Distribution of QoI Resistance in Populations of Tangerine-Infecting *Alternaria alternata* in Florida

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Abstract

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Chemical control, based on copper and quinone outside inhibitor (QoI) fungicides, has been essential for the management of brown spot of citrus, caused by *Alternaria alternata*. However, QoI control failures were detected recently in Florida. From 2008 to 2012, 817 monoconidial isolates of *A. alternata* from 46 citrus orchards were examined for sensitivity to azoxystrobin (AZ) and pyraclostrobin (PYR). Of the isolates, 57.6% were resistant to both fungicides, with effective concentration to inhibit 50% growth (EC₅₀) values greater than 5 µg/ml for AZ and 1 µg/ml for PYR. The mean EC₅₀ values for sensitive isolates were 0.139 and 0.020 µg/ml for AZ and PYR, respectively. The EC₅₀ values of both fungicides were highly correlated ($P < 0.0001$), indicating cross resistance. The proportion of resistant isolates differed sig-

nificantly ($P < 0.0001$) among cultivars and with QoI application frequency ($P < 0.0001$). However, resistance was not significantly related ($P = 0.364$) to disease severity in the field (low, moderate, and high) or isolate virulence ($P = 0.397$). The molecular basis for QoI resistance was determined for a subset of 235 isolates using polymerase chain reaction restriction fragment length polymorphism of the cytochrome *b* gene. All resistant isolates showed the point mutation G143A. Based on the presence of one or two introns, isolates were classified as profile I and profile II, respectively. The resistance frequency was significantly higher ($P < 0.0001$) in isolate profile II, suggesting a higher selection pressure for resistant population profile II.

Alternaria brown spot (ABS), caused by *Alternaria alternata* (Fr.:Fr.) Keissl., is a serious disease of tangerine cultivars (*Citrus reticulata* Blanco) and their hybrids in commercial citrus orchards in humid subtropical and semiarid growing regions (58). This fungus produces the host-selective ACT toxin (32,33), which plays an important role in host specificity and isolate virulence (23,34). Florida has 7,000 ha of tangerine and tangerine hybrids for fresh-fruit production (21). More than two-thirds of the tangerine cultivars planted in Florida are susceptible to ABS, including ‘Minneola’, ‘Nova’, and ‘Orlando’ tangelo; ‘Dancy’ and ‘Sunburst’ tangerine; ‘Murcott’ tangor, and some Dancy hybrids such as ‘Lee’ (56). The fungus can affect leaves, twigs, and young fruit, cause considerable yield loss, and produce blemishes on fruit that reduce marketability (56,58).

Management of ABS includes the use of cultural methods such as disease-free nursery stock, wide tree spacing, reduction of excessive vegetative growth, the elimination of overhead irrigation, and fungicide applications (16,57). Although cultural practices reduce disease severity, ABS control is largely dependent on fungicide applications (8,16,56). Copper-based fungicides (Fungicide Resistance Action Committee [FRAC] code M1), ferbam (FRAC code M3), and quinone outside inhibitors (QoIs; FRAC code 11) have been widely used in Florida over the last decade. Recently, Pristine (pyraclostrobin [QoI] + boscalid [FRAC code 7]) and Quadris Top (azoxystrobin [QoI] + difenoconazole [FRAC code 3]) were registered for use in citrus, increasing the options for fungicide rotations. To optimize fungicide applications for ABS control, a weather-based model (Alter-Rater) was developed (8,55); however, due to the complexity of some environmental

variables (difficulty in accurately measuring leaf wetness) used for the model, this has not been extensively adopted by growers. In Florida, growers may apply 2 to 15 fungicide sprays during the course of the growing season, depending on host susceptibility and weather conditions.

In general, QoI fungicides have a broad spectrum of activity against plant-pathogenic fungi. This includes the most important taxonomic groups; namely, Ascomycetes, Basidiomycetes, Deuteromycetes, and Oomycetes (5,6). QoI fungicides are classified as high risk for resistance development due to the site-specific mode of action. The QoI fungicides block electron transfer between cytochrome *b* and cytochrome *c* by binding to the quinol oxidation (Qo) site of the mitochondrial cytochrome *bc*₁ enzyme complex, which is a key component of the respiratory electron transfer chain (6,61). Resistance to QoI fungicides was first reported in *Blumeria graminis* f. sp. *tritici* (wheat powdery mildew) in Germany in 1988 (6). Thereafter, resistance was observed in many countries around the world among pathogens that affect many crops, including *Mycosphaerella fijiensis* in banana (52), *Plasmopara viticola* in grape (11), *Venturia inaequalis* in apple (35), *Podosphaera fusca* and *Pseudoperonospora cubensis* in cucumber (28), and *A. alternata* in pistachio (40).

A target site mutation of the cytochrome *b* gene, resulting in a specific amino acid substitution, prevents fungicide binding at the target site and causes QoI resistance (6). Three amino acid substitutions in the cytochrome *b* protein have been detected in QoI-resistant plant-pathogenic fungi and oomycetes (18). The amino acid substitution from glycine to alanine at position 143 (G143A) has been reported in most of the resistant pathogens, leading to a high level of resistance (resistance factor [RF] > 100). This mutation has been associated with QoI-control failures (20). The other two amino acid substitutions, from phenylalanine to leucine at position 129 (F129L) and glycine to arginine at position 137 (G137R), have been associated with moderate levels of resistance, and pathogen populations are usually controlled with recommended field rates of QoI fungicides (18,51).

In 2008, a commercial tangerine grower located in central Florida (Polk County) observed ABS control failure following several QoI applications. The isolates were screened for QoI sensitivity and a large proportion of isolates tested were QoI resistant (43).

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Several more suspected control failures were reported in 2009, leading to the hypothesis that QoI resistance of *A. alternata* could be widely distributed throughout the state. The objectives of this study were to determine (i) the baseline sensitivity to azoxystrobin and pyraclostrobin in *A. alternata* isolates never exposed to QoI fungicides, (ii) the in vitro sensitivity to azoxystrobin and pyraclostrobin of *A. alternata* isolates collected from commercial citrus orchards in Florida, and (iii) whether G143A was the mutation in the cytochrome *b* responsible for the resistance.

Materials and Methods

Isolate collection. From 2008 to 2009, five commercial tangerine hybrid orchards, encompassing eight blocks, were sampled from central Florida. Groves were sampled based on information provided by growers about ABS control failure using QoI fungicides. A more systematic statewide survey was initiated from 2010 to 2012 in commercial tangerine and tangerine hybrid orchards in 11 tangerine-producing counties, comprising 79% of the tangerine-production area in Florida. In 2010, 13 citrus orchards (121 ha) encompassing 18 blocks were sampled whereas, during 2011 and 2012, 12 orchards (125 ha in 23 blocks) and 18 orchards (345 ha in 29 blocks), respectively, were sampled. Disease severity was visually evaluated in the field based on symptom distribution and classified as low, moderate, and high. Low disease severity was characterized by small scattered lesions on old leaves, moderate disease severity corresponded to mild coalescing lesions on young leaves and fruit, and high disease severity showed abundant lesions in young and old leaves as well as fruit.

Samples consisted of 60 to 80 *Alternaria*-infected leaves or fruit arbitrarily selected per block from different trees. Samples were kept in sealed plastic bags on ice until pathogen isolation. Individual young lesions of approximately 4 mm² were surface disinfested with 50% ethanol for 30 s, followed by 5 min with 5% sodium hypochlorite, rinsed with sterile deionized water (SDW), blotted dry on sterile filter paper, and plated on potato dextrose agar (PDA; Becton Dickinson) amended with benomyl (10 µg/ml; DuPont Crop Protection), rifampicin (10 µg/ml; Fisher Scientific), and ampicillin (250 µg/ml; Fisher Scientific). Plates were incubated at 24°C for 5 to 7 days. Individual colonies of *A. alternata* were transferred to PDA overlaid with sterilized filter paper for long-term storage (45). Once the filter paper was colonized (5 to 7 days), it was lifted from the medium surface, placed in sterile coin envelopes, and dried for at least 6 h in a laminar-flow hood. Envelopes were stored at -20°C until further studies. Conidial production for either pathogenicity or fungicide sensitivity tests was induced by transferring 4-day-old mycelia from PDA to clarified V8 agar (100 ml of centrifuged V8 juice, 20 g of agar, and 10 g of CaCO₃ per liter). Cultures were incubated at room temperature under white fluorescent light for 7 to 12 days. Conidia were harvested by flooding the plates with 15 to 20 ml of SDW, gently rubbing the surface, and filtering through three layers of sterile cheesecloth.

Pathogenicity tests and monoconidial isolates. To differentiate between saprophytes and other *A. alternata* pathotypes, pathogenicity to tangerine was confirmed by inoculating immature leaves of Minneola tangelo, Dancy tangerine, or Murcott tangor in a detached leaf in vitro spray technique, as previously described (12). In total, 1,258 *A. alternata* isolates were tested for pathogenicity. For each fungal isolate, three replicate leaves were sprayed with 10⁵ conidia/ml using a chromatography sprayer (TLC Crown North American Professional Products). Leaves were placed in racks inside a clean 30-by-20-by-10-cm humid chamber and incubated at room temperature for 2 to 3 days. Pathogenicity was evaluated after the second or third day. Isolate virulence was determined by rating the leaf area covered by lesions on a scale of 1 to 3, where 1 = <25% leaf area diseased, 2 = between 25 and 50% leaf area diseased, and 3 = >50% leaf area diseased. An isolate was considered pathogenic if at least two leaves showed symptoms 3 days after inoculation.

To produce monoconidial cultures of pathogenic isolates, 100 µl of conidial suspension (5 × 10³ conidia/ml) was spread on 2% water agar (Becton Dickinson) and incubated in the dark at 24°C for 16 to 20 h. After incubation, one germinated conidium was transferred to PDA overlaid with sterilized filter paper for long-term storage, following the procedure described above (45).

Sensitivity of *A. alternata* isolates to azoxystrobin and pyraclostrobin. In total, 817 *A. alternata* monoconidial isolates were tested from the 2008 to 2012 sample collections. In vitro fungicide sensitivity was determined using the resazurin-based microtiter assay, as previously described (60). Briefly, technical-grade azoxystrobin (Syngenta Crop Protection) and pyraclostrobin (BASF Corp.) were dissolved in acetone at 10 mg/ml and 10-fold serially diluted. The final concentration of acetone in complete medium (7) was 0.1% by volume. Resazurin salt (Sigma-Aldrich) was dissolved in distilled water at 4 mM and filter sterilized (0.22-µm filter; Corning Inc.). Each isolate was added in triplicate into a 96-well, flat-bottom microplate (Corning Inc.). The final concentrations per well of either azoxystrobin or pyraclostrobin were 0, 0.001, 0.01, 0.05, 0.1, 0.5, 1 or 10 µg/ml and 0, 0.001, 0.005, 0.01, 0.05, 0.1, 1, or 10 µg/ml, respectively. The final conidia and resazurin concentrations (per well) were 4 × 10⁴ conidia/ml and 40 µM, respectively. Plates were covered with plastic lids, placed into a clean plastic humid chamber, and incubated in the dark at 27°C for 24 h, with shaking at 400 rpm. Fungal respiration was determined by measuring the resazurin reduction at 570 and 600 nm, as previously described (60). Absorbance was read with a microplate spectrophotometer (Bio-Rad). Isolate sensitivity was determined by the effective concentration needed to reduce resazurin by 50% (EC₅₀) using either sigmoidal or exponential decay functions (60). Isolates with EC₅₀ values for azoxystrobin and pyraclostrobin higher than 5 or 0.5 µg/ml, respectively, were classified as resistant. Isolates with EC₅₀ values lower than those values were classified as sensitive.

Baseline sensitivity of *A. alternata* to azoxystrobin and pyraclostrobin. In total, 40 *A. alternata* isolates were obtained from the

Table 1. Baseline sensitivity of *Alternaria alternata* isolates from different citrus hosts to azoxystrobin and pyraclostrobin

Isolate group	Location, county	Year	Host ^b	N ^c	Mean EC ₅₀ (µg/ml) ^a	
					Azoxystrobin	Pyraclostrobin
AR	Arcadia, DeSoto	1997	Sunburst tangerine	4	0.209 (0.098)	0.031 (0.010)
CPI	Immokalee, Collier	1996	Murcott tangor	3	0.209 (0.090)	0.036 (0.013)
EV	Lake Alfred, Polk	1996	Minneola tangelo	4	0.058 (0.017)	0.010 (0.003)
FM	Fort Meade, Polk	1996	Minneola tangelo	2	0.134 (0.032)	0.029 (0.007)
IMK	Immokalee, Collier	1997	Minneola tangelo	2	0.148 (0.192)	0.027 (0.033)
LOR	Lorida, Highlands	1997	Orlando tangelo	3	0.107 (0.112)	0.029 (0.015)
RAN	Immokalee, Collier	1997	Sunburst tangerine	5	0.070 (0.039)	0.015 (0.008)
SH	Polk City, Polk	1996	Minneola tangelo	6	0.178 (0.127)	0.032 (0.017)
VB	Vero Beach, Indian River	1997	Grapefruit	6	0.167 (0.121)	0.020 (0.013)
WP	West Palm Beach, Palm Beach	1996	Minneola tangelo	5	0.082 (0.043)	0.010 (0.006)

^a EC₅₀ = effective concentration needed to reduce fungal growth by 50%. Values in parentheses denote standard deviation.

^b Tangerine = *Citrus reticulata*, tangor = *C. reticulata* × *C. sinensis*, tangelo = *C. paradisi* × *C. reticulata*, and grapefruit = *C. paradisi*.

^c Number of isolates tested.

Dewdney lab culture collection (Table 1). These isolates were collected from tangerine hybrids and grapefruit groves where there was no history of QoI fungicide applications prior to 2000. Sensitivity to azoxystrobin and pyraclostrobin was determined using the resazurin-based microtiter assay, as described above.

DNA extraction and molecular detection of the G143A mutation. A subsample of 161 *A. alternata* isolates, phenotypically identified as QoI resistant, were arbitrarily selected to confirm the presence of the G143A mutation using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis (60). Additionally, 74 QoI-sensitive isolates were also arbitrarily selected to validate the lack of the G143A mutation. Genomic DNA was extracted from 3-day-old mycelia cultured on PDA overlaid with cellophane sheets. Mycelia were transferred into a 2-ml microcentrifuge tube containing a 5-mm-diameter stainless steel bead (Qiagen) and disrupted with a TissueLyser II (Qiagen) twice for 30 s and an oscillation frequency of 30 Hz/s. DNA was extracted using the DNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions. The total DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The cytochrome *b* gene of *A. alternata* isolates was partially amplified using the primers cytb2f (5'-CTA TGG ATC TTA CAG AGC AC-3') and DTRcytb2-INtr (5'-GTA TGT AAC CGT CTC CGT C-3'). PCR reactions were performed in a Peltier Thermal cycler (MJ Research Inc.) in a final volume of 25 μ l containing 1 unit of HotStart Taq *Plus* polymerase (Qiagen), 2.5 μ l of 10 $\times$ PCR buffer, 200 μ M each dNTP, 0.3 μ M each primer, and 0.5 to 20 ng of fungal DNA template. Cycling parameters were performed as previously described (60). PCR products were visualized with UV light after electrophoresis in 1.2% agarose gel with 1 $\times$ Tris-acetate-EDTA buffer stained with SYBR DNA gel stain (Invitrogen life Technologies).

PCR products were purified using the MiniElute PCR purification kit (Qiagen) following the manufacturer's instructions. Purified PCR (10 μ l) product was digested with restriction enzyme *Fnu*4HI (New England Biolabs) at 37°C for 1 h, according to the manufacturer's recommendations. Restriction fragments were separated and visualized by electrophoresis in 1.6% agarose gel.

Statistical analysis. Absorbance values from resazurin reduction tests were plotted against the log₁₀-transformed fungicide concentration, and EC₅₀ values (expressed as micrograms per milliliter) were calculated for each isolate using either sigmoidal or exponential decay functions (60) with SigmaPlot (ver. 11.0; Systat Software Inc.). EC₅₀ values were log₁₀-transformed prior to testing for normality using the Shapiro-Wilk test in PROC UNIVARIATE (ver. 9.3; SAS Institute) and graphically analyzed. Probabilistic distributions of baselines were calculated with InfoStat (ver. 2004; InfoStat). To determine whether EC₅₀ values of QoI fungicides from sensitive and resistant isolates were significantly higher than those from baseline isolates, a two-sample *t* test and a nonparametric Kruskal Wallis test were performed using PROC TTEST (SAS Institute) and InfoStat, respectively. The χ^2 tests were performed to examine whether the proportion of QoI-resistant isolates was distributed randomly over cultivars, field disease severity, virulence class, and number of QoI applications per year using PROC FREQ (SAS Institute). Cross-resistance between azoxystrobin and pyraclostrobin was determined by calculating a Pearson correlation coefficient using PROC REG and PROC CORR (SAS Institute).

Results

Baseline sensitivity to azoxystrobin and pyraclostrobin. The 40 monoconidial *A. alternata* isolates were log-normally distributed for both azoxystrobin ($W = 0.9621$, $P = 0.1969$) and pyraclostrobin ($W = 0.9451$, $P = 0.0516$). The range of EC₅₀ values for azoxystrobin was 0.012 to 0.356 μ g/ml, with mean and median EC₅₀ values of 0.135 and 0.098 μ g/ml, respectively (Table 1; Fig. 1A). Pyraclostrobin had a higher intrinsic activity than azoxystrobin. EC₅₀ values for pyraclostrobin were 0.003 to 0.053 μ g/ml, with mean and median EC₅₀ values of 0.023 and 0.020 μ g/ml, respectively (Table 1; Fig. 1B). The probability of an isolate

from the baseline population sampled showing an EC₅₀ value greater than 5 and 0.5 μ g/ml, proposed as discriminatory concentrations to differentiate sensitive and resistant isolates for azoxystrobin and pyraclostrobin respectively, was 2.01×10^{-6} for azoxystrobin and 8.74×10^{-6} for pyraclostrobin. A significant positive correlation ($r = 0.8001$, $P < 0.0001$) was observed between azoxystrobin and pyraclostrobin EC₅₀ values.

***A. alternata* isolate collection.** From 2008 to 2012, 2,653 *A. alternata* isolates were collected in Florida from 46 commercial citrus orchards encompassing 78 blocks. Of 2,653 isolates, 1,258 were tested for pathogenicity, of which 290 and 165 isolates were from Dancy and Sunburst tangerine, respectively; 21, 315, and 40 were from Lee, Minneola, and Orlando tangelo, respectively; and 427 were from Murcott tangor. More than 92% of Dancy, Lee, Minneola, and Orlando isolates were pathogenic, whereas, with Murcott and Sunburst, the proportion of pathogenic isolates was 84 and 68%, respectively.

QoI resistance in Florida citrus orchards. Among the 817 field isolates evaluated, 471 (57.6%) were resistant to both azoxystrobin and pyraclostrobin (Table 2). A completely QoI-resistant population was observed in 19 orchards, encompassing 25 blocks; mixed populations of QoI-resistant and -sensitive isolates were observed in 24 orchards (39 blocks); whereas a completely sensitive population was observed in 9 orchards (14 blocks). The frequency of QoI-resistant isolates in orchards with mixed populations varied from 7 to 95%. The presence of QoI-resistant isolates was widespread and very common across tangerine-producing counties in Florida (Fig. 2).

Sensitivity distribution of *A. alternata* isolates to azoxystrobin and pyraclostrobin. The sensitivity distribution of

Fig. 1. Baseline sensitivity distribution of *Alternaria alternata* isolates ($n = 40$) to **A**, azoxystrobin and **B**, pyraclostrobin, based on effective concentration needed to reduce fungal growth by 50% (EC₅₀) values.

Table 2. Origin, history of fungicide exposure, and detection of quinone outside inhibitor (QoI) resistance in *Alternaria alternata* isolates collected in Florida citrus groves from 2008 to 2012

Isolate group	Location, county	Year	Exposures ^a	Host ^b	Severity ^c	Class ^d	Number of isolates tested	Number of resistant isolates ^e
1	Fort Meade, Polk	2010	1	Murcott tangor	Low	1.5	14	1
2	Haines City, Polk	2010	4	Minneola tangelo	Low	2.5	16	16
		2010	4	Murcott tangor	Low	2	14	6
3	Umatilla, Lake	2011	2	Minneola tangelo	NA	3	12	12
4	Tavares, Lake	2012	2	Minneola tangelo	Moderate	2	10	0
5	Fort Myers, Lee	2012	3	Murcott tangor	Moderate	2	24	5
		2012	3	Sunburst tangerine	Low	1	2	1
6	Avon Park, Highlands	2011	2	Minneola tangelo	Moderate	2	16	13
	Avon Park, Highlands	2011	2	Murcott tangor	Moderate	2	27	25
	Babson Park, Polk	2011	2	Murcott tangor	Low	2	15	14
	Fort Meade, Polk	2011	2	Murcott tangor	Moderate	2	16	14
	Frostproof, Polk	2009	2	Murcott tangor	High	2.5	10	5
	Frostproof, Polk	2011	2	Minneola tangelo	Moderate	2	16	6
	Frostproof, Polk	2011	2	Murcott tangor	Low	2	8	7
7	Crooked Lake, Polk	2010	1	Murcott tangor	Low	2	14	1
8	Bartow, Polk	2012	1	Murcott tangor	Moderate	2	11	8
9	Davenport, Polk	2010	2	Murcott tangor	High	3	15	2
10	Bartow, Polk	2012	1	Sunburst tangerine	Low	2	12	0
11	Winter Haven, Polk	2010	1	Dancy mandarin	High	3	15	8
12	Clermont, Lake	2012	2	Murcott tangor	High	2	12	6
		2012	2	Sunburst tangerine	Low	2	11	10
13	Felda, Hendry	2012	2	Murcott tangor	Moderate	3	12	6
14	Dundee, Polk	2009	2	Murcott tangor	High	2	23	23
15	Lake Wales, Polk	2010	2	Minneola tangelo	NA	3	13	13
16	Fort Pierce, St. Lucie	2012	2	Murcott tangor	Low	3	2	0
17	Fort Meade, Polk	2012	4	Murcott tangor	Moderate	2	9	9
		2012	1	Sunburst tangerine	Moderate	2	10	4
18	Fort Pierce, St. Lucie	2011	4	Minneola tangelo	Moderate	2	10	10
19	Fort Pierce, St. Lucie	2010	NA	Minneola tangelo	NA	3	14	14
20	Immokalee, Collier	2012	>1	Murcott tangor	Low	2	11	2
21	Vero Beach, Indian River	2011	4	Minneola tangelo	High	3	30	30
22	Vero Beach, Indian River	2011	NA	Sunburst tangerine	Low	2	3	2
23	Venus, Highlands	2012	2	Murcott tangor	Low	2.5	12	3
24	Immokalee, Collier	2012	2	Murcott tangor	Moderate	3	12	2
25	Lake Wales, Polk	2010	2	Murcott tangor	Moderate	2	15	5
26	Zolfo Springs, Hardee	2011	2	Minneola tangelo	High	2	10	10
27	St. Cloud, Osceola	2010	1	Sunburst tangerine	Moderate	1	9	5
28	Polk City, Polk	2012	>3	Sunburst tangerine	Moderate	2	12	8
29	Clermont, Lake	2012	1	Orlando tangelo	High	1	11	0
		2012	1	Sunburst tangerine	Moderate	1	11	3
30	Oviedo, Seminole	2009	>4	Minneola tangelo	High	3	2	2
31	Fort Meade, Polk	2010	0	Murcott tangor	Moderate	1	11	0
		2010	0	Sunburst tangerine	Moderate	2	10	0
32	Lake Wales, Polk	2011	3	Minneola tangelo	Moderate	2	11	11
33	Nokomis, Sarasota	2011	2	Minneola tangelo	Moderate	2	9	9
34	Vero Beach, Indian River	2011	4	Minneola tangelo	Low	2	10	10
35	Bartow, Polk	2012	2	Murcott tangor	Low	2	1	1
36	Fort Pierce, St. Lucie	2011	4	Minneola tangelo	High	2	23	23
		2011	4	Murcott tangor	Low	1	1	1
37	Eustis, Lake	2012	2	Minneola tangelo	High	2	10	0
		2012	2	Orlando tangelo	Moderate	2	9	0
38	Immokalee, Collier	2012	2	Sunburst tangerine	Moderate	2	20	17
39	Haines City, Polk	2010	>3	Murcott tangor	Moderate	3	16	14
		2010	>3	Orlando tangelo	Low	2	16	12
40	Winter Haven, Polk	2008	>6	Dancy mandarin	High	3	20	19
		2010	>5	Dancy mandarin	High	2	16	12
41	Vero Beach, Indian River	2011	NA	Minneola tangelo	Moderate	3	10	10
42	Haines City, Polk	2010	2	Lee citrus hybrid	High	2	9	0
		2010	2	Minneola tangelo	High	2	10	0
		2010	2	Murcott tangor	High	2	9	0
43	Wauchula, Hardee	2011	1	Murcott tangor	NA	2	12	12
44	Mt. Dora, Lake	2012	NA	Minneola tangelo	High	2	11	0
		2012	NA	Murcott tangor	Low	2	10	0
45	Grand Island, Lake	2012	2	Lee citrus hybrid	Moderate	3	12	1
		2012	2	Murcott tangor	Moderate	3	12	3
		2012	2	Sunburst tangerine	Low	2.5	12	0
46	Oviedo, Seminole	2009	>4	Minneola tangelo	High	3	6	5

^a QoI exposures per year; NA = not available.

^b Tangor = *Citrus reticulata* × *C. sinensis*, tangelo = *C. reticulata* × *C. paradisi*, tangerine or mandarin = *C. reticulata*, and citrus hybrid = *C. reticulata* × tangelo.

^c Field severity was evaluated based on symptom distribution, where Low = small scattered lesions on old leaves, Moderate = mild coalescing lesions on young leaves and fruit, and High = abundant lesions in young and old leaves as well as fruit; NA = not available.

^d Median virulence class. Virulence tested by spray inoculation of detached tangerine leaves and rated 48 to 72 h after incubation, where 1 = <25%, 2 = 25 to 50%, and 3 = >50% leaf area.

^e Resistant isolates grew on complete medium in resazurin-based microtiter assay amended with azoxystrobin at >5 µg/ml or pyraclostrobin at >0.5 µg/ml. Sensitive isolates did not grow at those fungicide concentrations.

A. alternata isolates showed a clear division between QoI-sensitive and -resistant populations (Fig. 3). The EC_{50} values of the sensitive populations for azoxystrobin and pyraclostrobin were 0.012 to 0.836 and 0.002 to 0.116 $\mu\text{g/ml}$, respectively, resulting in mean EC_{50} values of 0.139 and 0.020 $\mu\text{g/ml}$, respectively. The mean EC_{50} values of sensitive populations for both azoxystrobin and pyraclostrobin was not significantly different ($t = -0.51$, $P = 0.6095$ and $t = 1.79$, $P = 0.0739$, respectively) than the baseline populations. EC_{50} values of the resistant populations were 6.626 to >10 $\mu\text{g/ml}$ for azoxystrobin and 1.130 to >10 $\mu\text{g/ml}$ for pyraclostrobin, with a median EC_{50} value of >10 and 5.507 $\mu\text{g/ml}$ for azoxystrobin and pyraclostrobin, respectively. On the other hand, the median EC_{50} values of resistant populations was significantly higher ($H = 110.39$, $P < 0.0001$ for azoxystrobin and pyraclostrobin) than the baseline populations. RF, expressed as the ratio of the EC_{50} value of a pyraclostrobin-resistant isolate to the mean EC_{50} for pyraclostrobin baseline sensitive isolates, was unimodally distributed (Supplementary Figure S1). The RF was 50 to >439 , with a median of 241.5.

Cross-resistance to QoI fungicides. A χ^2 analysis of 817 QoI-resistant and -sensitive isolates ($P = 0.999$) and Pearson correlation analysis of 346 QoI-sensitive isolates showed that EC_{50} values for azoxystrobin and pyraclostrobin were highly correlated ($r = 0.866$, $P < 0.0001$), suggesting a high level of cross-resistance between the two QoI fungicides (Fig. 4).

QoI resistance associated with citrus hosts and orchard factors. The frequency of resistant isolates was significantly different ($\chi^2 = 97.534$, $P < 0.0001$) among cultivars (Fig. 5). Lee showed the lowest proportion of resistant isolates. The highest proportion of resistant isolates was found in Minneola and Dancy and they did not significantly differ ($\chi^2 = 0.051$, $P = 0.822$). Moreover, the pro-

portion of resistance isolates was not significantly different ($\chi^2 = 4.33$, $P = 0.115$) among Murcott, Orlando, and Sunburst (Fig. 5).

The proportion of resistant isolates was not significantly different (Table 3) among the field disease severity categories (low, moderate, and high) evaluated. In orchards with low disease severity, 47% of the isolates were resistant. In orchards with moderate disease severity, the proportion of resistant isolates was 55% whereas, in orchards with high disease severity, 60% of the isolates tested were resistant (Table 3).

No significant differences in the proportion of resistant isolates were observed when *A. alternata* virulence class was examined (Table 3). Nevertheless, in Minneola, the proportion of resistant isolates was significantly different ($\chi^2 = 24.554$, $P < 0.0001$) among virulence classes. Almost 90% of resistant isolates from Minneola were highly virulent whereas, in other cultivars, the proportion of resistance was evenly distributed (*data not shown*).

A positive correlation was found between number of QoI applications and frequency of resistance ($r = 0.938$; $P = 0.0056$). Resistant isolates were found more frequently in orchards with more than four QoI applications per year (Table 3). On the other hand, in orchards with no QoI exposure, no resistant isolates were detected. In general, the proportion of resistant isolates increased with number of QoI applications per year.

Detection of the G143A mutation. A PCR-RFLP analyses was used to differentiate between isolates carrying the wild-type GGT codon (glycine) at amino acid 143 and those with the mutant allele containing a GCT (alanine) codon in the *A. alternata* population. Two distinct cytochrome *b* gene structures were identified based on the presence of one or two introns (60). A 377-bp fragment was amplified in isolates carrying one intron, designated as profile I, whereas a 1,564-bp fragment was amplified in isolates carrying

Fig. 2. Geographic distribution of quinone outside inhibitor-sensitive and -resistant *Alternaria alternata* isolates collected in 46 commercial citrus orchards encompassing 78 blocks from 2008 to 2012.

two introns, designated as profile II. The restriction enzyme *Fnu*4HI generated two fragments in isolates carrying the mutant allele G143A. The length of the fragments was 123p and 254 bp for profile I isolates and 254 and 1,310 bp for profile II isolates (Fig. 6). The fragments of 377 and 1,564 bp remained undigested in wild-type isolates profile I and profile II, respectively (Fig. 6B). The amino acid substitution G143A was present in all 161 QoI-resistant isolates tested and absent in all 74 sensitive isolates tested.

Between sensitive and resistant isolates, the proportion of isolates carrying the cytochrome *b* profile I or profile II was significantly different ($\chi^2 = 118.824$, $P < 0.0001$). The proportion of profile II isolates was 54% in resistant isolates and only 1% in sensitive isolates (Fig. 7). The proportion of profile I isolates among the populations tested was less skewed. In all, 31% of sensitive isolates and 14% of resistant isolates were profile I (Fig. 7).

Discussion

QoIs have been used for more than a decade in Florida for ABS control (16). Due to their effectiveness against *A. alternata* and good weathering properties, growers adopted them very quickly. Despite their excellent characteristics, resistance to QoI fungicides has been detected in many plant pathogens within a few years of their introduction to a cropping system (22). QoI resistance in *Alternaria* populations has been identified in vegetables as well as fruit and nuts such as pistachio (40), pear (27), apple (38), and potato (44). In 2008, QoI resistance of tangerine-infecting *A. alternata* isolates was detected in Polk County, FL (43), making ABS management considerably more difficult and costly. Tangerine production in Florida is mainly for the fresh-fruit market; therefore, the production of disease-free fruit is paramount and is heav-

ily reliant on fungicides, thus increasing the risk for resistance development. This is particularly true for the site-specific fungicides such as the QoIs. Our study represents the first report of the statewide QoI sensitivity distribution in the tangerine-infecting *A. alternata* population of Florida using both phenotypic and molecular approaches.

In the current study, the baseline distribution of *A. alternata* to azoxystrobin and pyraclostrobin was established using never-exposed isolates collected before the introduction of QoIs to citrus. In fungicide resistance monitoring programs, the establishment of baseline sensitivity is of great importance. It can be used to monitor for changes in the sensitivity profiles of target populations exposed to the tested modes of action over time. Comparisons of new populations to the baseline can give evidence of increasing frequency of resistance in the population that could lead to future control failure (50). The baseline distribution of azoxystrobin and pyraclostrobin was log-normally distributed within a relatively narrow range. The mean EC_{50} value for azoxystrobin obtained in our baseline study (0.135 $\mu\text{g/ml}$) was a little higher than the mean EC_{50} value observed in other wild-type *Alternaria* spp. For 14 pistachio-infecting *A. alternata* isolates, the mean EC_{50} value was 0.023 $\mu\text{g/ml}$ (40) whereas, in 32 *A. solani* isolates from potato, the

Fig. 3. Sensitivity distribution of *Alternaria alternata* isolates ($n = 817$) collected in Florida citrus orchards from 2008 to 2012 to **A**, azoxystrobin and **B**, pyraclostrobin, based on effective concentration needed to reduce fungal growth by 50% (EC_{50}) values. Black circles denote EC_{50} values for individual isolates; while small gray dots denote resistant isolates with EC_{50} values $>10 \mu\text{g/ml}$.

Fig. 4. Correlation of effective concentration needed to reduce fungal growth by 50% (EC_{50}) values of azoxystrobin and pyraclostrobin in quinone outside inhibitor-sensitive *Alternaria alternata* isolates ($n = 346$).

Fig. 5. Distribution of sensitive ($n = 346$) and resistant ($n = 471$) *Alternaria alternata* isolates to azoxystrobin and pyraclostrobin collected during 2008 to 2012 from Florida tangerine and tangerine hybrids orchards. Isolates were considered resistant when they grew on complete medium in resazurin-based microtiter assay amended with fungicide at concentrations >5 or $>0.5 \mu\text{g/ml}$ for azoxystrobin and pyraclostrobin, respectively.

mean EC₅₀ value was 0.038 µg/ml (44,49). Some differences are expected because two different methods were used to determine the EC₅₀ values (resazurin-based microtiter test versus spore germination assay) and because of the intrinsic interspecies variation in fungicide sensitivity. Our baseline result for azoxystrobin did not match the study done by Mondal et al. (42), in which the mean EC₅₀ value from five wild-type *A. alternata* citrus isolates was >100 µg/ml. However, this is likely because Mondal et al. (42) determined the EC₅₀ with a mycelial growth inhibition test and it appears that *A. alternata* mycelium is insensitive to azoxystrobin but not to pyraclostrobin.

The sensitivity distribution of the *A. alternata* populations to azoxystrobin and pyraclostrobin from the 2008 to 2012 surveys shows two clear populations of QoI-sensitive and -resistant isolates. This type of distribution suggests a disruptive, monogenic resistance, as expected for site-specific fungicides like QoIs (11). Our results revealed that *A. alternata* isolates continued to be more sensitive to pyraclostrobin than azoxystrobin, consistent with previous studies on several pathogens from citrus and vegetable crops (2,10,42,48). A positive correlation was found between azoxystrobin and pyraclostrobin, demonstrating cross-resistance between these two QoI fungicides, as previously described in other systems (13,19,31,35,52). Based on the EC₅₀ values of sensitive and resistant populations, we established discriminatory concentrations for both QoI fungicides for rapid identification of resistant isolates. We suggest the discriminatory concentrations of 5 and 0.5 µg/ml for azoxystrobin and pyraclostrobin, respectively, which approximately corresponds to the lowest EC₅₀ values of resistant isolates. The mean resazurin reduction of the sensitive population exposed at concentrations of 5 or 0.5 µg/ml for azoxystrobin and pyraclostrobin, respectively, decreased to less than 25% while the resistant population, exposed at the same fungicide concentrations, maintained the same levels of resazurin reduction as non-QoI-amended media (higher than 90%). The EC₅₀ values for the pyraclostrobin-resistant population had a left-skewed distribution, where 88% of resistant isolates had an RF greater than 100 and all carried the G143A mutation in the cytochrome *b* gene.

Our study shows that *A. alternata* resistance to azoxystrobin and pyraclostrobin is widespread in commercial citrus orchards in Florida; QoI-resistant isolates were found in 64 of 78 blocks sampled. The proportion of resistant isolates was greater than 25% in 73% of the blocks sampled, indicating that QoI resistance is likely to continue to increase with continued selection. Similarly, selection for QoI resistance was observed in almond- and pistachio-infecting

A. alternata, where 36 of 41 orchards sampled in 2005 showed more than 90% azoxystrobin-resistant isolates (39). The resistant population was most prevalent in the Indian River region on the east coast of Florida. This region comprises 12% of the total tangerine production area, consisting mainly of Minneola tangelo and Murcott tangor. Minneola commands a high fresh-fruit market price but, of the commercial tangerine cultivars grown in this area, it is also the most susceptible to ABS (53,58). To obtain good fruit quality and premium prices, more fungicide applications are often needed to manage disease on this cultivar compared with less-susceptible, lower-value cultivars such as the Sunburst tangerine. In the Indian River region, growers have used QoI fungicides more than three times per year, suggesting that the QoI-resistant population has been selected over time, as previously described in pistachio-infecting *A. alternata* (40). In contrast, the central region (Polk, Lake, Highlands, and Osceola Counties) is the largest tangerine-production area, comprising 48% of the total tangerine production area, and the cultivar diversity is higher compared with other regions; therefore, fewer QoI applications per year occur. In Florida, disease management programs and, hence, QoI selection pressure has varied according to the level of cultivar susceptibility to ABS. Sunburst tangerine is one of the least susceptible cultivars; thus, growers used fewer fungicide applications for ABS control on Sunburst than on Minneola tangelo. In fact, four of the seven Sunburst producers in the central region used QoI fungicides no more than once per year. Despite the low numbers of applications on Sunburst, some groves still showed a considerable proportion of resistant isolates (>30%), suggesting that only a few QoI applications are needed to start the selection of a resistant population.

Susceptibility to ABS is a dominant trait from the Dancy mandarin parent while resistance to ABS is controlled by a recessive allele (15,26). Many of the grapefruit × tangerine and tangerine × sweet orange hybrids are the result of crosses with Dancy tangerine and, therefore, susceptible to ABS. We observed higher disease intensity in the most susceptible cultivars, Minneola and Dancy, compared with the less susceptible cultivars, Murcott or Sunburst. In fact, 97% of isolates recovered from brown spot lesions on Minneola were pathogenic, whereas only 68% of Sunburst isolates were pathogenic, consistent with previous reports (45). Cultivar susceptibility to ABS was closely associated with QoI-resistance frequency and may be contributing to decreased efficacy of fungicide programs in some citrus orchards. The most susceptible cultivars, Minneola and Dancy, had a higher proportion of QoI-resistant isolates than the other cultivars. At least two factors could contrib-

Table 3. A χ^2 analysis of quinone outside inhibitor (QoI)-resistant isolates of *Alternaria alternata* collected in Florida from 2008 to 2012 according to field severity, virulence class, and number of QoI applications per year

Response variable, levels	Number of isolates tested	Number of resistant isolates ^a	χ^2	df ^b	P value
Field severity ^c					
Low	184	87	2.021	2	0.3641
Moderate	340	188	...	...	...
High	242	145	...	...	...
Virulence class ^d					
1	163	81	1.849	2	0.3970
2	341	192	...	...	...
3	300	186	...	...	...
QoI exposures ^e					
0	21	0	38.225	5	<0.0001
1	119	42	...	...	...
2	391	209	...	...	...
3	37	17	...	...	...
4	157	139	...	...	...
>5	44	38	...	...	...

^a Resistant isolates grew on complete medium in resazurin-based microtiter assay amended with azoxystrobin at >5 µg/ml or pyraclostrobin at >0.5 µg/ml. Sensitive isolates did not grow at those fungicide concentrations.

^b Degrees of freedom.

^c Field severity was evaluated based on symptom distribution, where Low = small scattered lesions on old leaves, moderate = mild coalescing lesions on young leaves and fruit, and high = abundant lesions in young and old leaves as well as fruit.

^d Virulence tested by spray inoculation of detached tangerine leaves and rated 48 to 72 h after incubation, where 1 = <25%, 2 = 25 to 50%, and 3 = >50% leaf area.

^e Number of applications per year.

ute to this finding: first, the abundant sporulation produced on Minneola leaf litter as well as infected leaves still in the canopy leading to increase the inoculum density and disease pressure (59); and, second, the selection pressure on QoI-resistant populations. Growers tend to apply fungicides more frequently when disease pressure is high, putting greater selection pressure on the pathogen population which, over subsequent generations, can lead to higher frequencies of resistance. Later, control failure was observed as a result of the dominance of resistant isolates in the population. Growers observed QoI-control failure when the proportion of resistance in a population was greater than 65%. On average, more than three QoI applications per year were made in Dancy and Minneola orchards while fewer than two were used in the less susceptible cultivars Murcott, Sunburst, Orlando, and Lee. Consequently, QoI selection pressure was higher in the most susceptible cultivars, increasing the frequency of resistant populations over time.

Regardless of cultivar, QoI resistance was not related to the disease severity levels observed in the field. This result implies that QoI resistance in the *A. alternata* population did not impose a negative effect on pathogenic fitness over time; thus, resistant isolates are able to compete under similar conditions with wild-type isolates. Previous studies indicated no fitness effects for QoI-resistant isolates of *Magnaporthe grisea* (3), *Plasmopara viticola* (14), *Erysiphe graminis* f. sp. *tritici* (13), or *A. alternata* (30), among others, and they successfully competed with the sensitive isolates for the same ecological niche. No relationship was observed between QoI exposures per year and field disease severity categories. Nevertheless, the greatest proportion of resistant isolates (60%) was obtained from orchards with high disease severity, which implies that resistant isolates were selected and a resistant population built up over time. Likewise, our results showed a lack

of relationship between resistant isolates and virulence. Peever et al. (47) found that virulence was similar in different citrus cultivars inoculated with Nova and grapefruit isolates. Disease severity and host specificity of *A. alternata* isolates depend on the production of the host-specific ACT toxin (32,46), controlled by a gene cluster

Fig. 7. Frequency distribution of *Alternaria alternata* isolates sensitive ($n = 74$) and resistant ($n = 161$) to azoxystrobin and pyraclostrobin according to cytochrome *b* profiles. Profile I isolates had one intron at amino acid F164. Profile II isolates had two introns at amino acid positions A126 and F164. Isolates were considered resistant when they grew on complete medium in resazurin-based microtiter assay amended with fungicide at concentrations >5 or >0.5 $\mu\text{g/ml}$ for azoxystrobin and pyraclostrobin, respectively, and showed the G143A point mutation in the cytochrome *b* gene.

Fig. 6. Restriction-fragment-length-polymorphism analysis of the partial structure of the cytochrome *b* gene from *Alternaria alternata* isolates that are either quinone outside inhibitor sensitive or resistant. **A**, Restriction enzyme *Fnu4HI* produced two fragments of 123 and 254 bp in resistant isolates (G143A) carrying the *cytb* profile I whereas, in isolates carrying the *cytb* profile II, the fragments obtained were of 254 and 1,310 bp. Boxes indicate exons and lines indicate introns. Lengths of exons and introns are not to scale. **B**, Detection of the G143A mutation with the restriction enzyme *Fnu4HI*, followed by electrophoresis on 1.6% agarose gels. Lane M = 100-bp DNA ladder (Invitrogen Life Technologies); lanes 1, 2, 3, 4, 6, 9, 10, 11, 13, 14, 15, 16, 17, 18, 19, 21, 22, 23, 24, 25, 26, 27, 28, 30, and 38 denote resistant isolates carrying the *cytb* profile II; lanes 5, 7, 8, 12, 20, and 37 denote resistant isolates carrying the *cytb* profile I; and lanes 29, 31, 32, 33, 34, 35, and 36 denote sensitive isolates carrying the *cytb* profile I.

involved in the biosynthesis of the toxin (1). Therefore, QoI resistance and virulence are two unrelated mechanisms present in *A. alternata* populations and not necessarily expected to be related to each other.

Only the amino acid substitution of glycine for alanine at codon 143 (G143A) in the cytochrome *b* was found in the QoI-resistant population. This mutation has been identified in many plant pathogens (22) and confers a high level of resistance (RF higher than 100). The G143A mutation is related to control failure of QoI fungicides (20), such as observed for *A. alternata* in pistachio (40), *V. inaequalis* in apple (37), *E. necator* in grape (41), *Cercospora beticola* in sugar beet (9), *Botrytis cinerea* in multiple crops (17,29,36), and others. Overall, the G143A mutation was observed in isolates with EC₅₀ values greater than 6 µg/ml for azoxystrobin and greater than 1 µg/ml for pyraclostrobin, which corresponded to an RF greater than 50. The RF lower than 100 observed in 12% of resistant isolates could be attributed to the method used to determine the EC₅₀ value. Because the resazurin-based microtiter assay is a very sensitive test, the resazurin reduction measurement could be affected by the conidial germination rate of individual isolates, giving a lower EC₅₀ value (60). In fact, the rate of resazurin reduction of some of the resistant isolates with an RF below 100 was lower than 62%, compared with the normal range (between 85 and 95%) observed in the majority of all isolates. A lower conidial germination rate could cause the underestimation of the overall dye reduction produced by a specific isolate, decreasing the EC₅₀ value.

Two *A. alternata* genotypes, profile I and profile II, were evenly distributed in Florida. The genotype designation was based on the presence of one or two introns in the amino acid region of 103 to 268 in the cytochrome *b* gene (60). Profile I isolates had only one intron at amino acid position 164 (F164). Profile II isolates had two introns, the first one at amino acid position 126 (A126) and the second one at position F164 (60). The cytochrome *b* gene structures of *A. alternata* from sunflower and *A. solani* from potato were characterized previously (24). No introns were observed in the cytochrome *b* of *A. alternata*, whereas four introns at positions A126, G143, V146, and F164 were observed in the cytochrome *b* of *A. solani* (24). The high level of identity (>82%) observed between the sequence of both *A. alternata* cytochrome *b* introns (A126 and F164) and their homologs in *A. solani* leads us to a hypothesis of recombination events within this particular region of the *A. alternata* genome (*unpublished data*). Heterogeneity in the cytochrome *b* gene (isolates with two or three introns) has been described previously in *B. cinerea* from cucumber and tomato (4); however this is the first description of cytochrome *b* heterogeneity in an imperfect fungus such as *Alternaria*. Genetic variation of *A. alternata* populations in citrus was documented previously using random amplified polymorphic DNA markers and *endo-polygalacturonase* gene sequencing (46). Two well-defined phylogenetic lineages were identified in a single grove of Minneola tangelo in Florida (54), revealing an unexpected level of genetic variability within this clonally reproducing species. Nevertheless, the implications of cytochrome *b* heterogeneity and the genetic diversity of *A. alternata* populations are unknown. Sequence analysis of the cytochrome *b* gene in several fungal plant pathogens revealed differences in the intron/exon structure among species (20). Furthermore, the presence of a bi-group I intron between amino acids 143 and 144 appears to prevent the G143A mutation in the cytochrome *b* (24,25). However, our *A. alternata* population did not have an intron in that location; therefore, the intrinsic risk to evolve G143A mutation is high as observed in this study. Interestingly, the majority of resistant isolates that we evaluated (127 of 161) belong to isolate profile II. However, it is unknown whether cytochrome *b* structure is related to the evolution of QoI resistance in tangerine-infecting *A. alternata*, or why the frequency of the mutant allele was higher in isolates carrying two introns. Further studies are needed to elucidate the effect of QoI fungicides on the population structure of both groups of isolates.

In summary, after testing a large collection of *A. alternata* isolates for azoxystrobin and pyraclostrobin sensitivity, we concluded

that QoI resistance is widespread in Florida and a real concern for tangerine producers. QoI-control failure detected in some groves was produced by the dominance of a resistant population possessing the amino acid substitution G143A. Therefore, regardless of ABS susceptibility, growers must be careful with the use of QoI fungicides to avoid the selection and development of resistance, especially in orchards where resistance has not yet been identified. Our study also identified two groups of isolates with different cytochrome *b* gene structures, where the majority of resistant isolates were in the group carrying two introns. Finally, more effective disease management strategies, such as rotation of fungicides with different modes of action and the exclusion of QoI fungicides where resistance has been detected, are fundamental in ABS control programs. Newer fungicides (difenoconazole and boscalid) in mixtures with QoI have been registered recently and can be used in rotation with copper and ferbam for ABS control. However, further research is needed to establish baseline sensitivities for those new active ingredients so that they can be monitored for resistance development.

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