Widespread QoI Fungicide Resistance Revealed Among *Corynespora cassiicola* Tomato Isolates in Florida

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Abstract

Target spot of tomato caused by *Corynespora cassiicola* is one of the most economically destructive diseases of tomato in Florida. A collection of 123 isolates from eight counties in Florida were evaluated for sensitivity to azoxystrobin and fenamidone based on mycelial growth inhibition (MGI), spore germination (SG), detached leaflet assays (DLAs), and sequence-based analysis of the cytochrome b gene (*cyt b*). Cleavage of *cyt b* by restriction enzyme (*Fnu4HI*) revealed the presence of a mutation conferring a glycine (G) to alanine (A) mutation at amino acid position 143 (G143A) in approximately 90% of the population, correlating with quinone outside inhibitor (QoI) resistance based on MGI (<40% at 5 µg/ml), SG (<50% at 1 and 10 µg/ml), and DLA (<10% severity reduction). The mutation conferring a phenylalanine (F) to leucine (L) substitution at position 129 (F129L) was confirmed in moderately resistant isolates (#9, #19, and #74)

based on MGI (40 to 50% at 5 µg/ml), SG (<50% at 1 µg/ml and >50% at 10 µg/ml), and DLA (>10% and <43% severity reduction) for both QoI fungicides, whereas sensitive isolates (#1, #4, #7, #28, #29, #46, #61, #74, #75, #76, #91, #95, and #118) based on MGI (>50% at 5 µg/ml), SG (>50% at 1 µg/ml and 10 µg/ml), and DLA (>50% severity reduction) correlated to non-mutation-containing isolates or those with a silent mutation. This study indicates that QoI resistance among *C. cassiicola* isolates from tomato is widespread in Florida and validates rapid screening methods using MGI or molecular assays to identify resistant isolates in future studies.

Keywords: chemical, *Corynespora cassiicola*, disease management, fungicide resistance, quinone outside inhibitor, *Solanum lycopersicum*, vegetables

Target spot of tomato, caused by *Corynespora cassiicola*, was first reported in Florida in 1972 (Blazquez 1972) and has become one of the state's most economically important fungal tomato pathogens. Within the last decade, the disease has been identified more frequently in the state, with increasing aggressiveness reported by tomato growers (MacKenzie et al. 2018; Schlub et al. 2007). Target spot can cause blighting of foliage, fruit damage, and postharvest rot if left unmanaged (Dixon et al. 2009; Fajola 1979). No viable host resistance is currently available in commercial tomato lines (Schlub et al. 2007); therefore, the most common method for control is through the use of protective fungicides throughout the season (MacKenzie et al. 2018; Pernezny et al. 1996; Schlub et al. 2007).

Pernezny et al. (1996) reported that target spot could reduce marketable yields of tomato by as much as 33% in the absence of protective fungicides. *Corynespora cassiicola* is recognized as a high-risk pathogen for fungicide resistance development (FRAC 2014), and resistance to several fungicide groups has been identified in the field and greenhouse (Date et al. 2004; Ishii 2006; Miyamoto et al. 2007, 2009). Among these fungicide groups are the quinone outside inhibitors (QoIs). QoIs are categorized as high risk for resistance development because of a highly site-specific mechanism of pathogen inhibition (FRAC 2015), and they specifically bind the quinol

oxidation site of the cytochrome *bc1* complex (complex III) in the electron transport chain to inhibit normal respiration (Fernández-Ortuño et al. 2008; Vincelli 2002).

Azoxystrobin is currently the oldest QoI fungicide labeled for use on target spot of tomato (Vallad et al. 2017). It was registered in the United States by Zeneca Agrochemicals (Fresno, CA) in 1996, and in 2000, after the merger of Zeneca Agrochemicals and Novartis Agribusiness (Basel, Switzerland) to Syngenta AG (Basel, Switzerland), it was labeled for use on tomato as Quadris (Bartlett et al. 2002). It was initially efficacious for target spot control, but Pernezny et al. (2002) reported reduced performance for azoxystrobin in Florida field trials conducted in the fall of 2001. Since then, numerous field trials have reported azoxystrobin resistance (Paret et al. 2013; G. E. Vallad, personal communication). Another QoI fungicide called fenamidone was registered for use on tomato by Bayer CropScience AG (Monheim am Rhein, Germany) not long after azoxystrobin, but fenamidone is not currently labeled for use against target spot in Florida (Vallad et al. 2017). Fenamidone has a molecular structure distinct from that of azoxystrobin (Bartlett et al. 2002), although both azoxystrobin and fenamidone belong to the same Fungicide Resistance Action Committee (FRAC) group (#11) based on biochemical mode of action (FRAC 2017).

Complete resistance to multiple QoI fungicides has been commonly attributed to a single-nucleotide polymorphism in the *cyt b* gene conferring an amino acid change from G to A at position 143 (G143A) (Ma and Michailides 2005). The resistant phenotype associated G143A mutation has been documented in >20 pathogens worldwide (Ishii 2006), and the restriction enzyme *Fnu4HI* (= *ItaI*) can be used to rapidly identify the G143A mutation via PCR-restriction fragment length polymorphism (PCR-RFLP) (Ishii 2006, 2009). Several minor mutations (F129L and G137R) have also been identified that elicit a quantitative and more moderate form of resistance (Sierotzki et al. 2007). A lack of cross-resistance to QoI fungicides has been identified in F129L mutants (Pasche et al. 2005), although the G143A mutation appears cross-resistant against all currently tested QoI chemistries (FRAC 2015). Resistance to QoI fungicides (FRAC group 11) could equate to a loss of efficacy in 9

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out of 19 systemic fungicides labeled for the control of target spot on tomato in Florida based on active ingredient (Vallad et al. 2017).

QoI resistance development in *C. cassiicola* has been identified in several cucumber studies (Date et al. 2004; Ishii et al. 2007; Miyamoto et al. 2009, 2010) and reported on tomato in Florida (Adkison 2012; Paret et al. 2013). Sensitivity shifts to azoxystrobin have been identified in many other crops and pathogens (Keinath 2009; Ma and Michailides 2003; Mondal et al. 2005; Pasche et al. 2004; Vawdrey 2008); however, no in-depth studies have been performed on *C. cassiicola* isolates from tomato to date. Grower demand has prompted this study through an increasing presence of *C. cassiicola* in Florida and the devastating consequences of inadequate chemical control in disease-favorable conditions. This study was conducted to characterize QoI resistance, determine the prevalence among *C. cassiicola* associated with outbreaks of target spot of tomato in Florida, and elucidate the molecular mechanisms of resistance.

Materials and Methods

Collection and storage of fungal isolates. A total of 123 monoklonal isolates of *C. cassiicola* were collected from eight counties in Florida between 2015 and 2017 (Fig. 1). Isolations were as single spores from sporulating leaf lesions or by plating surface-sterilized symptomatic tissue from fruits, leaves, and stems on a general isolation media, consisting of a half-strength potato dextrose agar (HS-PDA; 9.75 g of PDA, 1.6 g of agar) containing 100 µg/ml of streptomycin and 250 µg/ml of ampicillin, based on active ingredient (a.i.). All subsequent rates in this study were also based on a.i.

Fig. 1. Distribution of 123 *Corynespora cassiicola* isolates collected from 2015 to 2017 throughout eight important tomato-producing counties in Florida (numbers in parentheses indicate the number of isolates collected for that county).

All *C. cassiicola* isolates were identified based on their morphology, including obclavate to cylindrical pseudoseptate conidia with a highly distinctive hilum on its end, or at the point of conidiophore attachment (Ellis 1957; Qi et al. 2011). Thirteen representative isolates (Table 1) from different hosts and locations were selected to confirm *C. cassiicola* identity based on molecular characterization by sequencing the ribosomal DNA internal transcribed spacer (ITS). Genomic DNA from each isolate was used to perform PCRs with the primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). The final volume for the PCR was 25 µl containing: 25 ng of genomic DNA, 10× PCR buffer, 50 mM of MgCl₂, 200 µM dNTPs, 1 U of *Taq* DNA polymerase, and 5 µM of each primer. The PCR cycling conditions were initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing for 30 s at 51°C, extension at 72°C for 1 min; with a final extension at 72°C for 10 min. The amplified fragments were visualized on 1% agarose gel and then submitted to Elim Biopharmaceuticals, Inc. (Hayward, CA) for sequencing. Resulting forward and reverse sequences were aligned and the consensus sequence used to run BLASTn searches. A BLAST search in GenBank revealed that all 13 sequences showed 100% identity with *C. cassiicola* (Table 1). These sequences were also aligned with *C. cassiicola* sequences recovered from tomato plants previously identified by Dixon et al. (2009) (accession numbers FJ852574, FJ852575, FJ852678, FJ852679, and FJ852695 to FJ852697) and Sumabat et al. (2018) (accession numbers MF320502 to MF320506) and showed >99.6% identity, confirming that our isolates belong to the species of *C. cassiicola* (Table 1).

For long-term storage, isolates were transferred to half strength (HS)-PDA and grown for 10 to 21 days (25 ± 2°C and 12:12 photoperiod) with sterile filter paper squares placed surrounding the colony. When the mycelial mat fully colonized the plate, filter papers were removed, dried in a desiccator for 4 to 6 weeks, and stored in sterile envelopes at -20°C. Short-term isolate storage consisted of 5-mm HS-PDA agar plugs taken from the leading edge of 5- to 10-day-old colonies and stored in 15-ml Falcon tubes of sterile deionized (DI) water at room temperature. Isolates collected from cucumber (*n* = 3), cotton (*n* = 2), and sesame (*n* = 1) in Florida were also included in this study as a sensitivity check.

Mycelial growth inhibition assays. Salicylhydroxamic acid (SHAM) is often included in *in vitro* experiments with QoI fungicides to inhibit the alternative oxidase pathway (Bradley and Pedersen 2011; Luo and Schnabel 2008; Secor and Rivera 2012; Sierotzki et al. 2000; Weber and Hahn 2011). An experiment was conducted with 10 *C. cassiicola* isolates (2, 4, 7, 10, 17, 19, 20, 22, 24, and 25) to determine the effect of SHAM on mycelial growth at 100 µg/ml, which consisted of six reps and was conducted twice. Those 10 isolates were incorporated into a larger group of 29 *C. cassiicola* isolates representative of geographic location of isolation and differences in colony morphology and were subjected to HS-PDA amended with nine fungicide concentrations (0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, and 100 µg/ml) of commercial formulations of

Table 1. Representative *C. cassiicola* isolates used for species identification, confirmed by internal transcribed spacer (ITS) sequences

Isolate number	Isolate name	Host	State	County	Year collected	ITS sequence accession #
2	GEV3125	Tomato	Florida	Hendry	2015	MH806849
4	GEV3127	Tomato	Florida	Hendry	2015	MH806850
7	GEV3130	Tomato	Florida	Manatee	2015	MH806851
13	GEV3136	Tomato	Florida	Hillsborough	2011	MH806852
19	GEV3142	Tomato	Florida	West Palm Beach	2016	MH806853
20	GEV3143	Tomato	Florida	West Palm Beach	2016	MH806854
22	GEV3145	Tomato	Florida	Martin	2016	MH806855
24	GEV3148	Tomato	Florida	Collier	2016	MH806856
28	GEV3154	Cotton	Florida	Gadsden	2015	MH806857
29	GEV3155	Sesame	Florida	Suwannee	2015	MH806858
89	GEV3219	Cucumber	Florida	Hillsborough	2015	MH806859
90	GEV3220	Cucumber	Florida	Hillsborough	2015	MH806860
91	GEV3221	Cotton	Florida	Gadsden	2015	MH806861

azoxystrobin (22.9% a.i.) and fenamidone (44.4% a.i.) based on a.i. in the presence of SHAM (100 µg/ml). Azoxystrobin was included in the media in the presence and absence of SHAM in a separate experiment to determine potential inhibition of the alternative oxidase pathway *in vitro* (McIntosh 1994) at 100 µg/ml.

A stock solution of 50,000 µg/ml was prepared by dissolving 50 mg of SHAM per milliliter of 100% ethanol, which was added to amended and unamended media at 0.2% by volume (100 µg/ml SHAM). Separate fungicide stock solutions containing 100 and 10,000 µg/ml of azoxystrobin and fenamidone were prepared in sterile DI water for dilution to final fungicide concentrations ranging from 0.01 to 0.3 µg/ml and 1 to 100 µg/ml, respectively. Discriminatory dose screening used a stock solution of 2,500 µg/ml for dilution to one concentration (5 µg/ml) of each separate fungicide for the entire isolate population. SHAM (100 µg/ml) and fungicide stocks were added to the media at 55°C, poured at approximately 20 ml per Petri dish (15 × 85 mm), and allowed to cool before transfers.

Isolate plugs were recovered from sterile water storage and grown for 4 to 7 days on HS-PDA at 25 ± 2°C and a 12:12 photoperiod of fluorescent light. Plugs (5 mm) were taken from the leading edge of each isolate colony and placed face down on fungicide-amended media. Four different isolates were grown on each plate for 4 days, and colony diameter measurements were taken. Plug size was subtracted from the final value, and the effective concentration at which 50% mycelial growth inhibition (MGI) is achieved (EC₅₀) was calculated. A discriminatory dose of 5 µg/ml for both fungicides was used to differentiate sensitive and resistant isolates based on percentage MGI. This experiment consisted of three replicates and was conducted twice. The two *C. cassiicola* isolates from cotton (28 and 91) and one from sesame (91) were included as possible sensitive isolates, because previous studies did not identify QoI-sensitive isolates from tomato (Adkison et al. 2012).

Spore germination assay. Four plugs from sterile water storage were transferred to full-strength PDA (39 g of PDA/liter) for 5 to 7 days in 25 ± 2°C and a 12:12 photoperiod under fluorescent light. Plates were scraped with the flat end of a steel spatula spoon to remove all mycelia and placed face-up under 24-h fluorescent light for 4 to 8 days, approximately 60 cm from the light source. Spores were collected by cutting 10- to 15-mm squares from the media of sporulating cultures and shaking them in a sealed container with sterile DI water and one drop of Tween 20/100 ml. The spore suspension was filtered through a single layer of cheesecloth and quantified with a hemocytometer.

Before fungicide sensitivity assays for SG, the presence and absence of SHAM (100 µg/ml) was evaluated on 10 *C. cassiicola* tomato isolates (2, 4, 7, 10, 17, 19, 20, 22, 24, and 25). Total germ tube length was measured for 25 total spores in a repeated experiment for a total of 50 spores of each isolate to determine any potential deleterious effects of SHAM. Methods for SG were modified from Madhavi and Murthy's (2001) study on *C. cassiicola* rubber isolates. Fungicides were prepared at three separate concentrations of 0, 1, and 10 µg/ml for each isolate. The final concentration of SHAM was 100 µg/ml across all treatments, with a final spore concentration of 10³ spores per milliliter. Mixtures were prepared in 1.6-ml microcentrifuge tubes and vortexed for 5 s before 50 µl of each suspension was pipetted onto autoclaved cavity microscope slides. Each microscope slide contained two 18-mm-diameter cavities with 0.8-mm depressions. Slides were placed in a humidity chamber with no cover slide, sealed, and incubated for 12 h (6-h fluorescent light and 6-h dark photoperiod) at 27°C. Before the measurements were taken, all slides were placed in the refrigerator to limit further germ tube growth during the experiment. Observations were made on a compound microscope, with measurements taken with a digital camera equipped with the CellSens program.

For the experiment, total germ tube length was recorded for the first 25 conidia observed (12 from one cavity, 13 from the other), and the experiment was conducted four times for a total of 100 measurements per isolate-treatment-dose combination.

Sequence-based analyses of *cytb* gene. All fungal DNA isolations were performed via a modified Dellaporta method (Dellaporta et al. 1983). Fungal colonies were grown on HS-PDA for 7 to 10 days,

when mycelia was scraped with a sterile toothpick and placed in a sterile 2-ml tube with Dellaporta buffer (100 mM Tris [pH 8], 500 mM of NaCl, 50 mM of EDTA, and 10 mM of β-mercaptoethanol) and 10% (vol/vol) sodium dodecyl sulfate for homogenization. A 4-mm grinding ball was added to the tube and placed in a tissue homogenizer for two cycles of 30 s each (1,500 rpm). Samples were then frozen at -80°C for 10 min and incubated at 65°C for 15 min to release DNA from fungal cells, followed by adding potassium acetate (8 M), used for protein precipitation; isopropanol was used for nucleic acid precipitation, and 70% ethanol was used for cleaning the DNA pellet. The pellet was then resuspended in 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, Na₂) and stored at -20°C.

PCR amplification with *cytb* was initially undertaken with earlier described primers for *C. cassiicola* on cucumber, RSCBF1 and RSCBR2 (Ishii et al. 2007). No amplification could be achieved with these primers in our population; therefore, new primers specific for *cytb* analysis were designed (Table 2) based on predicted locations from the whole genome sequence of *C. cassiicola* isolate UM 591 collected from a contact lens (NCBI Assembly accession number GCA_000603925.1). PCR (25 µl reaction volume) included Promega (Madison, WI) GoTaq Green Master Mix 1X (1.25 units *Taq* DNA polymerase, 1.5 mM of MgCl₂, 200 µM dNTPs, and 1× reaction buffer), forward and reverse primers (4 µM each), and DNA (50 ng). PCR tubes were placed in a thermal cycler with an initial denaturation of 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s, completed by a final elongation at 72°C for 5 min. PCR products were digested with 150 units of *Fnu*4HI (= *Ita*1) at 37°C for 16 h (Ishii 2009; Ishii et al. 2007) and loaded onto 1.5% agarose gel with 1X GelRedTM (Biotium, Inc., Fremont, CA) in 1× Tris-borate-EDTA buffer, separated by electrophoresis, and visualized by transillumination with ultraviolet light. Before *Fnu*4HI exposure, PCR products from *cytb* analysis were sent to Elim Biopharmaceuticals Inc for DNA sequencing, and BioEdit (version 7.2.6.1) was used for sequence assembly (Hall et al. 2011). Sequences were aligned with the predicted *cytb* gene from *C. cassiicola* isolate UM 591 (NCBI Assembly accession number GCA_000603925.1).

Detached leaflet assay. Tomato seedlings (FL-47) were grown in 128-cell flats for 4 weeks and transplanted to 15-cm pots to grow for an additional 4 weeks. Twenty-four h before leaflet excision, the fourth newest leaf was dipped in Quadris 2.08FL (0.44 kg [a.i.]/ha) or Reason 500SC (0.29 kg [a.i.]/ha) based on the highest labeled broadcast volume for Florida tomatoes (280 liter/ha). Controls were dipped in sterile DI water. The first fully developed pair of leaflets from the main stem were excised at the petiole and placed abaxial side up in humidity chambers with wetted paper towels. Unraveled cotton balls were wetted and placed over excised petioles for maximum water retention. Each humidity chamber contained six treated and four untreated leaflets, and each leaflet was inoculated with four 7-µl droplets. Six isolates were chosen based on MGI, SG, and mutation analyses that were indicative of sensitivity (7 and 125), moderate resistance (19 and 79), and resistance (2 and 93). The six isolates were randomly applied to detached leaflets at 10⁴ spores per milliliter, pipetted onto each abaxial leaflet surface. Water controls were concurrently randomized. Each isolate applied to three treated and two untreated leaflets and replicated five times for a total of 15 treated and 10 untreated leaflets per

Table 2. Sequences of primers designed for *cytb*^z analysis and primers from previously described *C. cassiicola* cucumber isolates

Primer name	Primer origin	Sequence (5' to 3')
cct-cytb-F	This study	TATTATGCGGGATGTAAATAATGG
cct-cytb-R	This study	TAATGAGAAGAATCTATTTAATGTA GCA
RSCBF1	Ishii et al. 2007	TATTATGAGAGATGTAAATAATGG
RSCBR2	Ishii et al. 2007	AACAATATCTTGTCCAATTCATGG

^z *cytb* is a gene located in the region of the cytochrome *bc*1 complex associated with quinone outside inhibitor fungicide binding and inhibition.

isolate per experiment, and the experiment was conducted twice. Disease severity was calculated based on lesion diameter (in millimeters) after 1 week in humidity.

Data analysis. EC₅₀ values for MGI were calculated by regressing relative growth inhibition against the log₁₀ of the fungicide concentrations and fitting them to a sigmoidal curve to interpolate the 50% inhibition intercept in GraphPad Prism 7. Independent experiments were assessed for homogeneity of variance with the covtest function in PROC GLIMMIX before combining. Discriminatory dose and SG data are observed as mean percentage inhibition relative to the control. A *t* test was used to determine significance of detached leaf assays, and significant relationships were determined for $\pm$ SHAM assays via Fisher's protected LSD ($\alpha = 0.05$). All data were evaluated in SAS 9.4.

Results

Mycelial growth inhibition. Initial assays conducted with 10 *C. cassiicola* tomato isolates in the presence or absence of SHAM revealed a uniform reduction in mycelial growth across all isolates

in the presence of SHAM (Table 3), but no isolates were severely inhibited. The MGI assays conducted in the absence of SHAM identified EC₅₀ values >100 μ g/ml for azoxystrobin in all 29 preliminary isolates (Table 4). Preliminary screening in the presence of SHAM dramatically reduced EC₅₀ values of seven isolates (1, 4, 7, 9, 19, 28, and 29) for azoxystrobin (Table 4). Two characteristic groupings of sensitive (<100 μ g/ml) and resistant (>100 μ g/ml) isolates were revealed based on EC₅₀ values for both fungicides. EC₅₀ values for seven isolates (1, 4, 7, 9, 19, 28, and 29) appeared sensitive to azoxystrobin (+SHAM) and fenamidone (+SHAM), with EC₅₀ values <100 μ g/ml. Azoxystrobin- and fenamidone-sensitive isolates exhibited mean EC₅₀ values of 7.11 ± 12.3 (range = 0.22 to 36.7 μ g/ml) and 13.0 ± 10.56 (range = 3.86 to 36.0 μ g/ml), respectively. The remaining 22 isolates were resistant, with EC₅₀ values >100 μ g/ml for both fungicides (Table 4).

Upon observation of separations in preliminary dose-response results from 29 isolates (Fig. 2), a discriminatory dose of 5 μ g/ml was used to differentiate sensitive (>40% inhibition) and resistant (<40% inhibition) isolates. Screening all 123 isolates (Table 5) with the

Table 3. Mycelial growth inhibition (MGI) and spore germination (SG) of 10 *C. cassiicola* tomato isolates^y in the presence and absence of salicylhydroxamic acid (SHAM)

Assay	Experiment	Water control ^z	Ethanol ^z	Ethanol + SHAM ^z
MGI, mm	1	38.0 a	38.0 a	33.0 b
MGI, mm	2	34.0 a	34.0 a	29.0 b
SG, μ m germ tube length	1	211.0 a	200.0 a	198.0 a
SG, μ m germ tube length	2	226.0 a	224.0 a	220.0 a

^y *C. cassiicola* isolates 2, 4, 7, 10, 17, 19, 20, 22, 24, and 25 were included in the assay.

^z Means in the same row followed by the same letter are not statistically different based on Fisher's protected LSD at the 95% level of confidence.

Table 4. Calculated EC₅₀ values for azoxystrobin and fenamidone based on mycelial growth inhibition (MGI) for 29 *Corynespora cassiicola* isolates collected from tomato in Florida

Isolate number	Isolate name	County of origin	Sensitivity rating ^y	EC ₅₀ (μ g/ml) ^x		
				Azoxystrobin – SHAM ^z	Azoxystrobin + SHAM	Fenamidone + SHAM
1	GEV3124	Hendry	S	>100	0.2	8.5
2	GEV3125	Hendry	R	>100	>100	>100
3	GEV3126	Hendry	R	>100	>100	>100
4	GEV3127	Hendry	S	>100	1.8	9.7
5	GEV3128	Hendry	R	>100	>100	>100
6	GEV3129	Hendry	R	>100	>100	>100
7	GEV3130	Manatee	S	>100	0.9	4.5
8	GEV3131	Manatee	R	>100	>100	>100
9	GEV3132	Manatee	S	>100	7.7	19.8
10	GEV3133	Manatee	R	>100	>100	>100
11	GEV3134	Manatee	R	>100	>100	>100
12	GEV3135	Hillsborough	R	>100	>100	>100
13	GEV3136	Hillsborough	R	>100	>100	>100
14	GEV3137	Collier	R	>100	>100	>100
15	GEV3138	Collier	R	>100	>100	>100
16	GEV3139	Collier	R	>100	>100	>100
17	GEV3140	Collier	R	>100	>100	>100
18	GEV3141	Palm Beach	R	>100	>100	>100
19	GEV3142	Palm Beach	S	>100	36.7	36.0
20	GEV3143	Palm Beach	R	>100	>100	>100
21	GEV3144	Martin	R	>100	>100	>100
22	GEV3145	Martin	R	>100	>100	>100
23	GEV3147	Collier	R	>100	>100	>100
24	GEV3148	Collier	R	>100	>100	>100
25	GEV3150	Collier	R	>100	>100	>100
26	GEV3151	Collier	R	>100	>100	>100
27	GEV3153	Hillsborough	R	>100	>100	>100
28	GEV3154	Gadsden	S	>100	0.7	8.6
29	GEV3155	Suwannee	S	>100	1.8	3.9

^x EC₅₀ = effective concentration at which mycelial growth is inhibited by $\geq 50\%$ relative to the control. Azoxystrobin was derived from Quadris 2.08 FL and fenamidone from Reason 500 SC.

^y R, resistant (EC₅₀ >100 μ g/ml); S, sensitive (EC₅₀ <100 μ g/ml).

^z SHAM, salicylhydroxamic acid.

5- $\mu\text{g/ml}$ discriminatory dose of azoxystrobin and fenamidone in the presence of SHAM revealed eight new sensitive isolates (46, 61, 74, 75, 76, 91, 95, and 118) in addition to the seven isolates (1, 4, 7, 9, 19, 28, and 29) from preliminary screening with EC_{50} values $<100 \mu\text{g/ml}$ for both fungicides. The 15 sensitive isolates had mean MGI of $59.1 \pm 7.0\%$ (range = 43.8 to 68.7%) and $54.8 \pm 8.1\%$ (range = 41.1 to 69.2%) to azoxystrobin and fenamidone, respectively. The remaining 108 isolates had a mean inhibition of $17.3 \pm 7.2\%$ (range = no inhibition to 36.3% inhibition) for azoxystrobin and $9.36 \pm 5.5\%$ (range = no inhibition to 27.8% inhibition) for fenamidone and were regarded as resistant (Table 5). Interestingly, two isolates (9 and 19) from preliminary azoxystrobin and fenamidone screening and one isolate (74) from the discriminatory dose assay had between 40 and 50% mycelial inhibition for both fungicides in the presence of the discriminatory dose. All other isolates regarded as sensitive had $>50\%$ inhibition (Table 5). Therefore, these three isolates (9, 19, and 74) were considered moderately resistant based on discriminatory dose screening.

Spore germination. Initial assays conducted with 10 *C. cassiicola* tomato isolates in the presence and absence of SHAM revealed no difference in germ tube elongation across repeated experiments (Table 3). Based on results of the MGI assays, 12 sensitive (1, 4, 7, 28, 29, 46, 61, 75, 76, 91, 94, and 118), three moderately resistant (9, 19 and 74), and three resistant isolates (2, 17, and 119) were selected for SG assays. All three resistant isolates exhibited $<50\%$ germ tube inhibition for both 1 and 10 $\mu\text{g/ml}$ in the presence of both fungicides. Twelve sensitive isolates were confirmed as sensitive, with $>50\%$ inhibition to 1 and 10 $\mu\text{g/ml}$ of azoxystrobin, with germ tube inhibition ranging from 51.8 to 91.2% for 1 $\mu\text{g/ml}$ and a range of 61.3 to 97.1% for 10 $\mu\text{g/ml}$. Eleven out of 12 sensitive isolates were confirmed as sensitive to fenamidone, with a germ tube inhibition range of 51.3 to 83.4% for 1 $\mu\text{g/ml}$ and a range of 65.1 to 93.9% for 10 $\mu\text{g/ml}$. Three isolates (9, 19, and 74) for azoxystrobin and four isolates (4, 9, 19, and 74) from fenamidone were considered moderately resistant in SG assays with $<50\%$ inhibition at 1 $\mu\text{g/ml}$ and $>50\%$ inhibition at 10 $\mu\text{g/ml}$. Germ tube growth inhibition ranged from 27.4 to 31.1% and 62.0 to 66.0% for 1 and 10 $\mu\text{g/ml}$ of azoxystrobin, respectively. Relative tube inhibition ranged from 19.6 to 33.6% and 54.7 to 67.5% for 1 and 10 $\mu\text{g/ml}$ of fenamidone, respectively.

PCR-RFLP analysis and sequencing. Amplification of the *cytb* region with the primer pair cct-cytb-F and cct-cytb-R resulted in a 346-bp fragment from genomic DNA. Digestion of the *cytb* amplified product and visualization by electrophoresis produced two fragments (approximately 225 and 125 bp) among 108 isolates, and the remaining 15 isolates were left undigested as a single 346-bp fragment. The 15 isolates lacking the *Fnu4HI* restriction site in *cytb* corresponded to isolates rated as either sensitive or moderately resistant in MGI and SG assays. The 18 isolates included in SG assays were sequenced to confirm the presence of G143A in resistant isolates

Fig. 3. Detached leaflet treated with Quadris Flowable (Syngenta; azoxystrobin 22.9% a.i.) 24 h before inoculation with three *Corynespora cassiicola* tomato isolates (19, 93, and 125). One sensitive isolate (S) (125), one moderately resistant isolate (MR) (19), one resistant isolate (R) (93), and a water control (W) were applied to the abaxial leaf surface of the leaflet as 7- μl droplets. Detached leaflet responses to *C. cassiicola* infection are marked accordingly in the figure. Pictures were taken after 1 week in a humidity chamber.

Fig. 2. Dose-response curves from preliminary sensitivity screening of 29 *Corynespora cassiicola* isolates (Table 4) and arbitrary selection of a discriminatory dose (5 $\mu\text{g/ml}$) indicated by the vertical black line. The horizontal black line indicates 40% MGI, which was used to distinguish sensitive isolates ($>50\%$) from moderately resistant (40 to 50%) and resistant isolates ($>50\%$) in discriminatory dose screening for azoxystrobin and fenamidone.

Table 5. Percentage mycelial growth inhibition (%MGI) results of 123 *Corynespora cassiicola* isolates collected from tomato in Florida on half-strength PDA amended with a discriminatory dose (5 µg/ml) of azoxystrobin or fenamidone compared with a nonamended control and *Fnu4HI* amplicon digestion results

Isolate number	Isolate name	County of origin	Sensitivity rating ^y	<i>Fnu4HI</i> ^z	% MGI ^x	
					Azoxystrobin + SHAM	Fenamidone + SHAM
1	GEV3124	Hendry	S	ND	68.7	69.2
2	GEV3125	Hendry	R	D	20.6	12.8
3	GEV3126	Hendry	R	D	12.7	10.3
4	GEV3127	Hendry	S	ND	60.2	59.8
5	GEV3128	Hendry	R	D	15.5	13.1
6	GEV3129	Hendry	R	D	2.1	0.0
7	GEV3130	Manatee	S	ND	64.2	64.3
8	GEV3131	Manatee	R	D	11.4	6.0
9	GEV3132	Manatee	MR	ND	45.6	41.1
10	GEV3133	Manatee	R	D	4.4	13.1
11	GEV3134	Manatee	R	D	1.8	10.7
12	GEV3135	Hillsborough	R	D	8.7	11.6
13	GEV3136	Hillsborough	R	D	14.9	11.6
14	GEV3137	Collier	R	D	13.6	8.3
15	GEV3138	Collier	R	D	17.6	12.7
16	GEV3139	Collier	R	D	6.1	8.9
17	GEV3140	Collier	R	D	12.0	1.6
18	GEV3141	Palm Beach	R	D	14.5	6.2
19	GEV3142	Palm Beach	MR	ND	43.8	41.8
20	GEV3143	Palm Beach	R	D	10.0	10.5
21	GEV3144	Martin	R	D	12.7	8.2
22	GEV3145	Martin	R	D	12.4	5.7
23	GEV3147	Collier	R	D	10.2	4.9
24	GEV3148	Collier	R	D	14.3	9.2
25	GEV3150	Collier	R	D	11.9	11.6
26	GEV3151	Collier	R	D	16.2	11.8
27	GEV3153	Hillsborough	R	D	19.2	17.7
28	GEV3154	Gadsden	S	ND	64.2	62.2
29	GEV3155	Suwannee	S	ND	63.1	58.2
30	GEV3156	Hendry	R	D	21.1	6.2
31	GEV3157	Hendry	R	D	28.1	11.9
32	GEV3158	Hendry	R	D	19.8	11.7
33	GEV3159	Hendry	R	D	22.5	6.1
34	GEV3160	Hendry	R	D	22.1	7.5
35	GEV3161	Hendry	R	D	20.7	5.1
36	GEV3162	Hendry	R	D	17.6	5.8
37	GEV3163	Hendry	R	D	17.9	6.0
38	GEV3164	Hendry	R	D	24.3	8.7
39	GEV3165	Hendry	R	D	19.7	12.9
40	GEV3166	Hendry	R	D	17.0	7.4
41	GEV3167	Hendry	R	D	11.8	5.6
42	GEV3168	Hendry	R	D	15.3	6.5
43	GEV3169	Hendry	R	D	18.0	5.3
44	GEV3170	Hendry	R	D	21.0	4.8
45	GEV3171	Hendry	R	D	14.4	11.6
46	GEV3172	Hendry	S	ND	56.3	51.2
47	GEV3173	Hendry	R	D	19.2	6.1
48	GEV3174	Hendry	R	D	15.5	8.5
49	GEV3175	Hendry	R	D	16.9	5.9
50	GEV3176	Hendry	R	D	16.0	8.8
51	GEV3177	Hendry	R	D	2.1	5.5
52	GEV3178	Manatee	R	D	13.6	4.4
53	GEV3179	Manatee	R	D	8.6	6.3
54	GEV3180	Manatee	R	D	14.2	5.5
55	GEV3181	Manatee	R	D	14.8	6.0
56	GEV3182	Manatee	R	D	12.4	6.9
57	GEV3183	Manatee	R	D	22.5	11.1
58	GEV3184	Manatee	R	D	12.0	4.1
59	GEV3185	Manatee	R	D	11.3	8.8
60	GEV3186	Manatee	R	D	24.8	10.6

(Continued on next page)

^x % MGI was calculated for isolates exposed to the discriminatory dose (5 µg/ml) relative to the controls. Azoxystrobin and fenamidone discriminatory doses were derived from Quadris 20.8 FL and Reason 500 SC, respectively. SHAM, salicylhydroxamic acid.

^y Based on percentage inhibition values of >50% for sensitive (S), between 40 and 50% for moderately resistant (MR) isolates, and <40% for resistant (R) isolates.

^z *Cytb* amplicon is digested (D) or not digested (ND) by *Fnu4HI*, a mutation-specific restriction enzyme modified to cleave amplicons containing the G143A substitution, responsible for complete QoI resistance.

Table 5. (Continued from previous page)

Isolate number	Isolate name	County of origin	Sensitivity rating ^y	<i>Fnu4HI</i> ^z	% MGF ^x	
					Azoxystrobin + SHAM	Fenamidone + SHAM
61	GEV3187	Manatee	S	ND	65.0	54.2
62	GEV3188	Manatee	R	D	21.6	4.4
63	GEV3189	Collier	R	D	7.0	11.3
64	GEV3190	Collier	R	D	28.5	7.6
65	GEV3191	Collier	R	D	8.9	3.5
66	GEV3192	Collier	R	D	22.0	10.1
67	GEV3193	Collier	R	D	15.1	4.4
68	GEV3194	Collier	R	D	19.6	6.5
69	GEV3195	Collier	R	D	7.4	14.3
70	GEV3196	Collier	R	D	15.7	10.1
71	GEV3198	Palm Beach	R	D	18.4	3.3
72	GEV3199	Palm Beach	R	D	19.7	14.1
73	GEV3200	Palm Beach	R	D	15.8	5.6
74	GEV3202	Palm Beach	MR	ND	47.8	42.5
75	GEV3203	Palm Beach	S	ND	63.0	53.5
76	GEV3204	Martin	S	ND	55.8	56.0
77	GEV3205	Martin	R	D	36.3	5.9
78	GEV3206	Martin	R	D	24.3	6.6
79	GEV3207	Collier	R	D	14.0	15.6
80	GEV3208	Collier	R	D	13.3	5.7
81	GEV3209	Collier	R	D	17.0	7.2
82	GEV3210	Collier	R	D	9.3	4.2
83	GEV3211	Collier	R	D	18.3	3.1
84	GEV3212	Collier	R	D	16.2	6.7
85	GEV3213	Collier	R	D	15.0	5.3
86	GEV3214	Collier	R	D	12.2	9.0
87	GEV3215	Collier	R	D	13.2	6.1
88	GEV3216	Collier	R	D	13.7	7.8
89	GEV3219	Hillsborough	R	D	23.9	4.2
90	GEV3220	Hillsborough	R	D	23.2	2.8
91	GEV3221	Gadsden	S	ND	60.0	50.1
92	GEV3222	Hillsborough	R	D	18.2	7.8
93	GEV3223	Hillsborough	R	D	22.7	17.8
94	GEV3224	Hillsborough	R	D	9.1	4.4
95	GEV3225	Hillsborough	S	ND	66.3	60.7
96	GEV3226	Hillsborough	R	D	26.0	15.1
97	GEV3227	Hillsborough	R	D	18.4	8.4
98	GEV3228	Hillsborough	R	D	17.5	6.1
99	GEV3229	Hillsborough	R	D	18.6	3.9
100	GEV3230	Hillsborough	R	D	34.6	17.8
101	GEV3231	Collier	R	D	31.3	4.4
102	GEV3232	Hillsborough	R	D	17.4	6.2
103	GEV3233	Hillsborough	R	D	20.1	8.3
104	GEV3234	Hillsborough	R	D	29.0	17.0
105	GEV3235	Hillsborough	R	D	29.0	20.2
106	GEV3236	Hillsborough	R	D	16.6	25.0
107	GEV3237	Hillsborough	R	D	28.7	18.0
108	GEV3238	Hillsborough	R	D	29.8	20.2
109	GEV3239	Hillsborough	R	D	20.7	12.6
110	GEV3240	Hillsborough	R	D	34.5	18.9
111	GEV3241	Hillsborough	R	D	24.1	9.4
112	GEV3242	Hillsborough	R	D	21.5	13.0
113	GEV3243	Hillsborough	R	D	31.8	19.2
114	GEV3244	Hillsborough	R	D	27.4	18.4
115	GEV3245	Hillsborough	R	D	19.0	10.5
116	GEV3246	Hillsborough	R	D	29.4	19.9
117	GEV3247	Hillsborough	R	D	30.0	27.8
118	GEV3248	Hillsborough	S	ND	56.7	56.8
119	GEV3249	Hillsborough	R	D	19.4	14.1
120	GEV3250	Hillsborough	R	D	20.8	15.3
121	GEV3251	Hillsborough	R	D	17.6	12.7
122	GEV3252	Hillsborough	R	D	7.1	12.3
123	GEV3253	Hillsborough	R	D	20.7	15.6

and potentially observe the presence of any minor mutations (Table 6). Sequence alignment of the predicted *cytb* gene from the whole-genome sequence of *C. cassiicola* UM591 revealed several different single-nucleotide polymorphisms. A nucleotide change from GGT to GCT, corresponding to amino acid 143 in *cytb*,

conferred a substitution of G with A in all three isolates (2, 17, and 119) rated resistant to azoxystrobin and fenamidone in MGI and SG assays. A nucleotide change of TTC to TTA corresponding to amino acid position 129 caused an F-to-L substitution and was identified in isolates 9, 19, and 74 that were characterized as moderately

Table 6. Characterization of fungicide resistance based on mean spore GTI, restriction enzyme digestion, and sequencing results of 18 *C. cassiicola* isolates used in SG assays

Isolate number	Azoxystrobin		Fenamidone		<i>Cytb</i> ^y	<i>Fnu4HI</i> ^z
	Dose (µg/ml)	% GTI ^x (StDev)	Dose (µg/ml)	% GTI (StDev)		
1	1	90.2 (±4.2)	1	75.1 (±10.0)	WT	ND
	10	92.9 (±5.4)	10	88.5 (±8.6)		
28	1	61.5 (±6.5)	1	57.9 (±4.7)	S-162	ND
	10	71.2 (±4.7)	10	65.1 (±9.2)		
97	1	91.2 (±5.1)	1	83.4 (±5.7)	WT	ND
	10	97.1 (±3.2)	10	93.9 (±7.1)		
75	1	83.5 (±6.0)	1	58.3 (±12.1)	WT	ND
	10	88.7 (±6.6)	10	85.2 (±2.8)		
118	1	82.8 (±3.1)	1	63.9 (±10.5)	WT	ND
	10	90.7 (±5.8)	10	87.1 (±1.9)		
7	1	81.7 (±16.6)	1	61.7 (±13.9)	WT	ND
	10	90.0 (±8.7)	10	87.7 (±11.0)		
61	1	77.0 (±8.3)	1	60.2 (±6.9)	WT	ND
	10	83.0 (±8.5)	10	75.6 (±8.0)		
76	1	73.0 (±16.9)	1	54.4 (±5.2)	WT	ND
	10	87.6 (±7.6)	10	77.1 (±9.8)		
91	1	67.3 (±5.7)	1	51.3 (±4.9)	S-162	ND
	10	76.2 (±8.1)	10	73.9 (±6.9)		
46	1	67.8 (±6.4)	1	58.5 (±4.9)	S-162	ND
	10	72.4 (±5.6)	10	66.1 (±10.0)		
29	1	86.8 (±7.7)	1	71.9 (±10.9)	S-162	ND
	10	89.9 (±2.5)	10	85.1 (±3.6)		
4	1	51.8 (±7.8)	1	33.6 (±3.2)	S-162	ND
	10	61.3 (±9.3)	10	56.4 (±7.1)		
19	1	31.1 (±7.5)	1	22.5 (±9.4)	F129L	ND
	10	66.0 (±15.3)	10	67.5 (±10.9)		
74	1	27.4 (±15.5)	1	19.6 (±14.9)	F129L	ND
	10	60.7 (±8.1)	10	54.7 (±21.8)		
9	1	29.1 (±5.7)	1	31.0 (±6.3)	F129L	ND
	10	62.0 (±10.8)	10	60.4 (±10.3)		
2	1	4.2 (±4.5)	1	7.0 (±11.9)	G143A	D
	10	7.5 (±9.3)	10	3.7 (±5.4)		
17	1	7.9 (±10.4)	1	9.5 (±7.0)	G143A	D
	10	12.1 (±7.4)	10	15.6 (±16.9)		
119	1	21.6 (±11.8)	1	10.0 (±8.9)	G143A	D
	10	30.8 (±12.7)	10	11.8 (±7.3)		

^x GTI, mean germ tube length inhibition relative to the control (>50% to both, sensitive; <50% for both, resistant; <50% for 1 µg/ml and >50% for 10 µg/ml, moderately resistant). StDev, standard deviation from the mean of four experiments. Azoxystrobin and fenamidone concentrations were derived from Quadris 20.8 FL and Reason 500 SC, respectively.

^y WT, contained wild type (no mutation) based on alignment with predicted *cytb* from the whole-genome sequence (GenBank: JAQF000000000.1); S-162, silent mutation at amino acid position 162 in *cytb*; F129L, F to L substitution at position 129; G143A, G to A substitution at position 143.

^z *Fnu4HI*, restriction enzyme used to digest *cytb* amplicon at site corresponding to the G143A mutation; D, digested; ND, not digested.

resistant in MGI and SG assays for both fungicides. Isolate 4, which was identified as sensitive based on MGI and moderately resistant based on SG toward fenamidone, contained a previously undocumented silent mutation (S-162), which was also present in five isolates (4, 28, 29, 46, and 91) from cotton, sesame, and tomato, regarded as sensitive to both in vitro assays (Table 6). Seven tomato isolates (1, 7, 61, 75, 76, 95, and 118) lacked mutations in the *cytB* gene relative to the reference sequence.

Detached leaflet assay. Results from detached leaflet assays (DLAs; Fig. 3) identified two sensitive isolates (7 and 125), with no predicted amino acid substitutions present in *cytB*, that were inhibited by 72 and 74% in the presence of azoxystrobin and 48 and 65% in the presence of fenamidone. Two moderately resistant isolates (19 and 79) were inhibited by 26 and 31% in the presence of azoxystrobin and 36 and 43% in the presence of fenamidone. Two resistant isolates (2 and 93) were inhibited by 10% or enhanced by 3% in the presence of azoxystrobin, and in the presence of fenamidone both resistant isolates increased in disease severity by 3 and 15% (Table 7).

Discussion

Reduced performance of QoI fungicides (specifically, azoxystrobin) for the management of target spot on Florida tomatoes has been documented in several field trials over the last 15 years (Paret et al. 2013; Pernezny et al. 1996, 2002; G. E. Vallad, personal communication). It is difficult to find tomato fields not exposed to QoI fungicides in Florida, because these chemistries account for a

significant portion of commercial fungicides labeled for use on tomato. Because of widespread QoI use and no fungicide application history for our isolate population, a true baseline for QoI sensitivity could not be established in this study. However, a recent study conducted on *C. cassiicola* cucumber isolates revealed that sensitive EC₅₀ values based on MGI ranged from 0.68 to 2.30 µg/ml in the presence of azoxystrobin + SHAM (Duan et al. 2019). These values are not dissimilar to the EC₅₀ values of QoI-sensitive isolates exposed to azoxystrobin + SHAM in this study (0.2 to 1.8 µg/ml).

In the presence of SHAM (100 µg/ml), MGI based on a single discriminatory dose (5 µg/ml) was able to distinguish between resistant isolates containing the G143A substitution from moderately resistant isolates containing the F129L substitution, from sensitive isolates lacking any amino acid substitutions. SG assays could also clearly differentiate resistant, moderately resistant, and sensitive isolates in azoxystrobin and fenamidone screening. However, one isolate (4) did appear moderately resistant to fenamidone in SG assays but contained only a silent mutation relative to the reference sequence (S-162). Although it is known that QoI fungicides are more effective at inhibiting fungal SG than mycelium growth (Balba 2007; Mondal et al. 2005; Pasche et al. 2004), several studies have used an MGI assay to determine sensitivity to QoI fungicides, including one conducted on *C. cassiicola* cucumber isolates (Avila-Adame et al. 2003; Banno et al. 2009; Ishii et al. 2007).

Two QoI-sensitive isolates, two QoI-moderately resistant isolates, and two QoI-resistant isolates subjected to azoxystrobin and fenamidone in DLAs corroborated results from MGI, SG, and sequencing. In DLAs, disease severity of resistant isolates did not differ from that of untreated controls; however, sensitive and moderately resistant isolates had significantly lower disease severity than untreated controls for each separate fungicide formulation (Table 7). Therefore, isolate sensitivity of *C. cassiicola* to QoI fungicides from our population can be readily distinguished by MGI by use of a single discriminatory concentration or by restriction enzyme analysis, both more rapid approaches for isolate sensitivity characterization.

To date, no SG assays have been conducted on *C. cassiicola* in the presence of fungicides. Evaluation of SG in various other phytopathogenic fungi has been based on a binary (yes/no) system of germinated versus nongerminated conidia for the strobilurin fungicides (Fontaine et al. 2009; Hincapie et al. 2014; Kim et al. 2003; Pasche et al. 2004) because of their inherent ability to inhibit SG (Balba 2007; Vincelli 2002). However, some tomato isolates in our collection produced conidia readily, whereas others did not. Spores from some isolates had irregular tube lengths because of the variability in spore size and a lack of developmental uniformity among isolates. For example, a smaller, underdeveloped conidium could produce equal tube length to a much larger, more developed conidium from the same isolate. This created a challenge in the development of a binary evaluation for *C. cassiicola*; however, relative mean tube length provided clear distinctions of characteristic groupings. Therefore, mean tube length was calculated for each isolate-treatment-dose combination, and relative tube length inhibition (%) was generated

Table 7. Detached leaflet assay (DLA) results of six *C. cassiicola* tomato isolates conducted in the presence and absence of each individual quinone outside inhibitor (QoI) fungicide

Isolate number	Sensitivity ^y	Azoxystrobin (0.44 kg/ha) ^x		Fenamidone (0.29 kg/ha)	
		Untreated	Treated ^z	Untreated	Treated
2	R	3.8	3.9	3.9	4.5
93	R	4.2	3.8	3.9	4.0
19	MR	3.8	2.8*	4.2	2.4*
79	MR	3.9	2.7*	2.8	1.8*
7	S	3.1	0.8*	2.6	0.9*
125	S	1.8	0.5*	2.1	1.1*
Water	–	0.1	0.1	0.0	0.0

^x Fungicide concentrations are presented as kilograms of active ingredient (a.i.) per hectare at the highest labeled broadcast volume for field-grown tomatoes in Florida (280 liter/ha). Azoxystrobin and fenamidone mixtures were made from Quadris 20.8 FL and Reason 500 SC, respectively.

^y Sensitivity refers to mycelial growth inhibition (MGI) and spore germination (SG) results. R, resistant; MR, moderately resistant; S, sensitive.

^z Data are presented as lesion diameter in millimeters. Asterisks (*) indicate a significant difference in lesion diameter relative to the untreated control for each individual compound.

Table 8. Frequency of resistance to azoxystrobin and fenamidone and county distribution of 123 *C. cassiicola* isolates collected from 2015 to 2017 in Florida

County	No. of isolates	Host	Resistant	Moderately resistant	Sensitive	Frequency of resistance ^y
Hendry	28	Tomato	25	–	3	89.3%
Collier	27	Tomato	27	–	–	100%
Hillsborough	33	Tomato	31	–	2	93.9%
Hillsborough	3	Cucumber	3	–	–	100%
Manatee	16	Tomato	12	1	3	81.3%
Palm Beach	8	Tomato	3	2	3	62.5%
Martin	5	Tomato	4	–	1	80.0%
Gadsden	2	Cotton ^z	–	–	2	0%
Suwannee	1	Sesame ^z	–	–	1	0%
Total	123	–	108	3	12	87.8%

^y Frequency of resistance refers to isolates regarded as moderately resistant and resistant to quinone outside inhibitor (QoI) fungicides in that county based on mycelial growth inhibition (MGI) results corroborated by spore germination (SG), restriction enzyme digestion, and detached leaflet assays (DLAs).

^z Cotton and sesame *C. cassiicola* isolates were collected by Dr. Nicholas Dufault at University of Florida, Gainesville, FL.

by normalizing both doses (1 and 10 µg/ml) to the control for each separate experiment.

Fenamidone was introduced in this study to assess levels of resistance in a nonstrobilurin QoI fungicide compared with a strobilurin QoI fungicide, azoxystrobin. Although azoxystrobin and fenamidone have the same mode of action, they are structurally very different compounds (Bartlett et al. 2002). In addition, other components in the formulation could affect fungal growth; therefore, comparisons should not be made between fungicides, only relative differences between isolates to each fungicide.

Previous studies have identified several pathogens in which the G143A mutation cannot occur (Banno et al. 2009; Hily et al. 2011; Hincapie et al. 2014; Sierotzki et al. 2007) because of either the presence of a group I intron two nucleotides downstream of amino acid position 143 in the *cytb* gene (Hincapie et al. 2014) or a mutation in noncoding regions upstream of position 143 (De La Salle et al. 1982). Therefore, if the G143A mutation occurs, it will interfere with the splicing process, producing a deficient *cytb* and therein a lethal mutant (Burke 1988; Grasso et al. 2006). In this study, no mutations in noncoding sequences or the presence of type I introns after position 143 in *cytb* were identified in any of the sequenced isolates. This finding implies a lack of interference with the splicing process, and therefore *C. cassiicola* tomato isolates in our population with the G143A mutation have complete resistance to QoI fungicides and can exist in nature.

The in vitro results for the 123 *C. cassiicola* isolates collected for this study indicate widespread resistance to QoI fungicides in Florida. All 108 isolates with either the G143A or F129L substitution are distributed throughout six counties at a rate of ≥62.5%, up to 100% in one county (Table 8). The 12 isolates lacking any mutation or containing silent mutations were documented in all counties, indicating the presence of sensitive and resistant isolates in the same sampling locations throughout the state (Table 8). It is possible that sensitive *C. cassiicola* isolates are surviving in plant debris (Oliveira et al. 2012; Sarma and Nayudu 1971) or arriving from a distance by wind dispersal. Three isolates (26, 87, and 88) collected in early 2016 from organic tomato production fields in Immokalee, Florida still appeared resistant to QoI fungicides based on MGI and contained the G143A substitution in *CytB*.

The use of QoI fungicides in Florida tomato fields has sharply declined in the last decade, because of growers' reports of resistance and paralleled recommendations to limit further resistance development. Mixing or interchanging resistance-prone fungicides with fungicides from a different mode of action can limit the development of fungicide resistance (Staub 1991); however, several QoI fungicides (FRAC #11) are formulated as mixtures with different modes of action, including the succinate dehydrogenase inhibitors (SDHIs) (FRAC #7), demethylation inhibitors (FRAC #3), and one unknown mode of action (FRAC #27). These mixtures are often considered "broad spectrum" by chemical companies and growers. Widespread QoI resistance in Florida raises concern for the utility of these mixtures and selection of resistance to their fungicidal counterparts. In the presence of QoI resistance, the complimentary FRAC group becomes the solitary mechanism for control, which may accelerate resistance development to additional modes of action. Dual resistance to QoIs and SDHIs has been reported for *Botrytis cinerea* (Veloukas et al. 2014) and has potential to form in *C. cassiicola* based on reports of resistance in cucumber to SDHI fungicides via mutations in the SDH protein complex (Miyamoto et al. 2010) and studies that identified QoI resistance to *C. cassiicola* cucumber isolates based on mutations in *cytb* (Ishii et al. 2007).

Two studies have identified a great amount of morphological and genetic diversity among *C. cassiicola* isolates collected from tomato (Dixon et al. 2009; Sumabat et al. 2018). Diversity in colony morphology was also observed among *C. cassiicola* isolates in our population, which led to molecular identification using ITS from our eight most morphologically diverse tomato isolates and five non-host isolates, confirming *C. cassiicola* in all cases (Table 1). Our results align with studies performed on *C. cassiicola* and fellow Pleosporales pathogens such as *Alternaria alternata*, *Didymella*

(*Stagonosporopsis*) *bryoniae*, and *Venturia inaequalis* that have correlated complete QoI resistance with the G143A mutation (Finger et al. 2013; Ishii et al. 2007; Lesniak et al. 2011; Ma and Michailides, 2004). With the molecular mechanisms of QoI resistance elucidated (Gisi et al. 2002; Grasso et al. 2006; Kuck 2007) and a multitude of studies indicating a direct correlation between single point mutations in *cytb* and QoI resistance (Fernández-Ortuño et al. 2008; Ishii 2008; Ma and Michailides 2005), we feel confident in a characterization based on in vitro methods and corroboration of corresponding mutations.

This is the first large-scale characterization of *C. cassiicola* isolates from tomato conducted in Florida. Results from this study may also be applicable to the recent emergence of *C. cassiicola* in cotton and soybean throughout the southeastern United States, which similarly use QoI fungicides for disease management. The diverse nature of this pathogen and grower reliance on fungicides for target spot control on tomato and other crops suggest that additional studies are necessary to investigate resistance development in other labeled fungicide groups and ultimately limit disease losses through enhanced spray recommendations.

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