Reduced Sensitivity to Azoxystrobin of *Monilinia fructicola* Isolates From Brazilian Stone Fruits is Not Associated With Previously Described Mutations in the Cytochrome *b* Gene

Wagner V. Pereira, Departamento de Fitotecnia e Fitossanitarismo, Universidade Federal do Paraná, R. Funcionários, 1540, 80.035-050, Curitiba, PR, Brazil; **Isabela V. Primiano**, Departamento de Fitopatologia e Nematologia, Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo, 13418-900, Piracicaba, SP, Brazil; **Rafael G. F. Morales**, Departamento de Fitotecnia e Fitossanitarismo, Universidade Federal do Paraná, R. Funcionários, 1540, 80.035-050, Curitiba, PR, Brazil; **Natalia A. Peres**, University of Florida, Gulf Coast Research and Education Center, Wimauma 33598; **Lilian Amorim**, Departamento de Fitopatologia e Nematologia, Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo, 13418-900, Piracicaba, SP, Brazil; and **Louise L. May De Mio**, Departamento de Fitotecnia e Fitossanitarismo, Universidade Federal do Paraná, R. Funcionários, 1540, 80.035-050, Curitiba, PR, Brazil

Abstract

Quinone-outside inhibitor (QoI) fungicides are effective tools for pre-harvest control of brown rot of stone fruit. These fungicides have a very specific site of action so the risk of resistance selection is high. The sensitivity of *Monilinia fructicola* (G. Winter) Honey isolates to azoxystrobin (QoI) was investigated in 143 isolates collected between 2002 and 2011 from four Brazilian states in orchards with different frequencies of fungicide use (0 to 6 fungicides sprays/season). Sensitivity of the isolates to azoxystrobin was determined in vitro, by inhibition of mycelial growth and spore germination on fungicide-amended media or ex vivo by pathogen inoculation in untreated or treated fruit with azoxystrobin. Potential mutations in codons 143,

137, and 129 of the cytochrome *b* (*Cyt b*) gene and the occurrence of an intron immediately after codon 143 were analyzed in a subpopulation of the isolates. The *M. fructicola* population of São Paulo State was less sensitive to the fungicide than the population from the states of Paraná, Santa Catarina, and Rio Grande do Sul. The low sensitivity of the isolates was confirmed also by comparing to the sensitivity of the baseline isolates. Mutations in G143A, F129L, and G137R in *Cyt b* gene were not found. In addition, 58 isolates tested showed an intron after codon 143 in *Cyt b* gene. Our results indicate that other mechanisms of selection for low sensitivity to QoI fungicides should be investigated.

Brown rot is one of the most important fungal diseases of *Prunus* species worldwide (Mari et al. 2012; Willetts et al. 1977). Disease is caused by several species of fungi belonging to the genus *Monilinia*, of which *M. fructicola* is the most aggressive and prevalent in stone fruit in Brazil (May De Mio et al. 2008). The production of stone fruit is concentrated in southern Brazil, in the states of São Paulo (SP), Paraná (PR), Santa Catarina (SC), and Rio Grande do Sul (RS). Application of fungicides is the main strategy to control brown rot epidemics in all stone fruit producing regions in Brazil. Different fungicide groups, including methyl benzimidazole carbamates (MBCs), demethylation inhibitors (DMIs), dicarboximide, and quinone-outside inhibitors (QoIs), are recommended during flowering and preharvest to control the disease (May De Mio et al. 2011).

The QoI fungicides used in Brazil, such as azoxystrobin and pyraclostrobin, have been used mainly for peach rust (*Tranzschelia discolor*) control. QoI fungicides are not registered specifically to brown rot control, but its effect reducing sensitivity of *Monilinia* to azoxystrobin has been already observed (May De Mio et al. 2011). The producers of São Paulo State have been using an average of six azoxystrobin applications per season. Elsewhere in Brazil, a maximum of two applications per season are commonly used.

The QoI fungicides interfere with mitochondrial respiration by changing the energy patterns in the fungal cell. The fungicides block electron transfer between cytochrome *b* and cytochrome *c1* act on the ubiquinol oxidation site and hamper ATP production (Bartlett et al. 2002; Gisi et al. 2002). The high frequency of use of these fungicides increases selection pressure for resistant isolates (Chen et al. 2014). QoI fungicides, in general, have a recommended maximum of two to three sprays per season in several crops to avoid selection of

resistance isolates (Beresford et al. 1999). In recent years, decreased sensitivity of individuals in *M. fructicola* populations to QoI has been observed in the U.S. and China (Amiri et al. 2010; Chen et al. 2014; Luo and Schnabel 2008). Shifts in azoxystrobin sensitivity of *M. fructicola* populations (20 isolates) from Brazil indicated the risk of resistance (May De Mio et al. 2011). Some of the isolates with reduced sensitivity from these populations were shown to compete equally well with sensitive isolates. Typically, a resistant isolate may occur at an initial frequency in the order of 1 in 1,000 million spores or other structures of the pathogen. However, after the selection pressure by the fungicide, the number of resistant isolates can increase by reaching the ratio of 1 resistant in 100 sensitive isolates (Brent and Hollomon 1998). Thus, a large number of isolates from different regions should be evaluated to detect resistant isolates in the pathogen population. It is important to investigate the QoI sensitivity in a larger number of isolates (more than 100) representative of the *M. fructicola* populations in Brazilian stone fruits.

The reduced sensitivity to QoIs may be related to the emergence of individuals with mutations in the cytochrome *b* gene (*Cyt b*). Resistance to QoI fungicides has been attributed to three amino acid substitutions in the *Cyt b*. Changes from glycine to alanine at position 143 (G143A), from phenylalanine to leucine at position 129 (F129L), and from glycine to arginine at position 137 (G137R) have been associated to QoI resistance (Gisi et al. 2002; Klosowski et al. 2015; Semar et al. 2007). The G143A, F129L, and G137R mutations have been reported in different countries and crops such as *Cercospora beticola* in sugar beet in the U.S. (Bolton et al. 2013), *Phakopsora pachyrhizi* in soybean in Brazil (Klosowski et al. 2015), and *Pyrenopeziza teres* in barley in France (Semar et al. 2007). G143A is considered the most important in conferring high levels of resistance, whereas F129L and G137R mutations are less frequent and important as they confer resistance at low levels (Semar et al. 2007; Sierotzki et al. 2007).

The presence of an intron immediately following the G143A site has been shown to prevent the formation of the G143A mutation in some pathogens due to its inhibition of intronic splicing (Hily et al.

Corresponding author: L. L. May De Mio; E-mail: maydemio@ufpr.br

Accepted for publication 17 November 2016.

2011; Luo et al. 2010; Miessner and Stammler 2010). Thus, the presence of the intron could determine the chance of occurrence of resistant isolates (Luo et al. 2010). The intron has been reported in *M. fructicola* isolates from the U.S. (Hily et al. 2011) and China (Luo et al. 2010). In both locations, none of the mutations (G143A, F129 L, and G137R) was found. All *M. fructicola* isolates reported in China and the U.S. so far have the intron after codon 143, confirming an intraspecific homogeneity (Hily et al. 2011; Luo et al. 2010; Miessner and Stammler 2010). In addition to the causal agent of brown rot, the intron has been reported in basidiomycetes (Grasso et al. 2006), *Botrytis cinerea* (Banno et al. 2009), and *Phyllosticta* spp. (Hincapié et al. 2014; Stammler et al. 2013).

Brazilian isolates of *M. fructicola* with reduced sensitivity to QoI fungicides were not examined for mutations in the *Cyt b* gene and presence of an intron immediately following the G143A site. This study aimed to: i) evaluate the in vitro sensitivity of *M. fructicola* isolates from different growing seasons and regions of Brazil to azoxystrobin, ii) evaluate disease control ex vivo for isolates with different levels of azoxystrobin sensitivity, iii) verify the presence of an intron linked to codon 143 in the *Cyt b* region of *M. fructicola* isolates, and iv) analyze for the G143A, F129 L, and G137R mutations in the *Cyt b* gene in a subpopulation of the pathogen with different levels of azoxystrobin sensitivity.

Materials and Methods

***M. fructicola* isolates.** To assess the in vitro sensitivity of *M. fructicola* to azoxystrobin, 143 isolates were collected from spring 2002 to autumn 2011 (UFPR-LEMID Collection) from flowers, cankers on branches, symptomatic mature fruit, and mummified fruit in peach, nectarine, and plum orchards. The isolates were collected in four states of Brazil: 63 from SP, 30 from PR, 7 from SC, and 43 from RS. Single-spore cultures of each isolate were obtained and stored in potato dextrose agar (PDA) plug with fungal growth in sterile water at 4°C and/or in 10% glycerol until use (Castellani 1967). All isolates were previously identified as *M. fructicola* using primers and protocols developed by Hily et al. (2011).

In vitro sensitivity of *M. fructicola* isolates to azoxystrobin. The sensitivity to azoxystrobin (Priori SC; Syngenta Crop Protection, Greensboro, NC) of the 143 isolates of *M. fructicola* was evaluated on water agar (WA), WA amended with salicylhydroxamic acid (SHAM; Sigma-Aldrich, St. Louis, MO), and WA amended with SHAM and azoxystrobin. The fungicide was dissolved in sterile water, adjusted to 10 µg a.i./ml, and incorporated into the 2% WA to produce concentrations of 0, 0.3, or 1.0 µg/ml. SHAM was dissolved in 100% methanol, to obtain 100 mg/ml, and incorporated in WA. The methanol concentration in the WA media was 1 ml/liter. Azoxystrobin and SHAM were added to the autoclaved WA media cooled to 45°C. Conidia were obtained from 6-day-old colonies grown on PDA at 22°C and used for the production of spore suspensions. A 40 µl aliquot of the conidial suspension (4×10^5 conidia/ml) of each isolate was spread on the media of the different treatments. The plates containing the dispersed conidia on media were stored at 22°C for 9 to 12 h. The plates were divided into four equal quadrants and conidial germination was estimated in each quadrant. One hundred conidia per quadrant were evaluated. Conidia were considered germinated if

they produced germ tubes at least twice their width. The mean of the four quadrants of a Petri dish was used to represent a replicate, and each treatment was consisted of three replicates. The germ tube length was measured with the UTHSCSA Image Tool software (University of Texas, San Antonio). The incidence of germinated conidia was recorded to determine the percentage of conidial germination inhibition (*Gi*) for the isolate. This assay was carried out twice.

Determination of the EC₅₀ of *M. fructicola* isolates to azoxystrobin.

Twenty-four isolates with different levels of sensitivity to azoxystrobin were selected for this test. The fungicide was dissolved in sterile water and adjusted to 100 µg/ml. SHAM was dissolved in 100% methanol to yield a concentration of 100 µg/ml. The sensitivity of each isolate to azoxystrobin was determined using spore germination on 2% WA plus 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, or 7.4 µg of azoxystrobin/ml. Azoxystrobin and SHAM at 100 mg/ml were added to autoclaved the WA media cooled to 45°C. The methanol concentration in WA was 1 ml/liter. The conidial suspensions were prepared from 6-day-old colonies grown on PDA at 22°C under a 12-h photoperiod. The conidia were adjusted in sterile water to 10^4 conidia/ml. A 60 µl aliquot of the suspension was spread on WA amended with fungicide and SHAM or WA amended with SHAM (control). They were incubated at 22°C in the dark for 12 h. Evaluations were conducted as described in the previous section. Experiments were repeated using the same protocol.

Table 2. Average of germination (%) and germ tube length (µm) of 143 *Monilinia fructicola* isolates in water agar (WA) (control), WA amended with salicylhydroxamic acid (SHAM), and WA amended with SHAM and azoxystrobin (A) at 0.3 and 1 µg a.i./ml

Treatment	Germination (%) ^z	Germ tube length (µm) ^z
Control	91.9 a	902 a
SHAM	76.3 b	616 b
SHAM+A (0.3 µg/ml)	18.3 c	235 c
SHAM+A (1.0 µg/ml)	2.6 d	59 d

^z Means followed by the same letter in the same column are not significantly different based on Tukey's studentized range (honest significant difference) test ($P \leq 0.05$).

Table 3. In vitro sensitivity of *Monilinia fructicola* isolates to azoxystrobin, measured by percentage inhibition of conidial germination (*Gi*)

State ^x	No. of isolates	<i>Gi</i> (%)			
		0.3 µg/ml		1.0 µg/ml	
		Mean ^y	CI ^z	Mean ^y	CI ^z
SP	63	63.11 b	55.7–70.4	93.47 b	90.4–96.4
PR+SC	37	85.27 a	79.1–91.3	99.70 a	99.1–100
RS	43	87.97 a	83.5–92.4	99.69 a	99.2–100

^x SP: Sao Paulo, PR: Paraná, SC: Santa Catarina, RS: Rio Grande do Sul.

^y Means followed by the same letter in the same column are not significantly different based on Tukey's studentized range (honest significant difference) test ($P \leq 0.05$).

^z Confidence interval: $CI = X - t_{\alpha/2} \frac{s}{\sqrt{n}}$; $X + t_{\alpha/2} \frac{s}{\sqrt{n}}$

Table 1. Sequence, annealing temperature, extension times, and amplified region of each primer used for molecular characterization of *Monilinia fructicola* isolates

Primer	Sequence 5'-3'	Annealing temperature	Extension time	Amplified region	Reference
cola0-fwd	AGAGCACCTAGAACATTAGTT	52°C	1.5	Codon 129	This study
cola1-rev	AACCAAGCTTGAACCCGCT	52°C	1.5		
cola2-fwd	CGCGACAGGCTGGGTCACTGA	55°C	2.5	Codon 137, 143 and intron	This study
colaexon3-fwd	TTTACCTTACGGTCAAATGAGCCT	55°C	2.5	Codon 137, 143, and intron	Hily et al. 2011
colaexon4-rev	AACTCAACAATATCACCTCCAATTTCAT	55°C	2.5		
laxaexon3-fwd	TTACCTTACGGTCAAATGTCGCTA	55°C	2.5		
laxaexon4-rev	CAACAATATCTTGTCCAATTTCATGGT	55°C	2.5		

Brown rot control with azoxystrobin. The same 24 *M. fructicola* isolates used in the EC₅₀ assay with different levels of sensitivity to azoxystrobin were selected to evaluate the efficacy of fungicide control. The trial was conducted on fruit of two peach cultivars (Chimarrita and PS10711) that are highly susceptible to the pathogen. The fruits were surface-disinfested by immersion in 0.5% sodium hypochlorite solution (1 min), followed by immersion in 70% ethanol (1 min), and with three subsequent washes in sterile distilled water. The fruit were kept in a cool environment (23 to 25°C) to dry the excess water on the surface. This study used the method of Amiri et al. (2010) with some modifications. The fruit were immersed in sterile water (control) or fungicide solution using the recommended rate for use in the field (0.4 ml/liter) and stored at room temperature (23 to 25°C) for 12 h before inoculation. Conidial suspensions were prepared from spores produced on the surface of pre-inoculated fruit. An aliquot of 30 µl of a suspension containing 5×10^5 conidia/ml of each isolate was deposited on the surface of the fruit with a 9.5 mm deep wound. After inoculation, fruit were kept at 22°C for 5 days. Lesion diameters were measured daily in two perpendicular directions and the area under the lesion growth (AULG) calculated. For comparison among isolates, the difference of each AULG with and without fungicide for each isolate ($AULG_{IS} = AULG_{IS-treat} - AULG_{IS-cont}$) was calculated. Furthermore, the time between inoculation and appearance of sporulation on the surface of the fruit (latent period) was measured. Three single-peach replicates were used. The experiment was conducted twice in a completely randomized design.

Evaluation of the presence of an intron immediately after codon 143 of the *Cyt b* gene in *M. fructicola* isolates. To verify the presence of the intron after the codon 143 of the *Cyt b* gene, 58 isolates with different levels of sensitivity to the fungicide were selected. This analysis was conducted based on the methodology of Hily et al. (2011) with some modifications. Genomic DNA was extracted from these isolates using the DNA extraction kit Ultraclean Microbial (Mo Bio Laboratories, Carlsbad, CA), following the protocol established by the manufacturer. The DNA was quantified in a spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA) and the concentration was adjusted to 200 ng. The colaexon3-fwd and colaexon4-rev primers were used to amplify the codon 143 region of the *Cyt b* gene of the *M. fructicola* isolates (Table 1). PCR was conducted based on the method of Hily et al. (2011) with some modifications. A total volume of 12.5 µl contained 2 µl of the sample DNA, 10 µM of each primer (colaexon3-fwd and colaexon4-rev), 2.5 µl of the 10× PCR buffer solution, 1.5 mM of MgCl₂, 0.2 mM of dNTPs, and 0.4 units of Taq DNA polymerase. For the reaction, the thermocycler was programmed for one cycle of 30 s at 94°C, 45 s at 62°C, and 90 s at 72°C, followed by a cycle of 30 s at 94°C, 45 s at 60°C, and 90 s at 72°C, ending with 35 cycles of 30 s at 94°C, 45 s at 58°C, and 90 s at 72°C. The PCR products were visualized on 1.5% agarose gel in Tris-Borate-EDTA buffer (TBE) and stained with GelRed (Biotium Inc., Hayward, CA). DNA fragments were visualized by ultraviolet transillumination and photodocumented. If the isolates of *M. fructicola* showed an intron associated with the codon 143 of the *Cyt b* gene, a fragment of 1,258 bp was displayed;

Fig. 1. Frequency of the isolates in different classes of percentage inhibition of conidial germination of *Monilinia fructicola*. **A and B**, São Paulo population ($n = 63$); **C and D**, Paraná and Santa Catarina population ($n = 37$); **E and F**, Rio Grande do Sul population ($n = 43$). Discriminatory dose at 0.3 µg/ml: A, C, and E; 1 µg/ml: B, D, and F.

however, if the observed fragment was 92 bp, the absence of the intron (1,166 bp) was indicated.

Cyt b gene analysis to assess the potential mutations G143A, F129 L, and G137R and the association of the intron after codon 143. For the analyses of potential mutations in codons 143, 137, and 129 of the *Cyt b* gene and the occurrence of an intron immediately after the codon 143, nine isolates from the 2005–2011 UFPR-LEMID Collection (Paraná and São Paulo) and 10 isolates from São Paulo State collected in the 2012–13 season were used. The DNA of the 19 selected isolates was extracted from 10-day-old cultures, using the modified CTAB protocol (Doyle and Doyle 1987) or the Wizard DNA purification kit (Promega, Madison, WI), according to manufacturer's recommendations. Nucleic acids were quantified on the NanoDrop 1000 spectrophotometer. PCR was performed in 50-μl volumes containing 35.1 μl of water, 5 μl of 10x buffer, 1.5 μl of 50 mM MgCl₂, 1 μl of dNTP, 2.5 μl of each primer (10 uM), 0.4 μl of Taq (5 U.μl⁻¹), and 2 μl of DNA or water (control); conditions were 95°C for 2 min, followed by 29 cycles of 95°C for 30 s, 52 or 55°C for 30 s, 68°C for 1.5 or 2.5 min, and final extension of 5 min at 68°C (Table 1). PCR products were visualized on agarose gel at 1.5% and 5-μl aliquots of each sample were mixed with 1 μl of diluted GelRed (1:50) in the buffer.

An enzymatic cleaning, ExoSAP-IT (USB Corp., Cleveland, OH), of all PCR products was performed. Five microliters of each PCR sample were mixed with 2 μl of ExoSAP-IT and this mixture was incubated at 37°C for 15 min and then at 80°C for 15 min. After this incubation period, 15 μl of water was added, totaling 22 μl. Before sending for sequencing (Genewiz, South Plainfield, NJ), each sample was divided into 10 μl for the forward primer and 10 μl for the reverse primer. The reverse sequences were converted using the "Reverse Complement" program (https://www.ualberta.ca/~stothard/javascript/rev_comp.html) and all nucleotide sequences were aligned using the "Clustal Omega" program (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The consensus sequences were defined and compared

with sequences of the cytochrome *b* of *M. fructicola* available in GenBank, using the program BioEdit version 7.2.5.

Data analysis. Germination inhibition (*Gi*) was calculated by the following equation: $Gi = (G_{tc} - G_t) / G_{tc} \times 100$, where G_{tc} and G_t are the average frequencies of germinated conidia in treatments without and with fungicide, respectively. Analysis of variance (ANOVA) was performed on the data of sensitivity of *M. fructicola* isolates to azoxystrobin and the treatment means were compared by Tukey's Student Range test (honest significant difference, $P < 0.05$). The EC₅₀ of *M. fructicola* isolates to azoxystrobin was calculated by linear regressions between *Gi* and the log₁₀ of the fungicide concentration. The values *Gi*, area under the lesion growth of each isolate (AULG_{IS}), latent period (LP), and EC₅₀ estimated for each isolate were utilized in the multivariate analysis. The data were subjected to cluster analysis after standardization of the variables (the mean of the values for each variable was subtracted from each variable value and the result was divided by the standard deviation of the values for each variable) using the Minkowski distance and Ward's method as a clustering technique. Confidence interval (*CI*) was calculated by the following equation:

$$CI = X - t_{\alpha/2} \frac{s}{\sqrt{n}}; X + t_{\alpha/2} \frac{s}{\sqrt{n}}$$

where X is average of the observed values, $t_{\alpha/2}$ is tabulated value for bilateral t test with 95% confidence, s is standard deviation, and n is the number of observed values. All statistical analyses were conducted with the R software version 3.1.3 (R Development Core Team 2015).

Results

A significant decrease in germination and germ tube growth of conidia on WA with SHAM was observed when compared with the control without SHAM (Table 2). The average spore germination and the size of the germ tube in WA (control) was 91.87% and 902 μm,

Table 4. Percentage inhibition of conidial germination (*Gi*), area under lesion growth (AULG), latent period (LP), and concentration for 50% inhibition of conidial germination (EC₅₀) of *Monilinia fructicola* isolates

Isolates		<i>Gi</i> (%)		First assay		Second assay		EC ₅₀ (μg/ml)
		0.3 μg/ml	1 μg/ml	AULG _{IS} ^y	LP (days)	AULG _{IS} ^y	LP (days)	
Low sensitivity	SP06-213	22.05	82.42	6.86	2.3	1.29	2.0	0.23
	SP07-317	44.89	83.86	7.00	2.3	4.13	2.3	0.28
	SP08-343	24.70	79.36	7.19	2.5	2.99	2.0	0.36
	SP08-345	13.06	79.16	6.84	2.5	2.59	2.5	0.45
	SP09-829	28.52	84.27	7.06	2.3	1.00	2.0	0.17
	SP09-834	5.42	54.88	12.03	2.0	1.57	2.0	0.39
	SP09-839	31.16	54.51	6.02	2.0	0.71	2.0	0.32
	SP09-860	19.87	77.23	7.86	2.3	1.85	2.0	0.51
	SP09-885	29.65	50.70	7.07	2.0	3.74	2.0	0.63
	SP09-888	26.30	82.35	11.04	2.3	2.37	2.0	0.35
	SP09-940	23.75	85.75	14.89	4.8	2.18	2.0	0.26
	SP09-945	76.46	83.13	5.29	2.0	1.27	2.0	0.27
	SP09-951	42.72	70.26	14.08	4.5	1.33	2.3	0.33
	SP09-956	42.53	79.94	16.46	5.3	2.58	2.0	0.31
	Average	30.08 b	75.48 b	9.12 a	2.76 b	2.03 a	2.07 b	0.35 b
	CI ^z	(20.78; 39.36)	(68.79; 82.17)	(7.14; 11.11)	(2.14; 3.38)	(1.46; 2.60)	(1.96; 2.16)	(0.29; 0.41)
High sensitivity	SP08-340	100.00	100.00	16.37	4.3	2.30	3.0	0.06
	RS08-311	88.48	95.02	9.04	5.0	3.41	4.0	0.12
	RS08-329	100.00	100.00	8.80	3.0	3.19	4.0	0.13
	RS08-335	100.00	100.00	9.43	4.0	2.67	2.0	0.11
	RS10-979	47.51	100.00	13.87	5.0	2.47	4.0	0.17
	RS11-140	74.72	100.00	13.29	3.8	5.15	4.0	0.12
	PR02-100	81.66	100.00	14.13	5.5	3.59	3,0	0.05
	PR08-190	80.05	100.00	16.85	5.0	3.23	3.0	0.42
	PR11-13	67.32	100.00	9.29	3.5	1.25	2.0	0.37
	Average	82.19 a	99.45 a	12.34 a	4.34 a	3.03 a	3.22 a	0.17 a
	CI ^z	(68.67; 95.71)	(85.17; 100)	(9.85; 14.83)	(3.70; 4.98)	(2.21; 3.85)	(2.68; 3.83)	(0.07; 0.27)

^y AULG_{IS} = AULG_{IS-treat} - AULG_{IS-cont}

^z Confidence Interval:

$$CI = X - t_{\alpha/2} \frac{s}{\sqrt{n}}; X + t_{\alpha/2} \frac{s}{\sqrt{n}}$$

respectively. The conidia exposed to SHAM had a mean germination and germ tube length of 76.3% and 616 μm , respectively (Table 2).

The mean percentage inhibition of conidial germination (G_i) at 0.3 $\mu\text{g/ml}$ of azoxystrobin for the SP population was 63.1% with a confidence interval of 55.7 to 70.4% (Table 3). At this concentration, isolates of this population were distributed in all G_i classes (Fig. 1A). Populations from southern Brazil showed similar behavior, with germination inhibition above 85% (Table 3, Fig. 1C and E). At 1 $\mu\text{g/ml}$ of azoxystrobin, the average G_i for the SP population was 93.5% (Table 3). Two groups of sensitivity were observed in this population: one group with G_i ranging from 50 to 90% and another from 90 to 100% (Fig. 1B). The average G_i of the PR/SC and RS populations was greater than 99% (Table 3, Fig. 1D and F). There was no significant difference between G_i values of the PR/SC and RS populations ($P > 0.05$). However, the G_i values of these populations were significantly different ($P < 0.05$) from the values found in the SP population at 0.3 and 1 $\mu\text{g/ml}$ of azoxystrobin (Table 3).

M. fructicola isolates from stone fruits were initially classified into two groups according to the G_i at 0.3 and 1 $\mu\text{g/ml}$: isolates with high or low sensitivity to azoxystrobin (Table 4). The average time for sporulation of isolates with low sensitivity was 2.8 and 2.1 days in the first and second assay, respectively. The group of isolates with higher sensitivity showed average sporulation times of 4.3 and 3.2 days in the first and second assay, respectively (Table 4). The average EC_{50} of the group of isolates with lower sensitivity to fungicide was 0.35 $\mu\text{g/ml}$, significantly higher than the EC_{50} value of the group with higher sensitivity of 0.17 $\mu\text{g/ml}$ (Table 4). There was no difference in the AULG of the treatment compared with the AULG of the control (without fungicide) and there was no significant difference between the groups of isolates with higher and lower sensitivity to the fungicide (Table 4).

Fig. 2. Dendrogram of *Monilinia fructicola* isolates in groups according to combined analysis of latent period, effective concentration for 50% inhibition of conidial germination, percentage of conidial germination inhibition, and area under the lesion growth of each isolate. The dotted line represents the Minkowski distance value used to separate the two clades of isolates.

The dendrogram separated the isolates into two groups according to combined analysis of latent period and EC_{50} of conidial germination (Fig. 2). A group of eight isolates had greater sensitivity to the fungicide. This group was composed of isolates from all regions, mostly from PR and RS. Another group consisted of 16 isolates with low sensitivity to the fungicide, all of which, except for the isolate PR11-13 from PR, came from SP (Fig. 2).

All *M. fructicola* isolates (subpopulation of 58 isolates) yielded fragments of 1,258 bp, which corresponded to the presence of introns associated with the G143 codon in the *Cyt b* gene (Fig. 3). The cola 2-fwd primer was designed and used with colaexon4-rev to amplify the codons 137 and 143 and the 1,166 bp intron (fragments from 1,305 to 1,316 bp) (Fig. 4A). Besides this primer, a pair of primers, cola0-fwd and cola1-rev, were designed based on sequences deposited in the GenBank to amplify codon 129 (608-bp fragment). All isolates of *M. fructicola* had identical sequences (Fig. 4). No isolate showed any mutation in codons 129, 137, and 143, and a group I intron was located after codon 143 (Figs. 5 and 6). The intron-exon organization was the same as that of *Cyt b* sequences of other *M. fructicola* isolates in GenBank, showing 100% identity with accession numbers GU933643 (Miessner and Stammler 2010), GQ304941 (Luo et al. 2010), and GU952815 (Villani and Cox 2009). The sequences for Brazilian isolates SP05160 and 4A are available in GenBank under accession numbers KM610206 and KM610207, respectively.

Discussion

In this study, differences in the sensitivity of *M. fructicola* to azoxystrobin were found between sampled regions in Brazil. The population from SP has a lower sensitivity to the fungicide than the populations from PR, SC, and RS. The analysis of the *Cyt b* region showed that isolates from all these regions in Brazil have an intron associated with codon 143, indicating that mutations in this codon are unlikely to select for less sensitive individuals (Fig. 3). Other possible changes in *Cyt b* gene, such as F129 L and G137R, were not found in a subpopulation of the pathogen evaluated in this study. Our results reinforce a high intraspecific homogeneity of *M. fructicola* populations as previously observed in populations of U.S. (Hily et al. 2011) and China (Luo et al. 2010).

Low sensitivity of *M. fructicola* isolates to azoxystrobin in Brazil has been observed in areas with high spray frequency (3 to 6/season), whereas high sensitivity was detected in areas with low frequency of application (less than 2) (May De Mio et al. 2011). Sensitivity of *M. fructicola* has also been reported in other countries such as the United States (Luo and Schnabel 2008) and China (Chen et al. 2014).

Baseline sensitivity to azoxystrobin (EC_{50}) in *M. fructicola* populations was 0.15 $\mu\text{g/ml}$ in the U.S. (Amiri et al. 2010) and 0.05 to 0.13 $\mu\text{g/ml}$ in Brazil (May De Mio et al. 2011). In SP, the average EC_{50} of *M. fructicola* isolates was 0.35 $\mu\text{g/ml}$, clearly indicating a decrease in the sensitivity of this population to the fungicide. *M. fructicola* isolates collected in the states of PR,

Fig. 3. Analysis of the cytochrome *b* gene for the presence of an intron associated with the G143 codon in *Monilinia fructicola* isolates. PCR fragments using primers (colaexon3-fwd and colaexon4-rev) that anneal on both sides of the G143 codon are presented for representative isolates of *M. fructicola* with different levels of sensitivity to azoxystrobin.

SC, and RS showed EC₅₀ values close to the baseline values (Table 4), indicating that the pathogen population is more sensitive to the fungicide compared with the population in São Paulo. In the United States, Amiri et al. (2010) investigated the sensitivity of 157 *M. fructicola* isolates collected between 2006 and 2008 and found mean EC₅₀ values

ranging from 0.05 to 0.6 µg/ml in South Carolina and Georgia, with conidial germination assays. In mycelial growth assays, the same authors determined an EC₅₀ of 0.9 and 1.2 µg/ml. In China, EC₅₀ values ranged from 0.02 to 1.94 µg/ml for mycelial growth (Chen et al. 2014).

Fig. 4. A, Amplified DNA fragments with the cola2-fwd and colaexon4-rev primers for cytochrome *b* gene (*Cyt b*) region including codons 137, 143, and intron (1,166 bp) of *Monilinia fructicola* isolates with different levels of sensitivity to azoxystrobin and **B**, DNA fragments amplified with cola0-fwd and cola1-rev primers for *Cyt b* region including codon 129 of *M. fructicola* isolates with different levels of sensitivity to azoxystrobin. Water was used as the control.

Fig. 5. A, Sequence of codon 129 of the cytochrome *b* gene (*Cyt b*) from *Monilinia fructicola* obtained by the primer pairs cola0-fwd and cola1-rev. Text highlighted in gray indicates part of the intron (566 bp), arrows indicate the position of the sequence of the primer (5'-3'), bold and underlined text indicates the codon associated with partial resistance (F129) to QoIs. **B**, *Cyt b* partial nucleotide sequences from representative *M. fructicola* isolates sensitive or with reduced sensitivity to azoxystrobin. Codon 129 is underlined and in bold.

Fig. 6. A, Sequence of regions including codons 137 (G137), 143 (G143), and the intron of the cytochrome *b* gene (*Cyt b*) from *Monilinia fructicola* isolates with different levels of azoxystrobin sensitivity. Text highlighted in gray indicates the intron (1,166 bp), arrows indicate the position of the sequence of the primer (5'-3'), bold and underlined text indicates the codon associated with partial (G137) or total (G143) resistance to QoIs. **B**, Partial sequences of the *Cyt b* nucleotide from representative reduced sensitivity and sensitive isolates. Codons 137 and 143 are underlined and in bold and part of the intron is highlighted in gray.

The reduction in fungal sensitivity to azoxystrobin can be qualitative or quantitative. The qualitative type confers full resistance and is commonly associated with mutations in the *Cyt b* gene, and the quantitative type changes the sensitivity of the isolates to the fungicide and may not confer practical resistance to QoIs (Köller et al. 2004). Qualitative resistance may be preceded by a quantitative one, as observed in *Venturia inaequalis* isolates resistant to trifloxystrobin and kresoxim methyl (Köller et al. 2004). The mutation at codon 143 (G143A) of *Cyt b* is the resistance mechanism most associated with the complete absence of disease control in the field by QoI fungicides of various pathogenic fungi, such as *Magnaporthe oryzae* (Castroagudín et al. 2015; Kim et al. 2003). All *M. fructicola* isolates analyzed in this study showed no qualitative resistance, and the same intron-exon organization that has been described in previous studies with *M. fructicola* isolates in the U.S., China, Australia, and New Zealand (Hily et al. 2011; Luo et al. 2010; Miessner and Stammler 2010).

The occurrence of practical resistance has been studied in the laboratory, for example, on detached fruit (ex vivo). According to Chen et al. (2014), fruit inoculated with less sensitive *M. fructicola* isolates, after azoxystrobin treatment, had more brown rot symptoms (practical resistance). In the same experiment, the authors confirm that fruit inoculated with sensitive isolates had no symptoms of the disease. Likewise, Amiri et al. (2010) observed the same ratio comparing isolates of different sensitivity levels. Our results showed differences on the latent period of isolates with high and low sensitivity. The isolates with high sensitivity to fungicide had an average sporulation delay of about 1.4 days. Burnett et al. (2010) evaluated the antispore capacity on treated or nontreated fruit with azoxystrobin and noted a 15.9% reduction in sporulation on fruit treated with the fungicide, which supports the results of this study.

In conclusion, there was a clear change in sensitivity levels of *M. fructicola* isolates to azoxystrobin in Brazil. The common mutations related to resistance to QoIs (G143A, F129L, and G137R) were not found, suggesting that further studies should be directed to another reduced sensitivity mechanism. Moreover, monitoring of the isolates in the orchards should be continued.

Acknowledgments

We thank Karla Kudlawiek for technical assistance, Dr. Cristiano Nunes Nesi for statistical support, and stone fruit producers of the Rio Grande do Sul, Santa Catarina, Paraná, and São Paulo states. This work was supported by the National Counsel of Technological and Scientific Development (CNPq) [grant number 79041/2010-5 Universal/CNPq] and Coordination for the Improvement of Higher Education Personnel (CAPES) [Grant n.2829/2010]. We also thank CAPES for the post-doctoral fellowships of the Wagner V. Pereira and Rafael G. F. Morales and FAPESP (2014/09472-5) for the master grant to Isabela V. Primiano. The opinions, assumptions, conclusions, and recommendations expressed in this paper are those of the authors and do not necessarily reflect the views of FAPESP.

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