Baseline Sensitivity of *Guignardia citricarpa* Isolates from Florida to Azoxystrobin and Pyraclostrobin

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Abstract

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Citrus black spot (CBS), caused by *Guignardia citricarpa*, is an emerging disease in Florida. Fungicide applications are the main control measure worldwide. The in vitro activity and baseline sensitivity of *G. citricarpa* isolates to quinone outside inhibitor (QoI) fungicides (azoxystrobin and pyraclostrobin) were evaluated. The effective concentration needed to reduce mycelial growth or spore germination by 50% (EC₅₀) was determined for 86 isolates obtained from Florida counties where CBS is found. The effect of salicylhydroxamic acid (SHAM) plus azoxystrobin and pyraclostrobin was also assessed for mycelial growth and conidial germination. The mean EC₅₀ for mycelial growth for azoxystrobin was 0.027 µg/ml and that for pyraclostrobin

was significantly lower at 0.007 µg/ml ($P < 0.0001$). Similarly, the mean EC₅₀ for conidial germination for azoxystrobin was 0.016 µg/ml and that for pyraclostrobin was significantly lower at 0.008 µg/ml ($P < 0.0001$). There was no effect of SHAM on inhibition of mycelial growth or conidial germination by the QoI fungicides but SHAM slightly affected mycelium inhibition by pyraclostrobin. Cytochrome *b* was partially sequenced and three group 1 introns were found. One intron was immediately post G143, likely inhibiting resistance-conferring mutations at that site. It is surmised that the QoI resistance risk is low in the Florida *G. citricarpa* population.

Citrus black spot (CBS), caused by *Guignardia citricarpa* Kiely, anamorph *Phyllosticta citricarpa* (McAlpine) Van der Aa, was found in Collier County, FL in April 2010 (47). Most commercially grown citrus species, including grapefruit, tangerine, sweet orange, and lemon, are susceptible to CBS (11). The disease causes extensive blemishes on the fruit rind affecting the appeal for the fresh market, although the internal quality remains unaffected. In addition, premature fruit drop may occur, reducing crop yield (6,54).

The pathogen is found in many of the subtropical citrus production zones and is considered a very economically important citrus disease with severe crop losses because fruit are no longer marketable and can abscise prematurely. The disease was first described in New South Wales, Australia in 1895 (5,41). It has since spread to the subtropical regions of many citrus-producing countries such as Argentina, Brazil, China, Ghana, Indonesia, Kenya, Mozambique, Philippines, South Africa, Taiwan, Uruguay, Uganda, Venezuela, Zambia, and Zimbabwe (11,41,61). The disease, however, has not been reported in Mediterranean countries or production regions (5,41,61).

Currently, in Florida, three counties (Collier, Hendry, and Polk) are confirmed to have black spot and sections have been designated as quarantine areas by the United States Department of Agriculture Animal and Plant Health Inspection Service. Conditions to restrict the interstate movement of regulated articles (citrus fruit and plant parts such as leaves, budwood, and nursery stock) have been implemented. In addition to regulatory measures, control strategies must be undertaken. An economic assessment estimated the cost of

CBS control to be at least \$220 million annually in the United States; however, due to preventive copper sprays already used to control citrus canker, the actual cost may be not as high as predicted (27). In addition to the cost of control measures, it was forecast that there could be up to \$847 million of losses in Florida from CBS (27).

The humid subtropical conditions with high summer rainfall in Florida are favorable for the development of CBS (32). Predictive models used to determine the risk of CBS establishment after pathogen introduction to the United States found that Florida conditions are highly favorable for the disease and Texas is at moderate risk of introduction (41,61). Other areas, such as California, have a low risk for disease development due to the lack of rainfall and prolonged leaf wetness periods that promote the disease (41,61).

Hard spot is the most diagnostic preharvest symptom of CBS. The lesions are 3 to 10 mm in diameter, circular, depressed, with brick red to black margins and gray-white necrotic centers. On green fruit, the lesions may be surrounded by a yellow halo but can have a green halo on mature fruit (9,32). Pycnidia are often found at the center of the lesion as slightly elevated black dots but ascocarps are never formed in fruit lesions (9,32). False melanose, early virulent spot, virulent spot, and cracked spot are other fruit symptom types of black spot (9,32). Fruit remain susceptible to infection for 4 to 5 months after petal fall (32). Leaf symptoms are rare and most often appear on lemon. Fruit peduncles can also show symptoms. When symptoms are present on leaves, lesions are small, round, and red-brown, sometimes with a yellow halo. As they age, a necrotic center forms, surrounded by a dark-brown margin. Leaves remain susceptible from development for up to 10 months (55).

Black spot control is based mainly on preventive applications of fungicides during the period of fruit susceptibility (48); nevertheless, cultural practices have been implemented in Florida to reduce inoculum and pathogen spread (17). In other areas where CBS is present, the disease is controlled with fungicides from different mode of action groups such as the benzimidazoles, quinone outside inhibitors (QoIs; strobilurins), dithiocarbamates, and copper products (15,48). Currently in Florida, only QoI fungicides and copper are registered for the control of black spot. Monthly applications of copper and QoIs (azoxystrobin, pyraclostrobin, or trifloxystrobin)

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are recommended from early May to mid-September but there is a label limit of four QoI applications in a season. For this reason, it is suggested to reserve QoI fungicides for times when there is concern about copper phytotoxicity (temperatures exceeding 34°C), especially when applied in orchards whose fruit is destined for the fresh market (17).

QoI fungicides block electron transport at the quinol-oxidizing site of the cytochrome *b* complex (complex III) in the mitochondria, disrupting ATP production (8,22). Spore germination is the fungal growth stage that is particularly sensitive to QoIs (8). Several plant pathogens can avoid the toxic effects of QoI fungicides by the expression of the alternative oxidase pathway (28). The alternative oxidase pathway takes place in the inner mitochondrial membrane (57) and it can be inhibited by salicylhydroxamic acid (SHAM) and *n*-propyl gallate (46,50). For this reason, SHAM is usually added to QoI fungicides when tested in vitro (18). The addition of SHAM to azoxystrobin in in vitro tests with *Sclerotinia sclerotiorum* synergistically improved pathogen inhibition, compared with when azoxystrobin was used alone (18). Similarly, the mixture of SHAM and azoxystrobin in in vitro tests with *Colletotrichum capsici* and *Botrytis cinerea* showed a synergistic effect on mycelium inhibition (28).

The mode of action of QoI fungicides is highly specific, and many different pathogens have lost sensitivity to this group of fungicides due to a single-point mutation that leads to a change from glycine (G) to alanine (A) at amino acid residue 143 in the cytochrome *b* gene (G143A; 21,22,25). A second amino acid substitution shown to reduce sensitivity to QoI fungicides is the replacement of phenylalanine with leucine at position 129 (F129L; 40). A third amino acid substitution from glycine to arginine at the position 137 (G137R) was detected recently in *Pyrenophora tritici-repentis*, conferring reduced sensitivity to QoI fungicides comparable with that of the F129L substitution (51).

Azoxystrobin (Abound 2.08F; Syngenta Crop Protection) and pyraclostrobin (Headline; BASF Crop Protection), two members of the QoI class of fungicides, have been reported to provide good control of black spot in the field as well as in in vitro studies (1,19,35,44,48). Due to the site-specific mode of action of QoI fungicides, the potential for resistance development must be considered. Although *G. citricarpa* resistance to QoIs has not been reported, in vitro studies determined that mycelial growth was not completely inhibited even at high concentrations of azoxystrobin (43). On the other hand, the same author pointed out that the variation in sensitivity to the fungicide is possibly related to the genetic variability of *G. citricarpa* isolates.

Despite prolonged exposure to QoI fungicides, some fungi have yet to develop the high levels of resistance conferred by the G143A substitution (23,24,34). In these fungi, it has been shown that a group I intron is often found inserted after the G143 codon whereas, in species where resistance occurs, no intron is present (23). Such an intron was found in a study on a small number of *G. citricarpa* isolates and *G. bidwellii*, a species closely related to *G. citricarpa*, and resistance has yet to occur for either fungus (34,52). Little is known about the provenance of the Florida *G. citricarpa* population and whether the presence of the intron may vary, as it does in *B. cinerea* (7). QoI fungicides are currently key to CBS control in Florida; therefore, a resistance risk assessment is extremely important for the long-term management of the disease.

The baseline sensitivity of other Florida citrus pathogens such as *C. acutatum*, *Alternaria alternata*, *Elsinoë fawcettii*, *Diaporthe citri*, and *Mycosphaerella citri* to azoxystrobin and pyraclostrobin have been determined (36). Most isolates of the different pathogens were sensitive to the QoIs except for *A. alternata*, which has mycelia tolerant to azoxystrobin (36). More recently, resistance of *A. alternata* to QoI fungicides was reported (58), resulting in control failures.

Because black spot is an emerging disease in Florida, the establishment of baseline sensitivity of *G. citricarpa* isolates from Florida to QoI fungicides will help to monitor pathogen shifts in sensitivity to these fungicides. This baseline information could con-

tribute to the long-term effectiveness of current spray programs in Florida. The objective of this research was to evaluate QoI fungicides for in vitro activity and to determine the baseline sensitivity of *G. citricarpa* isolates from Florida.

Materials and Methods

Fungal isolates and storage. Eighty-six isolates of *G. citricarpa* from Collier, Hendry, and Polk Counties, where CBS is currently present, were evaluated for their in vitro sensitivity to the fungicides azoxystrobin and pyraclostrobin. The isolates were taken from hard spot, early virulent spot, and pycnidia. Briefly, symptomatic fruit were washed, surface disinfested in 5% NaOCl, rinsed in sterile deionized water (SDW), and air dried for 2 to 3 h. The lesions were excised, placed in 50% ethanol for 30 s, then in 5% NaOCl, then rinsed with SDW for 7 min and dried in a laminar air-flow hood. Sections of the lesions were placed on carrot agar (CA; 42) or potato dextrose agar (PDA) and incubated at room temperature with 12 h of light. When pycnidia were used for isolations, they were placed on moistened sterile filter paper and incubated overnight. Pycnidia were selected under the stereomicroscope and individually placed on CA. All isolations were incubated for 5 to 6 days. Isolates with typical morphology were placed on an indicator medium, oatmeal agar, and observed for a yellow halo (5). Each isolate was also subjected to polymerase chain reaction (PCR) identification with the primer sets NP-Br-ITS-Gc and NP-Br-ITS-Gm, as described by Peres et al. (42). Single-spore isolates were produced for all assays. For single-spore isolation, isolates cultured under a 12-h light cycle for 2 to 3 weeks were flooded with SDW containing 0.02% Tween 20. The resulting suspensions were washed with SDW by centrifuging at 5,000 rpm for 5 min. This washing process was repeated twice. The final conidial concentration was adjusted to 10⁶ spores/ml using a hemocytometer. Conidial suspensions (20 µl each) were spread on PDA and incubated at 25°C for 3 days. Germinated conidia were then picked up using a flame-sterilized needle under a stereomicroscope and transferred to PDA. For long-term storage, all isolates were kept on sterile filter paper in sealed plastic containers containing CaSO₄ desiccant at -20°C. For mycelium and conidia production, *G. citricarpa* isolates were transferred to half-strength PDA and grown for 14 days at 25°C.

Mycelium growth inhibition assay. Commercial formulations of azoxystrobin (Abound) and pyraclostrobin (Headline SC) were used. The fungicides were diluted in SDW to prepare stock solutions of the active ingredient at 100 and 1 mg/ml. From the 1 mg/ml stock, 3.5, 35, and 350 µl were added and, from the 100 mg/ml stock, 35 and 350 µl were added to molten half-strength PDA (3,500 ml) after cooling to 55°C to obtain final concentrations of active ingredient at 0.001, 0.01, 0.1, 1, and 10 µg/ml. Unamended medium was used as the control.

Amended PDA (20 ml) was poured into 100-mm-diameter petri dishes using a sterile bottle top dispenser (Fisherbrand). In all, 86 isolates were evaluated for sensitivity to the QoI fungicides. Mycelial plugs, 3 mm in diameter, from the actively growing area of the fungal colony were placed in the center of each plate. Three replicates were used for each fungicide concentration. Plates were incubated at 25°C for 14 days under continuous light, and colony diameter was determined as the average of two perpendicular measurements for each of the 86 isolates. The diameter of the mycelial plug was subtracted from the average colony diameter for each replicate. The percent inhibition was calculated relative to the growth of the unamended control for each isolate growing in the fungicide-amended plates.

Effect of SHAM and QoI fungicides on mycelial growth. Fifteen *G. citricarpa* isolates were tested to determine whether SHAM affected the response of fungal growth to azoxystrobin and pyraclostrobin. SHAM was dissolved in methanol at 0.1 mg/ml. The amount of methanol in the media was 0.1% (vol/vol). Half-strength PDA (900 ml) was amended with 9 and 90 mg of SHAM to obtain final concentrations of 10 and 100 µg/ml. The effect of SHAM at 10 and 100 µg/ml on fungal growth was evaluated in

combination with final fungicide concentrations of 0, 0.001, 0.01, 0.1, 1, and 10 µg/ml. Percent growth inhibition was based on comparison with the growth in SHAM plates with no fungicide. Inoculation method and measurements were done as described above.

Spore inhibition assay. Isolates of *G. citricarpa* were cultured on half-strength PDA for 14 days. Then, conidia were washed off using 4 ml of sterile water with 0.02% Tween 20. The suspension was centrifuged at 5,000 rpm for 5 min, resuspended, and centrifuged three more times each for 5 min and the conidial concentration was adjusted to 10⁶ spores/ml using a hemacytometer.

The germination medium consisted of 2% 'Valencia' orange juice (pH 4.0) and was prepared to stimulate conidial germination (31). Germination was assessed on hydrophobic slides (Fisher Scientific) and each well contained 10 µl of liquid (33,37). Prior their addition to the wells, 10-fold serial dilutions of QoI fungicides of 0.001 to 1 µg/ml were prepared. Each well received 7.5 µl of 2% 'Valencia' juice, 1.25 µl of the corresponding fungicide concentration, and 1.25 µl of the adjusted conidial suspension. Control wells contained 1.25 µl of SDW instead of the fungicide. Hence, the suspension in each well had a 3:1 ratio of 'Valencia' orange juice to fungicide and conidia. Hydrophobic slides were placed into a petri dish filled with water at the bottom to prevent desiccation and incubated at 23°C for 20 h. Then, 100 conidia were observed under the microscope at ×400 to determine the percent germinated conidia. A conidium was considered germinated if the germ tube was equal to or longer than the length of the conidium. The percent spore inhibition was calculated for each of the 86 isolates, fungicide, and experiment combinations.

Effect of SHAM and QoI fungicides on spore germination. The same isolates used to determine the effect of SHAM on mycelial growth were used to evaluate the effect of SHAM on spore germination inhibition. Different SHAM concentrations were tested prior to the addition of QoI fungicides. A stock suspension of SHAM at 0.1 mg/ml was serially diluted to obtain final concentrations in each well of SHAM at 10, 25, 50, and 100 µg/ml. The content of methanol in each well was 0.1% (vol/vol). Each well received 7.5 µl of 2% 'Valencia' orange juice, 1.25 µl of the adjusted spore suspension, and 1.25 µl of the corresponding SHAM concentration. Control wells received 1.25 µl of SDW instead of SHAM. Thus, the suspension in each well had a 3:1 ratio of Valencia orange juice to medium.

The effect of SHAM at 10 µg/ml on spore germination was evaluated in combination with final QoI fungicide concentrations of 0, 0.001, 0.01, 0.1, and 1 µg/ml. To maintain a 3:1 ratio of 'Valencia' juice to SHAM, fungicide and conidia, the amount of medium added to each well was modified. Therefore, each well received 7.5 µl of 2% orange juice, 0.83 µl of each fungicide concentration, 0.83 µl of SHAM, and 0.83 µl of the adjusted conidial suspension. Control wells received 0.83 µl of SDW instead of the fungicide. After the slides were loaded with the suspension, the same procedure as described in the spore inhibition assay was followed. Percent inhibition was determined for each isolate–fungicide–experiment combination.

Azoxystrobin technical-grade versus commercial formulation and the effect of SHAM on mycelium inhibition. To test whether or not there was a difference between commercial formulation of azoxystrobin (Abound) or technical-grade active ingredient, 10 isolates of *G. citricarpa* were tested against the two forms

of the chemical. Azoxystrobin commercial formulation and technical-grade stock solutions of active ingredient at 100 µg/ml were diluted in water or acetone, respectively, and added to molten half-strength PDA at 0, 0.001, 0.01, 0.1, 1, and 10 µg/ml. In addition, the effect of SHAM at 100 µg/ml added to commercial formulation and technical-grade azoxystrobin was also tested on mycelium growth inhibition. The dilution method was as described for the effect of SHAM on mycelium growth assay. Inoculation method, measurements and calculation of percent inhibition were done as described for the mycelium inhibition assay.

Calculation of effective concentration to reduce growth by 50% and statistical analysis. For all experiments, there were three replications per isolate–fungicide concentration combination and each experiment was repeated twice. For mycelium inhibition assays, the effective concentration to reduce growth by 50% (EC₅₀) was determined by fitting a four-parameter logistic (sigmoidal) function for azoxystrobin and a three-parameter function for pyraclostrobin. On the other hand, for spore-inhibition assays, EC₅₀ was determined by fitting a three-parameter sigmoidal function for both QoIs. For all experiments, EC₅₀ values were calculated using SigmaPlot (ver. 10.0; Systat Software Inc.).

To test for the homogeneity of variance, the standardized residuals were plotted against predicted values. In addition, the normality of the data distribution was tested with univariate analysis. Because variances were equal, data from different experiments were pooled to calculate the mean EC₅₀ for each fungicide-isolate combination.

The effect of fungicide, isolate, experiment, and all the two-way interactions were investigated by an analysis of variance (ANOVA). Treatment means were separated using the least significant difference with PROC GLM (SAS 9.3; SAS Institute).

DNA extraction and sequence analysis of *cyt b*. The mycelium of isolate Gc12 was harvested from 5-day-old cultures on half-strength PDA overlaid with a layer of cellophane. After grinding the mycelia in liquid nitrogen, genomic DNA (gDNA) was extracted using the DNeasy Plant Mini kit (Qiagen), purified using phenol/chloroform/isoamyl alcohol (25:24:1), and sequenced using the Ion Torrent Personal Genome Machine (PGM; 318 chip; Life Technologies). The sequence data were then assembled using the de Bruijn graph algorithm (62) embedded in the CLC Genomic Workbench (v5.0.1; CLC Bio) and analyzed by BlastN against the GenBank database. To fill the gaps between contigs, primers targeting the end of the contigs were designed (Cytb3/Cytb12 and Cytb5/Cytb6, Table 1). PCR reactions were performed in 25-µl volumes containing 1 to 10 ng of gDNA, 0.2 µM primers, 0.2 mM each dNTP, 1× PCR buffer, and 1 U of HotStar Taq polymerase (Qiagen) with cycling conditions of 95°C for 5 min; followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and a final 5-min extension at 72°C. PCR products were cloned into the pGEN-T vector (Promega Corp.) and sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida). The partial *cyt b* sequence was deposited in GenBank (accession number KC788404).

To identify the coding sequence of the partial *cyt b*, total RNA was extracted using the RNeasy Plant Mini kit (Qiagen) from mycelia harvested as above and reverse transcribed with the oligo (dT)₂₃VN primer using the ProtoScript AMV First-Strand cDNA Synthesis kit (New England BioLabs), according to the manufacturer's instructions. The resulting cDNA was analyzed by PCR using the primer pair KES126/KES115 (34), and PCR products were cloned and sequenced as above.

All *G. citricarpa* isolates in this study were sequenced to identify potential mutations in the hot spot, a highly conserved region of the *cyt b* gene associated with QoI resistance where mutations, including G143, G137, and F129, may occur (23). Briefly, mycelia were crushed with pipette tips in tubes containing 20 µl of extraction buffer from the Extract-N-Amp Plant kit (Sigma-Aldrich). Following incubation at 95°C for 10 min, crushed mycelia were mixed with 20 µl of dilution buffer. PCR reactions were performed as above using primers pairs Cytb7/Cytb8 and Cytb11/Cytb12 (Table 1) targeting regions containing G143 and G137, and F129,

Table 1. Primers designed in the study to amplify the *cyt b* gene from *Guignardia citricarpa* genomic DNA

Primer	Sequence (5' to 3')
Cytb3	AGCTCCTAGAACACTTGTGTTGAAC
Cytb5	TTAGGTTGGTCGTACAGTGAAAGTGAG
Cytb6	TACCTATCTCACGACTAGCCAATCAC
Cytb7	GGGTGTAATTGCCAAATTACGGGA
Cytb8	CATTTCACCCTAATTTGTTTGGCG
Cytb11	TATGTACGAAAGGAAATGGGTAATCCG
Cytb12	TATGACATAGGTGCTCTGTTTAAGGC

respectively, and 10 samples from each primer pair were arbitrarily selected for sequencing. Sequence alignment and the percent homology were performed using MEGA v5.10 (53).

Results

Inhibition of mycelial growth. The EC_{50} of *G. citricarpa* isolates to azoxystrobin and pyraclostrobin was determined and the ANOVA showed that the fungicide effect was highly significant ($P < 0.0001$). No interactions between experiment, fungicide, and isolate were found (Table 2). The mean EC_{50} value of the 86 isolates for azoxystrobin was significantly higher ($P < 0.0001$) than the EC_{50} value for pyraclostrobin regardless of growth stage tested (Table 3). Azoxystrobin inhibited mycelial growth up to 73% at 1 $\mu\text{g/ml}$ but only 68% at 10 $\mu\text{g/ml}$ (Fig. 1A). This reduction in

Table 3. Effective concentration of azoxystrobin and pyraclostrobin fungicides to inhibit mycelial growth and spore germination by 50% (EC_{50}) of 86 *Guignardia citricarpa* isolates

Fungicide	Mean EC_{50} $\mu\text{g/ml}^z$	
	Mycelium inhibition	Spore inhibition
Azoxystrobin	(0.002–0.26) 0.027 aA	(0.001–0.03) 0.016 aB
Pyraclostrobin	(0.002–0.03) 0.007 bA	(0.001–0.03) 0.008 bB

^z Average of two independent experiments. Numbers in parentheses indicate minimum and maximum mean EC_{50} value of mycelium and spore inhibition for each fungicide. Mean separation within column followed by the same lowercase letter are not significantly different according to *t* test (least significant difference [LSD], $P \leq 0.05$). Mean separation within rows followed by the same uppercase letter are not significantly different according to *t* test (LSD, $P \leq 0.05$).

Table 2. *F* statistics and *P*-values for the effect of azoxystrobin and pyraclostrobin on mycelium growth and spore germination inhibition of 86 *Guignardia citricarpa* isolates^z

Factor	df	Mycelium growth inhibition		Spore germination inhibition	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Experiment	1	3.22	0.0762	1.07	0.3040
Fungicide	1	22.89	<u><0.0001</u>	158.0	<u><0.0001</u>
Isolate	85	0.99	0.5095	2.70	<u><0.0001</u>
Experiment $\times$ fungicide	1	3.65	0.0595	4.41	<u>0.0387</u>
Experiment $\times$ isolate	85	0.97	0.5554	1.14	0.2673
Fungicide $\times$ isolate	85	1.01	0.4790	2.75	<u><0.0001</u>

^z Significant effects are underlined.

Fig. 1. A and B, Inhibition of mycelial growth of *Guignardia citricarpa* by different concentrations of azoxystrobin and pyraclostrobin, respectively. Points represent the average of two experiments and three replicates for each concentration. C and D, Frequency distribution of the effective concentration of azoxystrobin and pyraclostrobin, respectively, needed to reduce mycelial growth by 50% (EC_{50}) of *G. citricarpa* isolates.

growth inhibition at the highest concentration was not observed with pyraclostrobin. At 10 µg/ml, growth inhibition was 91% for pyraclostrobin (Fig. 1B).

For azoxystrobin, 87% of the isolates had an EC₅₀ between 0.009 and 0.03 µg/ml (Fig. 1C). For pyraclostrobin, however, the EC₅₀ values were more evenly distributed over the range and 59% of isolates were between 0.003 and 0.006 µg/ml, whereas another 31% of the isolates were between 0.007 and 0.01 µg/ml (Fig. 1D).

Effect of SHAM on mycelial growth. The addition of SHAM to media amended with azoxystrobin had no effect on the EC₅₀ values for mycelial growth of the 15 *G. citricarpa* isolates at 10 µg/ml ($P = 0.7136$) or 100 µg/ml ($P = 0.0726$). However, the addition of SHAM to pyraclostrobin had a significant effect on the EC₅₀ values for mycelial growth at the same concentrations ($P = 0.017$ and 0.006 , respectively; Table 4). No other factors or interactions between factors were significant. Although SHAM increased growth inhibition with azoxystrobin at 10 µg/ml (Fig. 2A), pairwise comparison indicated that there was no significant difference in the EC₅₀ values and, for this reason, SHAM was not used to determine the baseline sensitivity of the 86 *G. citricarpa* isolates.

With the addition of SHAM to pyraclostrobin, a pattern of growth inhibition was observed that was similar to that with pyraclostrobin alone (Fig. 2B). The addition of SHAM at 10 µg/ml to the lowest concentration of pyraclostrobin inhibited growth more than SHAM at 100 µg/ml or pyraclostrobin alone. However, the EC₅₀ values for both SHAM concentrations were the same, and pairwise comparison indicated that the addition of SHAM to pyraclostrobin had an effect on the EC₅₀ values.

Inhibition of spore germination. Preliminary results from the spore germination test using azoxystrobin and pyraclostrobin at 0, 0.001, 0.01, 0.1, 1, and 10 µg/ml indicated that 10 µg/ml completely inhibited spore germination. Thus, the highest fungicide concentration, 10 µg/ml, was eliminated from all spore inhibition assays.

There was a significant effect for fungicide, isolate, and the fungicide-isolate and fungicide-experiment interactions (Table 2). Conidial germination was inhibited up to 91 and 94% by azoxystrobin and pyraclostrobin, respectively, at 1 µg/ml (Fig. 3A and B). No shift in the spore inhibition was observed at the highest concentration of azoxystrobin as occurred in the mycelium inhibition assay. For azoxystrobin, 66% of the isolates had an EC₅₀ between 0.009 and 0.01 µg/ml (Fig. 3C) whereas 44% of the isolates had an EC₅₀ between 0.003 and 0.006 µg/ml for pyraclostrobin (Fig. 3D).

Effect of SHAM on spore germination. Different SHAM concentrations were tested prior to the addition of the QoI fungicides. SHAM completely inhibited spore germination at 25, 50, and 100 µg/ml. Crystals formed on the slides when SHAM was added at 100 µg/ml. Apparently, there is a reaction between the acid in the orange juice and the high concentration of SHAM which induced crystal formation.

Table 4. Mean effective concentration of azoxystrobin and pyraclostrobin with and without salicylhydroxamic acid (SHAM) to inhibit mycelial growth and spore germination by 50% (EC₅₀) of 15 *Guignardia citricarpa* isolates

Experiment ^z	EC ₅₀ µg/ml ^y	
	Azoxystrobin	Pyraclostrobin
Mycelium		
No SHAM	0.017 a	0.008 a
SHAM (10 µg/ml)	0.016 a	0.005 b
SHAM (100 µg/ml)	0.013 a	0.005 b
Spore		
No SHAM	0.015 a	0.006 a
SHAM (10 µg/ml)	0.016 a	0.005 a

^y Mean separation within column followed by the same letter are not significantly different according to *t* test (least significant difference, $P \leq 0.05$) for each independent experiment.

^z Two independent experiments: mycelium and spore inhibition.

SHAM at 10 µg/ml was selected for further tests of activity on spore germination inhibition when added to the QoIs. An ANOVA indicated that SHAM had no effect on the sensitivity of conidial germination when added to azoxystrobin and pyraclostrobin ($P = 0.3849$ and 0.5887 , respectively; *data not shown*). Isolates had a significant effect ($P = 0.0007$) for azoxystrobin; however, interactions between factors were not significant for either fungicide (*data not shown*). The mean EC₅₀ value for the isolates tested with azoxystrobin only was 0.015 µg/ml and, with SHAM, was 0.016 µg/ml. When pyraclostrobin was used alone, the mean EC₅₀ was 0.006 µg/ml and the EC₅₀ mean with SHAM was 0.005 µg/ml (Table 4). The percent spore inhibition when azoxystrobin or pyraclostrobin was used alone was very similar to the inhibition when SHAM was added to the fungicides (Fig. 4A and B). Without SHAM, the maximum spore germination inhibition was 93% for azoxystrobin and 92% for pyraclostrobin. With SHAM at 10 µg/ml, the maximum spore germination inhibition was 95% and 97% for azoxystrobin and pyraclostrobin, respectively (Fig. 4A and B).

Azoxystrobin technical-grade versus commercial formulation and the effect of the different grades and SHAM on mycelium inhibition. No significant effect on the EC₅₀ values between azoxystrobin technical-grade versus commercial formulation was demonstrated by ANOVA ($P = 0.9939$; Table 5). Also, no significant effect of the experiment or isolate was found and interactions between factors were not significant (*data not shown*).

The percent mycelium inhibition by both azoxystrobin formulations was similar at the different concentrations. For commercial

Fig. 2. A and B, Effect of salicylhydroxamic acid (SHAM) on the activity of azoxystrobin and pyraclostrobin, respectively, on mycelial growth of *Guignardia citricarpa* isolates. Points represent the average of two experiments and three replicates for each concentration.

product at 1 µg/ml, there was a 74% inhibition but, when the concentration was increased to 10 µg/ml, inhibition was only 67%. The same trend was also observed when using technical grade: at 1 µg/ml, there was 76% inhibition of mycelial growth but, at 10 µg/ml, the inhibition was only 69% (Fig. 5A).

The effect of SHAM at 100 µg/ml added to azoxystrobin technical-grade and commercial formulation was also tested. There was no effect of the addition of SHAM to technical-grade or commercial formulation as determined by ANOVA ($P = 0.2006$). However, pairwise comparison of EC_{50} values from technical grade with or without SHAM were significant ($P = 0.0001$; Table 5), indicating that the addition of SHAM did affect the EC_{50} value of azoxystrobin technical grade.

Mycelial growth was stimulated when SHAM was added to the lowest rate of azoxystrobin commercial formulation. Nonetheless, mycelial growth inhibition by azoxystrobin commercial formulation at 1 µg/ml with SHAM was 88% but, at 10 µg/ml, the inhibition was slightly less at 81%. The growth inhibition by technical grade at 1 µg/ml with SHAM was 83% and, at 10 µg/ml, in contrast to the commercial formulation, the inhibition was higher, 91% (Fig. 5B). Despite SHAM, growth inhibition was higher when the maximum rate of azoxystrobin technical grade was used; it had no significant effect on the EC_{50} values of either formulation of azoxystrobin.

Sequence analysis of *cyt b*. BlastN analysis revealed that three contigs (1,129, 1,581, and 5,319 bp) shared high sequence identity with the *cyt b* gene of *G. bidwellii* (GenBank accession JF785545; E-value < $5e^{-109}$). After PCR amplification and sequencing using Cytb3/Cytb12 and Cytb5/Cytb6 primers, a partial *cyt b* gene of 8,546 bp was generated. In addition, the coding sequence of the partial *cyt b* gene was obtained using reverse-transcription PCR amplifying a 504-bp fragment encoding for the amino acid residues 99 to 266 (*data not shown*). The partial *cyt b* gene of *G. citricarpa* contains three introns between amino acid residues 99 and 259 (Fig. 6; Supplementary Figure 1). The first intron of 3,328 bp is inserted after the codon encoding a tyrosine residue at position 132 (Y132). The second intron of 943 bp is inserted after the codon encoding a proline residue at position 135 (P135). The third intron is inserted after the codon encoding a glycine residue at position 143 (G143) (Fig. 6). All three introns belonged to the group I intron family (12). To determine whether all *G. citricarpa* isolates possessed the intron after amino acid position 143, PCR reactions using Cytb7/8 and Cytb11/12 primers amplified 594- and 1,323-bp fragments, respectively, from all tested *G. citricarpa* isolates (*data not shown*). Sequencing results from 10 arbitrarily selected isolates also revealed 100% sequence identity with the corresponding sequence of isolate Gc12 in both regions and con-

Fig. 3. A and B, Inhibition of spore germination of *Guignardia citricarpa* by different concentrations of azoxystrobin and pyraclostrobin, respectively. Points represent the average of two experiments and three replicates for each concentration. C and D, Frequency distribution of the effective concentration of azoxystrobin and pyraclostrobin, respectively, to reduce spore germination by 50% (EC_{50}) of *G. citricarpa* isolates.

Fig. 4. A and B, Effect of salicylhydroxamic acid (SHAM) on the activity of azoxystrobin and pyraclostrobin, respectively, on spore germination of *Guignardia citricarpa* isolates. Points represent the average of two experiments and three replicates for each concentration.

Table 5. Mean effective concentration of azoxystrobin fungicide and technical-grade azoxystrobin, with and without salicylhydroxamic acid (SHAM), that inhibited mycelial growth of 10 *Guignardia citricarpa* isolates by 50% (EC_{50})

	EC_{50} µg/ml ^a	
	Azoxystrobin technical	Azoxystrobin commercial
No SHAM	0.017 aA	0.016 aA
SHAM	0.013 bA	0.014 aA

^a Mean separation within columns followed by the same lowercase letter are not significantly different according to *t* test (least significant difference [LSD], $P \leq 0.05$). Mean separation within rows followed by the same uppercase letter are not significantly different according to *t* test (LSD, $P \leq 0.05$).

firmed that no substitution was observed in these isolates (*data not shown*).

Discussion

In this study, we established the baseline sensitivity of *G. citricarpa* isolates from Florida to azoxystrobin and pyraclostrobin. EC_{50} values for mycelium inhibition of other citrus pathogens to azoxystrobin were higher than *G. citricarpa* when compared with our results (EC_{50} mean = 0.027 µg/ml). The mean EC_{50} of *E. fawcettii* was 0.06 µg/ml, the mean EC_{50} for *C. acutatum* was 0.40 µg/ml, the *D. citri* EC_{50} mean was 0.08 µg/ml, and the EC_{50} mean for *M. citri* was 1.62 µg/ml (36). Vega et al. (58) found a mean

Fig. 5. A, Inhibition of mycelium growth of *Guignardia citricarpa* by different concentrations of azoxystrobin technical-grade and commercial formulation. Points represent the average of two experiments and three replicates for each concentration. **B,** Effect of salicylhydroxamic acid (SHAM) on the activity of technical-grade and commercial formulation of azoxystrobin on mycelial growth of *Guignardia citricarpa* isolates. Points represent the average of two experiments and three replicates for each concentration.

EC_{50} of 0.44 µg/ml for QoI-sensitive conidia of *A. alternata* for azoxystrobin. Although Mondal et al. (36) reported EC_{50} values higher than 100 µg/ml from *A. alternata* mycelium, the values from sensitive isolates' conidia were much lower, indicating that mycelium of this pathogen is insensitive to azoxystrobin (36,58). In addition, the baseline for mycelium of *Plasmopara viticola* isolates showed a large range of EC_{50} values, from 0.04 to 0.78 µg/ml (59). The most similar baseline to *G. citricarpa* for azoxystrobin was from sensitive isolates of *C. graminicola*, which had a range of EC_{50} values from 0.05 to 0.1 µg/ml (4). In vitro activity of *G. citricarpa* to azoxystrobin was also evaluated in Brazil; even at higher doses of azoxystrobin (10 µg/ml), mycelium growth could not be totally inhibited and some isolates had a decrease in mycelium inhibition when compared with the lowest concentration of the fungicide (1 µg/ml; 43). Similar results were obtained in this study, where less inhibition was observed at 10 µg/ml with all the isolates tested. On the other hand, the same authors reported complete inhibition of sporulation by azoxystrobin.

Few reports on in vitro activity of pyraclostrobin are available. In this study, isolates of *G. citricarpa* were highly sensitive to pyraclostrobin (EC_{50} mean = 0.007 µg/ml). Mondal et al. (36) established the baseline sensitivity of five fungal citrus pathogens and their EC_{50} values to pyraclostrobin were greater than the values reported in this study for *G. citricarpa*.

Spore germination is a fungal stage particularly sensitive to QoIs (8). In this study, the EC_{50} values for conidia of *G. citricarpa* to azoxystrobin were lower (EC_{50} mean = 0.016 µg/ml) than the EC_{50} values reported for conidial germination of *Erysiphe graminis* f. sp.

Fig. 6. Partial structure at the exon-intron junction in the cytochrome *b* gene of *Guignardia citricarpa*. Boxes indicate exons and lines indicate introns. Lengths of exons and introns are not to scale.

tritici on cereals, which ranged from 0.022 to 0.235 µg/ml (14). Our results showed that the baseline for mycelial growth was higher than for spore germination, implying that *G. citricarpa* conidia are more sensitive to QoI fungicides. In other studies, spore germination has also been shown to be more sensitive than mycelium to pyraclostrobin. The EC₅₀ values of pyraclostrobin for conidia of *E. necator* were similar to our results (EC₅₀ mean = 0.008 µg/ml), ranging from 0.0016 to 0.010 µg/ml with a mean of 0.0044 µg/ml (60).

Some fungal plant pathogens evade the toxic effects of QoI fungicides by the alternative respiration pathway, which allows the generation of ATP without electron transport through complex III and, thus, allows the fungus to survive even at high doses of fungicide (38,63). It has been reported that residual growth of *Magnaporthe grisea* sustained by alternative respiration was involved in the spontaneous emergence of QoI-resistant cytochrome *b* target site mutants (3). Nevertheless, for several fungal species, this rescue mechanism had little effect in the field when QoI fungicides were used to control the disease (38,63). It has been proposed that, for those fungal species, plant antioxidants such as flavones present in the host can silence this mechanism during infection by quenching the reactive oxygen (2). Therefore, differences between *in vitro* and *in vivo* for the same pathogen are likely to be found (38). For this reason, the alternative respiration should be blocked for some pathogens by using SHAM during *in vitro* experiments.

In the current study, SHAM did not affect the activity of azoxystrobin. Neither mycelial growth nor spore germination was significantly affected by the addition of SHAM to the medium. Similar results were obtained with *C. graminicola* and *Penicillium digitatum*, where the addition of SHAM did not increase the activity to azoxystrobin (4,30). Our results also indicated that the addition of SHAM to pyraclostrobin did affect the activity on mycelium growth, although the EC₅₀ values were similar. However, spore germination was not affected by SHAM. As in our study, the addition of SHAM to pyraclostrobin significantly reduced the colony diameter of the citrus pathogen *D. citri*, even though EC₅₀ values could not be calculated due to reversed inhibition at low doses of the fungicide (36). Thus, our results indicate that *G. citricarpa* is not using the alternative respiration pathway.

Interestingly, in our study, azoxystrobin at 10 µg/ml showed lower mycelium growth inhibition than at 1 or 0.1 µg/ml. Thus, to evaluate whether or not the product formulation was influencing these results, technical-grade was compared with commercial formulation. There were no differences between the two formulations. The addition of SHAM slightly increased the inhibition at 10 µg/ml; however, it did not affect their EC₅₀ values. Although there is no evidence that *G. citricarpa* is using alternative respiration, it is hypothesized that the pathogen may be activating this pathway with azoxystrobin specifically at 10 µg/ml and SHAM is blocking this pathway, or else the mycelium is not taking up the fungicide well at higher concentrations and SHAM increases the permeability.

Overall, *G. citricarpa* isolates were very sensitive to azoxystrobin and pyraclostrobin. In other regions with CBS, azoxystrobin and pyraclostrobin were tested for field efficacy with positive results (1,19,35,44,48).

Analysis of the *cyt b* gene structure showed that a group I intron is located directly after G143 in the Florida *G. citricarpa* isolates (13). The group I introns always have the nucleotide T directly upstream from the 5' splice site and the nucleotide G before the 3' splice site (12,13). Studies in *Saccharomyces cerevisiae* revealed that a mutation from a GGT (glycine) to GAT (aspartic acid) at the codon 256 of the *cyt b* gene, which was two nucleotides upstream

from the 5' splice site of a group I intron, significantly interfered with pre-mRNA maturation processes, leading to a deficient and lethal protein (10,16). The same exon/intron boundary with group I introns has been found in other filamentous fungi such as *A. solani* (23), *B. cinerea* (7), *G. bidwellii* (34), *Monilia fructicola* (26), *Puccinia* spp. (24), and *Pyrenophora teres* (51), in which resistance to QoI fungicides conferred by the G143A substitution has not been reported yet. Recently, Stammler et al. (52) found that there were introns in the *G. citricarpa cyt b* gene but they were not identified. The size of the introns found in this study and by Stammler et al. (52) were very close but could not be compared for homology because the sequence was not available in GenBank. According to Stammler et al. (52), the risk of a G143A mutation in the *cyt b* gene causing resistance to QoI fungicides is low for *G. citricarpa* due to the presence of an intron, and it is possible to conclude that there is a low risk for the Florida *G. citricarpa* population as well. It was recently reported that, in yeast, rescue mechanisms that could potentially reestablish respiration close to wild-type levels may exist for the G143A mutation in the presence of a group I intron (56). The three hypothesized mechanisms were the deletion of the intron, overexpression of genes coding for mitochondria metal ion carriers in the nucleus, or a second mitochondrial mutation to restore exon/intron splicing (56). The rescue mechanisms are untested in the field but it indicates that the inhibition of the G143A mutation is not absolute.

Although F129L and G137R mutations in the *cyt b* gene have been reported to confer a low level of resistance to QoI fungicides, the efficacy of QoI fungicides on disease control in the greenhouse or in the field is not significantly affected (39,49,51), indicating that the use of QoI fungicides to control *G. citricarpa* harboring a F129L or G137R mutation would be still effective.

When chemical control measures fail, it may be due to selection pressure for pathogen resistance. The high specificity of the mode of action of QoI fungicides together with sexual recombination that occurs in some fungal pathogens promote the selection of resistant strains after prolonged periods of pesticide use (10,29). Several pathogens have become insensitive to QoI fungicides (20,21,25, 45,58) and the risk for *G. citricarpa* developing resistance is unknown. Because of the intron structure of the *cyt b* gene of *G. citricarpa*, there is a low probability for the G143A mutation which confers a high level of resistance. These results agree with the fact that QoI fungicides have been used extensively in countries where CBS has been present for many years but there are no reports of QoI resistance to date. The baseline study will help monitor any population shifts in the future that may occur, probably due to mutations other than the G143A.

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