using primers DTRcytb2 and DTRcytb2-INtr in sensitive isolates but two fragments of 95 and 254 bp were observed only in resistant isolates (Fig. 6).

Discussion

In this study, we compared a microtiter assay using the oxidation-reduction dye RZ with the standard conidia germination method to evaluate QoI-fungicide sensitivity in *A. alternata*. The standardization of media, conidia concentration, and incubation time is critical for the reproducibility of any microtiter assay (15,31). Culture media not only must have enough nutrients to

Table 3. Effective concentration needed to reduce resazurin or conidia germination by 50% (EC_{50}) for azoxystrobin and pyraclostrobin by isolate

Isolate	EC_{50} (µg/ml) ^a			
	Azoxystrobin		Pyraclostrobin	
	GT	RZ	GT	RZ
BL-17-4-3S	0.056	0.692	0.001	0.011
BLK-17-S-10S	0.502	0.385	0.012	0.019
CPI-ORI-2S	0.060	0.069	0.004	0.013
EV-3-1S	0.071	0.761	0.007	0.015
LJ-1-8-2S	>10	>10	5.214	3.142
LJ-3-3-2S	>10	>10	4.542	2.701
LOR-ORI-1-3S	0.254	0.594	0.004	0.007
R3Q26-F9-2S	>10	>10	5.162	1.094
R3Q33-1-1S	>10	>10	4.006	4.490
VB-RQF-3S	0.064	0.346	0.011	0.013
WM-1-2S	>10	>10	4.246	4.693
WM-1-3S	>10	>10	5.228	2.072

^a Average of two independent experiments. GT = germ tube assay and RZ = resazurin assay.

ensure the growth of isolates but also must prevent the activation of alternative pathways for energy production in the fungus, such as alternative respiration or anaerobic fermentation (54). Furthermore, some compounds present in the media can affect the solubility and stability of fungicides (45). It is highly recommended to use a chemically defined medium or synthetic medium for sensitive bioassays and avoid the use of undefined media, such as a natural media, that can give variable results (13,27). Thus, many synthetic media have been evaluated in medicine for detection of multidrug resistance in human disease-causing filamentous fungi and yeasts. The National Committee for Clinical Laboratory Standard has published a standard protocol for filamentous fungi and yeasts (36,37). However the diversity of fungi found in agricultural crops as well as the many registered fungicides makes the selection of a specific media for fungicide sensitivity assays difficult. Thus, an optimal medium must be evaluated prior to a bioassay for each fungal species.

Conidia germination and *A. alternata* growth in CM were optimal compared with MM, PDB, and YPD; thus, CM was chosen for our experiments. Although MM allowed germination and growth as well, their levels were suboptimal and lower values of RZ reduction were observed over a 24 h incubation period. Nutrient requirements for *A. alternata* growth are quite simple: glucose source (0.2 to 4%), nitrate salts (less than 1%), and an optimum carbon/nitrogen ratio (32); nevertheless, the addition of microelements, vitamins, and an extra nitrate source, present in CM, adequately supported growth of *A. alternata* during the first 24 h of the microtiter assay. CM also was pH stable post-sterilization, which ensured optimal RZ performance because RZ is pH sensitive (46). On the other hand, both PDB and YPD showed inconsistent RZ reduction levels produced by post-sterilization media acidification that caused RZ to become a colorless compound (9,28). Thus, media that develop a low pH post sterilization, such as PDB and YPD, should be avoided in RZ-based microtiter assays. However, media based on yeast extract and peptone have been used successfully in similar bioassays (16,55,56).

Conidia concentration is also an important variable to be considered in a microtiter assay. Cox et al. (12) and Rampersad (47) found a strong correlation between RZ reduction and conidia concentration in *Monilinia fructicola* and *Verticillium dahliae*, respectively, testing concentrations from 10^1 to 10^6 conidia/ml, with optimal final concentrations between 5×10^4 and 1×10^5 conidia/ml per microtiter well. In our study, the greatest percent RZ reduction was obtained with 10^5 conidia/ml that resulted in a final concentration of 5×10^4 conidia/ml for each microtiter well, which is consistent with previous reports (12,47). However, with a high cell density or prolonged incubation time, the RZ reduction reaches a plateau, caused by a second redox step, followed by a diminution of RZ fluorescence, where the RZ (blue color) is reduced first to resorufin (pink color) and, finally, to colorless hydroresorufin (10,28). A diminution of RZ fluorescence was observed in our study when incubation was extended to 48 h. Here, the presence of a colorless compound was clearly observed, especially when conidia concentration was greater than 10^4 conidia/ml (*unpublished data*). For an optimal result with the microtiter assay, RZ reduction must be measured during the logarithmic growth phase of the fungus (47). In our experiment, this phase occurred 24 h after the

incubation. Shorter incubation times gave suboptimal levels of RZ reduction and an underestimation of the dye-response curve (*unpublished data*).

EC₅₀ values obtained with the microtiter assay and the conidia germination test were not significantly different for the QoI fungicides evaluated. The conidia germination test estimates only the proportion of conidia germinated after exposure to specific fungicide concentrations, whereas the microtiter assay measures the metabolic activity of fungal growth during conidial germination and germ tube elongation. EC₅₀ values obtained by RZ-based microtiter assay also were consistent with values obtained in QoI

Fig. 3. Dose response of *Alternaria alternata* isolates to azoxystrobin based on resazurin microtiter assay. Columns 1–6 and 7–12 showed the typical resazurin reduction for sensitive or resistant isolates, respectively.

Fig. 4. Relationship between azoxystrobin and pyraclostrobin sensitivity (effective concentration needed to reduce fungal growth by 50% [EC₅₀] values as determined by the resazurin-based microtiter assay and the conidial germination assay. Circles represent azoxystrobin and stars represent pyraclostrobin.

Table 4. Analysis of variance for effective concentration needed to reduce resazurin or conidium germination by 50% (EC₅₀ values) for isolate

Factor ^a	df	SS	MS	F value	P value
Isolate	11	1,075.73	97.79	24.57	<0.0001
Fungicide ^b	1	247.65	247.65	62.21	<0.0001
Method ^c	1	2.08	2.08	0.52	0.4724
Isolate × Method	11	16.42	1.49	0.37	0.9616
Error	70	278.66	3.98	...	...
Total	94	1,634.89	...	...	...

^a Data were pooled from two independent experiments.

^b Azoxystrobin and pyraclostrobin.

^c Resazurin-based microtiter assay and conidia germination assay.

sensitivity tests for pistachio-infecting *A. alternata* and potato-infecting *A. solani* (2,24,39,40,48,49). Thus, an RZ-based microtiter assay can determine the sensitivity of field isolates to QoI fungicides. To date, no correlation has been made between the results of the microtiter assay and field control failures but this work is on-going and will be presented subsequently.

To monitor the QoI sensitivity of citrus-infecting *A. alternata* isolates over time, the baseline sensitivity to azoxystrobin and pyraclostrobin must be identified because it is unclear thus far. When Mondal et al. (34) conducted a baseline sensitivity study for *A. alternata* in citrus, they found EC₅₀ values greater than 100 µg/ml for azoxystrobin and 0.87 µg/ml for pyraclostrobin, based on mycelial growth inhibition tests, which demonstrated that *A. alternata* mycelium is insensitive to azoxystrobin. Moreover, in our study, we found mean EC₅₀ values of 0.443 µg/ml for azoxystrobin and 0.012 µg/ml for pyraclostrobin in isolates never exposed to QoI. The presence of resistant isolates also was confirmed in our study, because the mean EC₅₀ values between QoI-sensitive and -resistant isolates shifted more than 100-fold, which corresponded with values previously reported for different pathogens (40,48,57,58).

Azoxystrobin and pyraclostrobin are broad-spectrum fungicides that inhibit mitochondrial respiration by blocking the electron transfer in the *bc₁* cytochrome complex (5,6,65). Several fungal plant pathogens can avoid the deleterious effects of mitochondrial inhibitors through alternative oxidase (AOX; 21,22,66). The AOX reaction takes place within the inner mitochondrial membrane and involves a nuclear-encoded protein (64). This protein is sensitive to SHAM and *n*-propyl gallate (21). SHAM prevents AOX from masking QoI sensitivity in assays during saprophytic stages of mycelium growth or conidia germination. Avila-Adame and Köller (3) reported an isolate-dependent inhibition of conidia germination in *Magnaporthe grisea* of 3 to 14% in response to 100 µg/ml of SHAM. They also found a rescue factor (ratio between EC₅₀ values without SHAM and with SHAM for the same isolate) from azoxystrobin, at the same concentration of SHAM, of 7 to 140 for mycelia growth and greater than 580 for conidia germination. Thus, SHAM has a developmental regulation effect where conidia germination has shown a higher rescue potential than mycelia growth. In our study, we found an isolate-dependent dose response on fungal respiration caused by SHAM. The level of RZ reduction was 5 to 45% for SHAM at 25 and 200 µg/ml, respectively. This response was independent of whether the isolates were sensitive or resistant to azoxystrobin or pyraclostrobin; therefore, SHAM was not used for the microtiter assay.

The RZ microtiter technique cannot be used for fungi that do not readily sporulate in culture or fungi that are not able to grow in liquid media (63). However, it has several advantages over traditional assays. The time needed to measure the RZ test is reduced to less than 30 min for a group of 12 isolates after 24 h of incubation,

compared with conventional conidia germination tests that required between 45 min and 1 h per isolate after incubation. Another advantage is the evaluation of multiple isolates and multiple fungicide concentrations at the same time in a conventional microtiter plate. Furthermore, different platforms such as a spectrophotometer and fluorometer could be used to measure RZ reduction (based on the light absorbance and fluorescence properties of RZ) in response to the metabolic activity of the fungus (47).

RZ-based microtiter assays and conidia germination tests are methods developed to quantify fungicide sensitivity at a phenotypic level. To identify the genes responsible for resistant phenotypes, DNA-based methods must be used. The specific primers previously described (18) for the cytochrome *b* gene of *A. alternata* failed to amplify the gene of two isolates (one susceptible and one resistant), which required the development of a new set of primers. The presence of two introns, the first at amino acid position 126 (A126) and the second at amino acid position 164 (F164), was unexpected, because it had been reported that the *A. alternata* cytochrome *b* gene did not have any introns (18,29,30). Grasso et al. (18) reported that the presence of an intron between the codons 143 and 144 may prevent a QoI-resistant mutation at this position. However, the introns A126 and F164 in *A. alternata* do not appear to affect QoI resistance (4,18). Moreover, the gene structure of the cytochrome *b* was found to be diverse among isolates, due to the presence of introns, although the coding regions of the gene were highly conserved.

In our study, the amino acid substitution G143A was correlated with an azoxystrobin- and pyraclostrobin-resistant phenotype in *A. alternata*. This mutation was reported in other plant pathogen spe-

Fig. 6. Cleavable amplified polymorphic sequence analysis of the cytochrome *b* gene. The restriction enzyme *Fnu*4HI produced two fragments of 254 and 95 bp in resistant isolates. Fragments are indicated by arrows. Sizes of molecular mass standard are indicated to the left of the panel (100-bp marker; Promega Corp.). Lane 1 = EV-3-1S; lane 2 = VB-RQF-3S; lane 3 = LOR-ORI-1-3S; lane 4 = BL-17-4-3S; lane 5 = BLK-17-5-10S; lane 6 = LJ-1-8-2S; lane 7 = LJ-3-3-2S; lane 8 = R3Q26-F9-2S; lane 9 = R3Q33-1-1S; and lane 10 = WM-1-3S.

Fig. 5. Partial structure at the exon-intron junction in the cytochrome *b* gene of *Alternaria alternata*. Boxes indicate exons and lines indicate introns. Lengths of exons and introns are not to scale. **A**, Isolates BLK-17-S-10S, R3Q33-1-1S, LJ-1-8-2S, VB-RQF-3S, R3Q26-F9-2S, BL-17-4-3S, WM-1-3S, LOR-ORI-1-3S, LJ-3-3-2S, and EV-3-1S. **B**, Isolates WM-1-2S and CPI-ORI-2S.

cies (8,20). Additionally, a rapid molecular test was developed, based on the CAPS-PCR technique, to identify QoI-resistant isolates in *A. alternata* that contain a single intron. Moreover, due to the genetic variability of the cytochrome *b* gene of *A. alternata* isolates, a different technique, such as allele-specific PCR, could be validated for a rapid molecular diagnostic test. However, a new set of primers must be developed prior to the implementation of allele-specific PCR. Molecular techniques have also been used for a rapid detection of QoI-resistant isolates from pistachio leaf samples. Ma et al. (30) developed an allele-specific PCR assay for a rapid detection of QoI-resistant pistachio-infecting *A. alternata*; however, the presence of PCR inhibitors constitutes an obstacle for an accurate detection of resistant isolates. Thus, additional steps must be implemented during PCR to avoid the deleterious effects of PCR inhibitors (30).

In summary, the findings presented here showed that the colorimetric microtiter assay based on RZ dye is a simple, rapid, reliable, and accurate technique to measure the sensitivity of *A. alternata* to azoxystrobin and pyraclostrobin, and could be used for large-scale surveys for resistance screening as suggested for a similar assay (12). It seems likely that it could be adapted for other plant pathogens and fungicides. This method should be combined with molecular diagnostic tools to confirm the genotype associated with fungicide resistance.

Acknowledgments

We thank E. G. Johnson for assistance with sequence alignments and G. E. Vallad and N. A. Peres for critically reading this manuscript.

Literature Cited

- Akimitsu, K., Peever, T. L., and Timmer, L. W. 2003. Molecular, ecological and evolutionary approaches to understanding *Alternaria* diseases of citrus. *Mol. Plant Pathol.* 4:435-446.
- Avenot, H., Morgan, D. P., and Michailides, T. J. 2008. Resistance to pyraclostrobin, boscalid and multiple resistance to Pristine (pyraclostrobin + boscalid) fungicide in *Alternaria alternata* causing *Alternaria* late blight of pistachios in California. *Plant Pathol.* 57:135-140.
- Avila-Adame, C., and Köller, W. 2003. Impact of alternative respiration and target-site mutations on responses of germinating conidia of *Magnaporthe grisea* to Qo-inhibiting fungicides. *Pest Manage. Sci.* 59:303-309.
- Banno, S., Yamashita, K., Fukumori, F., Okada, K., Uekusa, H., Takagaki, M., Kimura, M., and Fujimura, M. 2009. Characterization of QoI resistance in *Botrytis cinerea* and identification of two types of mitochondrial cytochrome *b* gene. *Plant Pathol.* 58:120-129.
- Bartlett, D. W., Clough, J. M., Godfrey, C. R. A., Godwin, J. R., Hall, A. A., Heaney, S. P., and Maund, S. J. 2001. Understanding the strobilurin fungicides. *Pestic. Outlook* 12:143-148.
- Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. 2002. Review: The strobilurin fungicides. *Pest Manage. Sci.* 58:649-662.
- Bennett, J. W., and Lasure, L. L. 1991. Growth media. Pages 441-458 in: *More Gene Manipulations in Fungi*. J. Bennett and L. Lasure, eds. Academic Press Inc., San Diego, CA.
- Brent, K. J., and Hollomon, D. W. 2007. Fungicide resistance in crop pathogens: how can it be managed? FRAC Monogr. No.1. CropLife International, Brussels.
- Bueno, C., Villegas, M. L., Bertolotti, S. G., Previtali, C. M., Neumann, M. G., and Encinas, M. V. 2002. The excited-state interaction of resazurin and resorufin with amines in aqueous solutions. *Photophysics and photochemical reaction. Photochem. Photobiol.* 76:385-390.
- Byth, H. A., Mchunu, B. I., Dubery, I. A., and Bornman, L. 2001. Assessment of a simple, non-toxic alamar blue cell survival assay to monitor tomato cell viability. *Phytochem. Anal.* 12:340-346.
- Canihos, Y., Peever, T. L., and Timmer, L. W. 1999. Temperature, leaf wetness, and isolate effects on infection of *Minneola* tangelo leaves by *Alternaria* sp. *Plant Dis.* 83:429-433.
- Cox, K. D., Quello, K., Deford, R. J., and Beckerman, J. L. 2009. A rapid method to quantify fungicide sensitivity in the brown rot pathogen *Motilinia fructicola*. *Plant Dis.* 93:328-331.
- Cuenca-Estrella, M., and Rodríguez-Tuleda, J. L. 2001. Present status of the detection of antifungal resistance: the perspective from both sides of the ocean. *Clin. Microbiol. Infect.* 7:46-53.
- Dewdney, M. M., and Timmer, L. W. 2010. Florida citrus pest management guide: *Alternaria* brown spot. Univ. Fla. Inst. Food Agric. Sci. Gainesville. Publ. No. PP-147.
- Espinel-Ingroff, A., and Kerker, T. M. 1991. Spectrophotometric method of inoculum preparation for the in vitro susceptibility testing of filamentous fungi. *J. Clin. Microbiol.* 29:393-394.
- Fai, P. B., and Grant, A. 2009. A rapid resazurin bioassay for assessing the toxicity of fungicides. *Chemosphere* 74:1165-1170.
- Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Manage. Sci.* 58:859-867.
- Grasso, V., Palermo, S., Sierotzki, H., Garibaldi, A., and Gisi, U. 2006. Cytochrome *b* gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Manage. Sci.* 62:465-472.
- Invitrogen. 2009. Alamar blue assay technical bulletin. www.invitrogen.com
- Ishii, H. 2010. QoI fungicide resistance: current status and the problems associated with DNA-based monitoring. Pages 37-45 in: *Recent Developments in Management of Plant Diseases*. U. Gisi, I. Chet, and M. Gullino, eds. Springer, Dordrecht, The Netherlands.
- Joseph-Horne, T., and Hollomon, D. W. 2000. Functional diversity within the mitochondrial electron transport chain of plant pathogenic fungi. *Pest Manage. Sci.* 56:24-30.
- Joseph-Horne, T., Hollomon, D. W., and Wood, P. M. 2001. Fungal respiration: a fusion of standard and alternative components. *Biochim. Biophys. Acta* 1504:179-195.
- Kalina, M., and Palmer, J. M. 1968. The reduction of tetrazolium salts by plant mitochondria. *Histochemie* 14:366-374.
- Karaoglanidis, G. S., Luo, Y., and Michailides, T. J. 2011. Competitive ability and fitness of *Alternaria alternata* isolates resistant to QoI fungicides. *Plant Dis.* 95:178-182.
- Kohmoto, K., Akimitsu, K., and Otani, H. 1991. Correlation of resistance and susceptibility of citrus to *Alternaria alternata* with sensitivity to host-specific toxins. *Phytopathology* 81:719-722.
- Köller, W., Parker, D. M., Turechek, W. W., Avila-Adame, C., and Cronshaw, K. 2004. A two-phase resistance response of *Venturia inaequalis* populations to the QoI fungicides kresoxim-methyl and trifloxystrobin. *Plant Dis.* 88:537-544.
- Kuhajek, J. M., Jeffers, S. N., Slatery, M., and Wedge, D. E. 2003. A rapid microbioassay for discovery of novel fungicides for *Phytophthora* spp. *Phytopathology* 93:46-53.
- Larson, E. M., Doughman, D. J., Gregerson, D. S., and Obritsch, W. F. 1997. A new, simple, nonradioactive, nontoxic in vitro assay to monitor corneal endothelial cell viability. *Invest. Ophthalmol. Vis. Sci.* 38:1929-1933.
- Ma, Z., Felts, D., and Michailides, T. J. 2003. Resistance to azoxystrobin in *Alternaria* isolates from pistachio in California. *Pestic. Biochem. Physiol.* 77:66-74.
- Ma, Z., and Michailides, T. J. 2004. An allele-specific PCR assay for detecting azoxystrobin-resistant *Alternaria* isolates from pistachio in California. *J. Phytopathol.* 152:118-121.
- Meletiadi, J., Mouton, J. W., Meis, J. F. G. M., Bouman, B. A., and Verweij, P. E. 2002. Comparison of the Etest and the sensititre colorimetric methods with the NCCLS proposed standard for antifungal susceptibility testing of *Aspergillus* species. *J. Clin. Microbiol.* 40:2876-2885.
- Misaghi, I. J., Grogan, R. G., Duniway, J. M., and Kimble, K. A. 1978. Influence of environment and culture media on spore morphology of *Alternaria alternata*. *Phytopathology* 68:29-34.
- Mizutani, A., Yukioka, H., Tamura, H., Miki, N., Masuko, M., and Takeda, R. 1995. Respiratory characteristics in *Pyricularia oryzae* exposed to a novel alkoxyminoacetamide fungicide. *Phytopathology* 85:306-311.
- Mondal, S. N., Bhatia, A., Shilts, T., and Timmer, L. W. 2005. Baseline sensitivities of fungal pathogens of fruit and foliage of citrus to azoxystrobin, pyraclostrobin, and fenbuconazole. *Plant Dis.* 89:1186-1194.
- Mondal, S. N., Godoy da Silva, A., and Dewdney, M. M. 2009. Resistance to strobilurin fungicides in a population of *Alternaria alternata* causing *Alternaria* brown spot of citrus. (Abstr.) *Phytopathology* 99:S88.
- National Committee for Clinical Laboratory Standard. 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-second edition. Document M27-A2. National Committee for Clinical Laboratory Standards, Wayne, PA.
- National Committee for Clinical Laboratory Standard. 2002. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard. Document M38-A. National Committee for Clinical Laboratory Standards, Wayne, PA.
- O'Brien, J., Wilson, I., Orton, T., and Pognan, F. 2000. Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* 267:5421-5426.
- Pasche, J. S., Piche, L. M., and Gudmestad, N. C. 2005. Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. *Plant Dis.* 89:269-278.
- Pasche, J. S., Wharam, C. M., and Gudmestad, N. C. 2004. Shift in sensitivity of *Alternaria solani* in response to QoI fungicides. *Plant Dis.* 88:181-187.
- Peever, T. L., Canihos, Y., Olsen, L., Ibañez, A., Liu, Y.-C., and Timmer, L. W. 1999. Population genetic structure and host specificity of *Alternaria* spp. causing brown spot of *Minneola* tangelo and rough lemon in Florida. *Phytopathology* 89:851-860.
- Peever, T. L., Ibañez, A., Akimitsu, K., and Timmer, L. W. 2002. Worldwide phylogeography of the citrus brown spot pathogen, *Alternaria alternata*. *Phytopathology* 92:794-802.
- Peever, T. L., Olsen, L., Ibañez, A., and Timmer, L. W. 2000. Genetic differ-

- entiation and host specificity among populations of *Alternaria* spp. causing brown spot of grapefruit and tangerine $\times$ grapefruit hybrids in Florida. *Phytopathology* 90:407-414.
44. Pettit, R. K., Weber, C. A., Kean, M. J., Hoffmann, H., Pettit, G. R., Tan, R., Franks, K. S., and Horton, M. L. 2005. Microplate alamar blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. *Antimicrob. Agents Chemother.* 49:2612-2617.
 45. Pijls, C. F. N., Shaw, M. W., and Parker, A. 1994. A rapid test to evaluate in vitro sensitivity of *Septoria tritici* to flutriafol, using a microtitre plate reader. *Plant Pathol.* 43:726-732.
 46. Promega Corporation. 2009. CellTiter-blue cell viability assay technical bulletin. www.promega.com.
 47. Rampersad, S. N. 2011. A rapid colorimetric microtiter bioassay to evaluate fungicide sensitivity among *Verticillium dahliae* isolates. *Plant Dis.* 95:248-255.
 48. Rosenzweig, N., Atallah, Z. K., Olaya, G., and Stevenson, W. R. 2008. Evaluation of QoI fungicide application strategies for managing fungicide resistance and potato early blight epidemics in Wisconsin. *Plant Dis.* 92:561-568.
 49. Rosenzweig, N., Olaya, G., Atallah, Z. K., Cleere, S., Stanger, C., and Stevenson, W. R. 2008b. Monitoring and tracking changes in sensitivity to azoxystrobin fungicide in *Alternaria solani* in Wisconsin. *Plant Dis.* 92:555-560.
 50. Russell, P. E. 2004. Sensitivity baselines in fungicide resistance research and management. FRAC Monogr. No. 3. CropLife International, Brussels.
 51. Sierotzki, H., Frey, R., Wullschlegel, J., Palermo, S., Karlin, S., Godwin, J., and Gisi, U. 2007. Cytochrome *b* gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. *Pest Manage. Sci.* 63:225-233.
 52. Sierotzki, H., Stammler, G., and Mehl, A. 2011. Molecular methods for fungicide resistance detection. (Abstr.) *Phytopathology* 101:S229.
 53. SigmaPlot. 1993. Curve fitting tutorial. Pages 9-1 to 9-16 in: *Transforms and Curve Fitting*. United States.
 54. Spiegel, J., and Stammler, G. 2006. Baseline sensitivity of *Monilinia laxa* and *M. fructigena* to pyraclostrobin and boscalid. *J. Plant Dis. Prot.* 113:199-206.
 55. Stammler, G., Benzinger, G., and Speakman, J. 2007. A rapid and reliable method for monitoring the sensitivity of *Sclerotinia sclerotiorum* to boscalid. *J. Phytopathol.* 155:746-748.
 56. Stammler, G., and Speakman, J. 2006. Microtiter method to test the sensitivity of *Botrytis cinerea* to boscalid. *J. Phytopathol.* 154:508-510.
 57. Steinfeld, U., Sierotzki, H., Parisi, S., Poirey, S., and Gisi, U. 2001. Sensitivity of mitochondrial respiration to different inhibitors in *Venturia inaequalis*. *Pest Manage. Sci.* 57:787-796.
 58. Stevenson, K. L., Langston, D. B., Jr., and Seebold, K. W. 2004. Resistance to azoxystrobin in the gummy stem blight pathogen documented in Georgia. *Plant Health Progress*. Online publication. doi:10.1094/PHP-2004-1207-01-RS.
 59. Tamura, K., Dudley, J., Nei, M., and Kumar, S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596-1599.
 60. Timmer, L. W., Peever, T. L., Solel, Z., and Akimitsu, K. 2003. *Alternaria* diseases of citrus—novel pathosystems. *Phytopathol. Mediterr.* 42:99-112.
 61. To, W. K., Fothergill, A. W., and Rinaldi, M. G. 1995. Comparative evaluation of macrodilution and alamar colorimetric microdilution broth methods for antifungal susceptibility testing of yeast isolates. *J. Clin. Microbiol.* 33:2660-2664.
 62. Toté, K., Vanden Berghe, D., Levecque, S., Bénére, E., Maes, L., and Cos, P. 2009. Evaluation of hydrogen peroxide-based disinfectants in a new resazurin microplate method for rapid efficacy testing of biocides. *J. Appl. Microbiol.* 107:606-615.
 63. Tremblay, D. M., Talbot, B. G., and Carisse, O. 2003. Sensitivity of *Botrytis squamosa* to different classes of fungicides. *Plant Dis.* 87:573-578.
 64. Vanlerberghe, G. C., and McIntosh, L. 1997. Alternative oxidase: from gene to function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:703-734.
 65. Ypema, H. L., and Gold, R. E. 1999. Kresoxim-methyl: modification of a naturally occurring compound to produce a new fungicide. *Plant Dis.* 83:4-19.
 66. Ziogas, B. N., Baldwin, B. C., and Young, J. E. 1997. Alternative respiration: a biochemical mechanism of resistance to azoxystrobin (ICIA 5504) in *Septoria tritici*. *Pestic. Sci.* 50:28-34.