Sensitivity of *Monilinia fructicola* from Brazil to Tebuconazole, Azoxystrobin, and Thiophanate-Methyl and Implications for Disease Management

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Abstract

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The aim of this study was to investigate the sensitivity of *Monilinia fructicola* isolates to tebuconazole (demethylation inhibitor [DMI]), azoxystrobin (quinone outside inhibitor), and thiophanate-methyl (methyl benzimidazole carbamate) in 118 isolates collected from four states in Brazil from orchards with different histories of fungicide use. Sensitivity to fungicides was determined by inhibition of mycelial growth and spore germination on fungicide-amended media. Polymerase chain reaction was used to determine the frequencies of *M. fructicola* genotypes exhibiting high (HR) and low (LR) resistance to thiophanate-methyl among sampled populations. Resistance to tebuconazole was found in 15.8% of isolates collected from São Paulo State. The 50% effective concentration (EC₅₀) values varied from 0.01

to greater than 100 µg/ml. The EC₅₀ values for tebuconazole corresponded to its historic use frequency in the orchard; for instance, isolates from orchards with no DMI fungicide use had the lowest mean EC₅₀ value (0.04 µg/ml), while those collected from orchards where more than five DMI fungicide sprays were applied per season had a mean EC₅₀ value of 21.17 µg/ml. All isolates were sensitive to azoxystrobin but their EC₅₀, based on tests of inhibition of conidial germination, increased from 0.05 in 2002 to 0.44 µg/ml in 2008. The EC₅₀ values based on mycelial growth inhibition for thiophanate-methyl were >162 µg/ml, 1.99 to 12.5 µg/ml, and <1.0 µg/ml for HR, LR, and sensitive isolates, respectively.

Stone fruit (nectarine, peach, and plum) are grown on about 22,000 ha in Brazil (11), located mainly in the southern states of Paraná (PR), Santa Catarina (SC), and Rio Grande do Sul (RS) and the southeastern state of São Paulo (SP). The products are marketed as fresh fruit from September to January, depending on the cultivar and where the fruit are produced. Fruit for processing are mainly produced in Pelotas County of RS, while fresh fruit are produced in all of the aforementioned states.

Stone fruit production in Brazil is threatened by blossom blight and fruit rot caused by the ascomycete fungus *Monilinia fructicola* (G. Winter) Honey (18,20). Fungicide sprays are usually used to control this disease in commercial peach orchards of Brazil. A grower may apply three fungicide sprays during bloom and three more before harvest. However, the number of sprays per season may differ depending on weather conditions and location (18). Most Brazilian stone fruit growers in SP use quinone outside inhibitor (QoI) fungicides alternated with demethylation inhibitor (DMI) fungicides as part of their disease management program. They also continue to apply these fungicides after harvest to control peach rust caused by *Tranzschelia discolor* (1). Some peach producers use iprodione, procymidone, and thiophanate-methyl fungicides at least twice a season.

The frequent use of site-specific fungicides has the potential for fungicide resistance development in *M. fructicola* populations. *M. fructicola* isolates resistant to the methyl benzimidazole carbamate (MBC) fungicide benomyl have been found in the United States (17,19,32,34) and in other countries (9,23,24). Although benomyl was withdrawn from the market in 1992, stone fruit growers in Brazil and elsewhere have the option to use thiophanate-methyl,

another MBC, as a replacement for benomyl. DMI fungicide resistance has been detected in *M. fructicola* isolates from commercial peach orchards in Georgia (25), South Carolina, Ohio, New York, and New Jersey (5,14) but not in California (31). With respect to QoI fungicides, Amiri et al. (2) observed a shift toward reduced sensitivity to azoxystrobin after a comparison between isolate populations from 2006 and 2008. Where QoI use is frequent and selective pressure is high, the sensitivity of isolates may change over time, as was recently determined in other pathosystems (12,33), including fungal pathogens of perennial crops (3).

It is not clear whether *M. fructicola* has developed fungicide resistance in light of the aforementioned frequent use of site-specific fungicides for blossom blight and fruit brown rot control in commercial peach orchards in Brazil. Thus, the objectives of this study were to determine the sensitivity of *M. fructicola* isolates from Brazil to the DMI fungicide tebuconazole, QoI fungicide azoxystrobin, and MBC fungicide thiophanate-methyl. Furthermore, isolates resistant to thiophanate-methyl were characterized on a molecular level.

Materials and Methods

Isolates. In total, 118 isolates of *M. fructicola* were collected from spring of 2000 to fall of 2008 from blossoms, twig cankers, symptomatic mature fruit, and mummified fruit in peach, nectarine, and plum orchards. The isolates were identified to the species level as described previously (7) and came from four states in Brazil: 29 from SP, 30 from PR, 29 from SC, and 30 from RS (Fig. 1). To obtain single-spore isolates, a drop of a spore suspension (approximately 10⁶ conidia/ml) of each isolate was streaked onto a 90-mm petri dish containing acidified (2.5 ml of 25% [vol/vol] lactic acid per liter) potato dextrose agar (PDA-LA) (Microtech Scientific, Vista, CA). The petri dishes were incubated at 23 to 25°C for 1 day and single germinated conidia were transferred onto a fresh PDA-LA petri dish with the aid of a dissecting microscope (×20 magnification). Each isolate was stored in form of mycelial plugs in sterile water at 4°C until use (6).

Survey of fungicide sensitivity. All 118 isolates of *M. fructicola* were tested for sensitivity to tebuconazole, azoxystrobin, and

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thiophanate-methyl. Technical-grade tebuconazole (a.i. 97.5%; Bayer Corporation, Kansas City, MO) was dissolved in 100% ethanol, adjusted to a concentration of 10 mg a.i./ml, and added to PDA-LA cooled to 45°C after sterilization to produce final tebuconazole concentrations of 0, 0.005, 0.015, 0.045, 0.135, 0.405, and 1.2 µg/ml. A 4-mm mycelial plug of each isolate was removed from the edge of a 6-day-old colony on PDA-LA using a cork borer and placed separately in the center of a PDA-LA petri dish amended with one of the above fungicide concentrations. Two replicate petri dishes per concentration were used for each isolate. The cultures were incubated at 25°C for 6 days. The diameter of the colony was measured in two perpendicular directions, and the diameter of the 4-mm original mycelial plug was subtracted. Some isolates showed no significant reduction of growth at the above-mentioned fungicide concentrations. These isolates were further retested on media amended with tebuconazole at 0.005, 0.015, 0.045, 0.135, 0.405, 1.2, 3.6, 10.2, and 30.6 µg/ml.

Technical-grade azoxystrobin (Syngenta Crop Protection, Greensboro, NC) was dissolved in 100% acetone and adjusted to a

concentration of 1 mg/ml. Sensitivity of each isolate to azoxystrobin was determined using a spore germination method (21) on 2% water agar (WA) medium amended with azoxystrobin at 0, 0.0125, 0.05, 0.015, 0.45, 0.135, 0.405, and 1.2 µg/ml. Azoxystrobin and salicylhydroxamic acid (SHAM; 22) were added to autoclaved WA cooled to 45°C. SHAM (Sigma-Aldrich, St. Louis) was dissolved in 100% methanol at a concentration of 100 mg/ml. The concentrations of acetone and methanol in all WA media were 1 ml/liter. This assessment was performed with two subpopulations, using 12 isolates for the first subpopulation (3 for each of the four states) collected in 2008 (4 years after the introduction of azoxystrobin in stone fruit crops), 2 isolates collected in 2002, and 1 in 2004 from orchards with no azoxystrobin spray history (considered baseline isolates). This experiment was repeated twice.

No other baseline isolates of *M. fructicola* were available from Brazil; however, some of the 12 isolates tested (PR 152, PR 157, PR 158, and PR 159) were considered baseline isolates because they were collected before the use of the active ingredient or taken

Fig. 1. Map of São Paulo, Paraná, Santa Catarina, and Rio Grande do Sul states of Brazil and locations of *Monilinia fructicola* isolate collection.

from unsprayed backyard peach trees located 150 km away from commercial orchards.

Conidial suspensions were prepared in sterile water from 10-day-old PDA-LA cultures at 25°C and adjusted to 1×10^4 conidia/ml. A 0.1-ml aliquot of the conidial suspension of each isolate was spread on azoxystrobin-amended medium (plus SHAM) and control (with SHAM only) medium. Three petri dishes of each fungicide concentration were used per isolate. After incubating the petri dishes at 25°C in darkness for 12 h, 100 conidia per petri dish were examined microscopically and the percentage of germinated conidia was recorded per isolate. The 50% effective concentration (EC_{50}) value for each isolate was obtained using a linear regression between percentage of inhibition of spore germination and the corresponding log10 concentrations of the fungicide.

Thiophanate-methyl (Topsin M 70 WP; Elf Atochem North America, Inc., Philadelphia) was suspended in sterile water, adjusted to a concentration of 10 mg/ml, and added to PDA-LA medium at a final concentration of 1 µg/ml, the discriminatory dose for sensitive isolates (S). All 118 isolates were tested for high resistance (HR) and isolates that grew in 1 µg a.i./ml were tested further for the presence of the E198A mutation (FEA), conferring HR. Primers LRF and LRR2 were used to detect low resistance (LR) as described previously (16). All isolates with HR and 12% of those with LR were subjected to mycelium growth-inhibition tests using three replicate petri dishes of PDA-LA medium amended with thiophanate-methyl at 0, 1, 5, 10, 15, 100, and 500 µg/ml as described above. This experiment was repeated twice and, because equal variances between the two datasets were obtained, the data were combined. Isolates that grew with thiophanate-methyl at 1 mg/ml but lacked the allele for LR or HR are referred to as nonsensitive isolates (NS).

DNA extraction from single-spore cultures and detection of resistance alleles. To extract DNA from each of the 118 isolates, mycelium from 7-day-old cultures on PDA-LA medium was transferred to 40 ml of potato dextrose broth (PDB) and incubated for 6 days at 25°C. Mycelium was harvested, washed with sterile water four times, and dried for 2 h. DNA was extracted using the Fast-DNA kit by following the manufacturer's guidelines (Biogene Inc., Carlsbad, CA). Polymerase chain reaction (PCR) was performed for all 118 isolates to detect the HR and LR genotype as described previously (16,17).

Data analysis. For each concentration, the inhibition of colony growth (L_i) of isolate i was calculated by $L_i = (C_{ck} - C_i)/C_{ck} \times 100$,

where C_{ck} is the mean colony diameter for the control with no fungicide and C_i is the mean colony diameter for isolate i . The concentrations of fungicides were log10 transformed. Linear regressions of the L values on log concentration of the fungicide were performed with the REG procedure of SAS (version 8.0; SAS Institute, Cary, NC). The EC_{50} value for each fungicide was calculated from the corresponding regression equation for each isolate where the regression coefficient was significant at $P < 0.05$.

The EC_{50} values of *Monilinia* isolates for tebuconazole were classified into four groups by state, four groups by fungicide spray history with an equal interval, and another group by historical and current population. The fungicide history was defined as follows: no use of fungicide (0 sprays), 1 to 2 sprays per season, 3 to 4 sprays per season, and, more than 5 sprays per season. The isolates of *M. fruticola* collected from 2000 to 2004 were considered as the historic population while those collected from 2005 to 2008 (when there was an intensive use of tebuconazole started in 2005 in Brazil) were considered as the current population. The arithmetic mean EC_{50} and the frequency of isolates were calculated for each group. For each fungicide, comparisons were conducted with the NPAR1WAY procedure of SAS using the Kuiper Test to determine significant differences between two distributions of EC_{50} values, and using median scores (χ^2) to compare averages among populations collected in different years and different states of Brazil. To compare the EC_{50} values between the historic and current populations, analysis of variance was conducted and means were compared with the Fisher's least significant difference (LSD) test of SAS (version 8.0; SAS Institute).

Results

Resistance to tebuconazole and distribution of EC_{50} values by state and fungicide exposure. The distribution of EC_{50} values of the 118 isolate population of *M. fruticola* fit the log normal function (Kolmogorov-Smirnov $d = 0.23$, $P < 0.01$). Only four of the analyzed isolates had EC_{50} values between 10 and 20 µg/ml while only three isolates had EC_{50} values >100 µg/ml (Fig. 2). There was a significant difference ($P \leq 0.001$) in the mean EC_{50} value between the historical population of isolates collected from 2000 through 2004 and the current population of isolates collected from 2005 to 2008. The EC_{50} values ranged from 0.01 to 0.27 in 31 historical isolates, and from 0.01 to more than 100 µg/ml in 87 current population isolates. The majority (84%) of the historical population had $EC_{50} < 0.06$ µg/ml as compared with 46.5% of the current population. In the current population, 15.8% of the isolates were resistant to tebuconazole (using the discriminatory dose of propiconazole 0.3 µg/ml as described by Cox et al. [8]; Table 1; Fig. 2). The frequency of the historical population was significantly different from the corresponding frequency of the current population (Kuiper test, $K_a < 0.001$; Fig. 2A and B).

The mean EC_{50} value of 30 isolates from the SP region was significantly ($P < 0.002$) different from the values of the other three regions; isolates from SC were not different from PR ($P = 0.2443$; Table 2; Fig. 3). Isolates from the SP region had a mean EC_{50} value of 16.5 µg/ml and a range of 0.017 to 165 µg/ml, compared with isolates from the other regions, which ranged from 0.010 to 0.2

Fig. 2. Distributions of 50% effective concentration (EC_{50}) values for tebuconazole from **A**, historic and **B**, current populations of *Monilinia fruticola* isolates in Brazil. The Kuiper test (NPAR1WAY procedure of SAS) showed significant difference in the distribution of EC_{50} values between these two populations ($K_a = 0.001$).

Table 1. Means and ranges of 50% effective concentration (EC_{50}) values to tebuconazole in historic (2000 to 2004) and current (2005 to 2008) populations of *Monilinia fruticola* isolates collected from stone fruit orchards in Brazil

Population	EC_{50} (µg/ml)		No of isolates	% of isolates resistant ²
	Mean ¹	Range		
2000–2004	0.04 b	0.01–0.27	31	0.0
2005–2008	5.65 a	0.01–>10.2	87	15.8

¹ Numbers followed by different letters indicate significant differences at $P = 0.001$.

² Percentage of isolates with resistance to tebuconazole ($EC_{50} > 0.3$ µg/ml). Dosage used to discriminate propiconazole resistant from sensitive isolates according to Cox et al. (8).

(RS) and 0.01 to 0.1 (SC) to 0.014 to 0.5 µg/ml (PR). In SP, resistant isolates were detected at 43% (13 of 30 isolates) with EC₅₀ greater than 0.3 µg/ml, while only 3% were detected in PR. In RS and SC, no resistant isolates were detected and the maximum EC₅₀ values were 0.2 and 0.1 µg/ml, respectively (Fig. 3). The distributions of the isolates among the four regions were very distinct, although the Kuiper Test (used to distinguish two populations for asymptotic distribution) showed that the isolate distribution in SC was not statistically different from that of PR ($P = 0.3302$; Table 2; Fig. 3).

The mean EC₅₀ correlated well with the number of fungicide sprays historically applied to the orchard of origin. Values ranged from 0.04 µg/ml for 0 sprays, to 0.06 µg/ml for 1 or 2 sprays per season, to 0.10 µg/ml for 3 to 4 sprays per season, and to 21.17 µg/ml for areas with more than 5 sprays per season (Table 2; Fig. 4). Comparing the distribution of EC₅₀ values of isolates among the regions, the Kuiper Test demonstrated discrepancy only for more than 5 sprays ($P = 0.0001$) but the mean EC₅₀ values were also different between isolates from orchards with 1 to 2 and those with 3 to 4 sprays ($P = 0.0095$; Table 2; Fig. 4).

Sensitivity of *Monilinia* isolates to azoxystrobin using spore germination assays. The current (2008) population of isolates

showed significantly ($P \leq 0.0001$) less sensitivity to azoxystrobin compared with the historic population (2002 to 2004), with mean EC₅₀ values of 0.31 and 0.12 µg/ml, respectively. Statistical analyses showed that the sensitivity of isolates to azoxystrobin varied among populations in different states (Table 3). Two isolates were used as baseline references (PR100, collected in 2002 and PR 158, collected in 2004), which had EC₅₀ values of 0.05 and 0.13 µg/ml, respectively. The 12 isolates that received 1 to 3 sprays per season showed significantly higher EC₅₀ values, ranging from 0.19 to 0.44 µg/ml, compared with the EC₅₀ values of the baseline isolates.

Molecular identification of MBC resistance alleles and in vitro sensitivity to thiophanate-methyl. In all, 65 of 118 isolates (54.8%) were sensitive to thiophanate-methyl. Only 2.5% of the resistant isolates had the E198A allele conferring HR and 35.9% were identified as LR with primers LRF and LRR2. Nine resistant isolates did not test positive for any of the two mutations. The EC₅₀ for the HR and LR isolates ranged from 162 to >500 µg/ml and 1.99 to 12.5 µg/ml, respectively (Table 4). In total, 80.5% of SP isolates were sensitive to thiophanate-methyl compared with 34.5 and 56.7% of the isolates from SC and RS, respectively. One isolate with HR was found in RS and two were found in SP. In the PR region, 0, 53.3, and 46.7% were HR, LR, and S isolates. The distri-

Table 2. Mean 50% effective concentration (EC₅₀) values of *Monilinia fructicola* isolates to tebuconazole and significance values (P) comparing distribution functions and means between populations of isolates classified per locations or number of fungicide sprays per season

States of Brazil ^y	Mean EC ₅₀ (µg/ml) and P value between states ^x				Mean EC ₅₀ (µg/ml) and P value between fungicide use ^x				
	SP	PR	SC	RS	Sprays ^z	0	1-2	3-4	≥5
SP	16.86	0.0003	0.0002	0.0020	0	0.04	0.7730	0.0630	0.0001
PR	(0.0001)	0.06	0.2443	0.0361	1-2	(0.5955)	0.08	0.0095	0.0001
SC	(0.0001)	(0.3302)	0.04	0.0035	3-4	(0.2791)	(0.0645)	0.10	0.0001
RS	(0.0020)	(0.0101)	(0.0401)	0.07	≥5	(0.0001)	(0.0001)	(0.0001)	21.17

^x Numbers in bold represent the mean EC₅₀ (µg/ml) per state or historical fungicide use category. P values in parentheses were estimated with the Kuiper test (Ka) and indicate significance in comparing the population distribution on each state or the population classified based on the number of fungicide applications; P values without parentheses were estimated with a χ^2 and indicate significance comparing the means of EC₅₀ in each state or those based on the number of fungicide applications.

^y States of Brazil: PR = Paraná, SC = Santa Catarina, SP = São Paulo, and RS = Rio Grande do Sul.

^z Number of fungicide sprays per season.

Fig. 3. Distribution of 50% effective concentration (EC₅₀) values for tebuconazole from *Monilinia fructicola* populations collected from A, Santa Catarina; B, Paraná; C, Rio Grande do Sul; and D, São Paulo. The Kuiper test (NPAR1WAY procedure of SAS) showed significant difference in the distribution of EC₅₀ values between populations ($Ka < 0.05$); except for those between Paraná and Santa Catarina states ($Ka = 0.3302$).

bution of S isolates between years indicated a steady increase of S isolates from 20% in 2000 to 50% in 2005, after which the frequencies remained steady (Fig. 5).

Discussion

The DMI fungicide tebuconazole has been applied in stone fruit orchards to control brown rot caused by *Monilinia* isolates in the state of SP, Brazil, since 1995 and in other states of Brazil since 2004. Our results indicate a significant increase in the EC_{50} values for tebuconazole in the current population (2005 to 2008) compared with the EC_{50} values of the historic populations (2000 to 2004) of *M. fructicola* isolates. These data are in agreement with Zehr et al. (32), who reported that the EC_{50} values of *M. fructicola* isolates from South Carolina had increased within 3 years after 29 applications of propiconazole, a fungicide with a different active ingredient but that belongs in the same group of fungicides. In contrast, Yoshimura et al. (31) compared two populations of *M. fructicola* from California and found similar EC_{50} values for tebuconazole in the 1992-to-1998 population compared with isolates collected in 2002.

Reported baseline distributions of EC_{50} values to triazoles of *M. fructicola* varied with production regions. For example, Wilcox and Burr (30) from New York determined EC_{50} values for *M. fructicola* ranging from 0.003 to 0.034 $\mu\text{g/ml}$, with a mean of 0.007 $\mu\text{g/ml}$ for tebuconazole; Zehr et al. (32) in South Carolina reported a range of 0.02 to 0.15 $\mu\text{g/ml}$, with a mean of 0.03 $\mu\text{g/ml}$ for propiconazole; and Yoshimura et al. (31) in California reported a range of 0.001 to 0.0607 $\mu\text{g/ml}$ for tebuconazole. Our results show high EC_{50} values (0.01 to 0.27 $\mu\text{g/ml}$) for the isolates of the historical population; however, when only the isolates that never had come in contact with the fungicide were considered, the EC_{50} values were similar (0.01 to 0.09 $\mu\text{g/ml}$) to those reported for U.S. populations. Holb and Schnabel (10) reported that a baseline isolate (SDL71.01) collected in 2001 from a peach orchard in South Carolina that had never been exposed to DMIs and was located at least 8 km away from the nearest commercial orchard was sensitive to propiconazole, tebuconazole, and fenbuconazole, with EC_{50} values of 0.023, 0.018, and 0.019 $\mu\text{g/ml}$, respectively. Our results showed that 15.8% of the isolates in the current population were resistant to

tebuconazole and that the majority of resistant isolates were from SP, where more than five sprays of tebuconazole per season were used. This intensive use of DMI fungicides must have led to an accelerated rate of selection for resistance. Other studies document a slower selection process (25). The reason for this intensive use of tebuconazole in the SP region is that this fungicide is also applied to control peach leaf rust (*T. discolor*), thus giving a longer period of exposure for *M. fructicola* to the active ingredient of the fungicide, especially because the spray period can sometimes last from spring to early autumn. In some cases, tebuconazole has also been used in other Brazilian states but, in SP, the peach production area is located in a region with subtropical conditions and the weather is

Table 3. Description of *Monilinia fructicola* isolates, number of sprays of azoxystrobin per season, and the corresponding 50% effective concentration (EC_{50}) values

Isolate description				
State ^w	Name	Year	Applications ^x	EC_{50} ($\mu\text{g/ml}$) ^y
SP	346	2008	>5	0.4475 a
PR	190	2008	3–4	0.4320 a
PR	186	2008	1–2	0.2988 b
PR	185	2008	1–2	0.2333 c
SP	340	2008	>5	0.2063 c
SP	347	2008	>5	0.1920 cd
SC	546	2008	1–2	0.1526 de
SC	502	2008	ni	0.1463 ef
SC	329	2008	ni	0.1352 ef
PR	158 ^z	2004	0	0.1317 ef
RS	558	2008	0	0.1310 ef
RS	335	2008	0	0.1177 ef
RS	339	2008	0	0.1049 f
PR	100 ^z	2002	0	0.0500 e

^w States of Brazil: PR = Paraná, SC = Santa Catarina, SP = São Paulo, and RS = Rio Grande do Sul.

^x Number of azoxystrobin applications per season.

^y Different letters indicate significant differences at $P = 0.05$.

^z Isolates PR 100 (2002) and PR 158 (2004) were considered baseline isolates because they did not come in contact with QoI fungicides in the field.

Fig. 4. Distribution of 50% effective concentration (EC_{50}) values for tebuconazole from populations of *Monilinia fructicola* collected from Brazilian stone fruit orchards with **A**, no fungicide spray; **B**, 1 to 2 sprays per season; **C**, 3 to 4 sprays per season; and **D**, more than 5 sprays per season. The Kuiper test (NPAR1WAY procedure of SAS) showed significant difference in distribution of EC_{50} values between number of fungicide sprays ($Ka = 0.0001$).

hotter than the southern states, making the management of the pathogen ever more intensive than that in the other states.

The distribution of *M. fructicola* isolate populations from Brazilian states seemed to be influenced by the general disease management practiced in peach orchards. For instance, the mean value of EC₅₀ to tebuconazole was high (16.3 µg/ml) for isolates in SP but very low (0.04 to 0.07 µg/ml) for isolates in the other states. Incidentally, SP is the most northeasterly state (Fig. 1) and has a subtropical to tropical climate, where the fruit production cycle is shorter and the harvest is early in the season, beginning in September and finishing in October. The association of the favorable conditions with several diseases, and the absence of cold, may have contributed to the high brown rot pressure, necessitating frequent fungicide applications. Brannen et al. (4) controlled the DMI-insensitive *M. fructicola* population in Georgia by using two pre-harvest applications of increased rates of DMI fungicides, or applications of the premixed fungicide Pristine (boscalid plus pyraclostrobin) followed by propiconazole. Although sprays with increased rates of DMI fungicides are recommended for the subtropical conditions in Brazil, there is a major concern over fungicide resistance, which needs to be closely monitored in the near future, mainly because the resistance mechanism to DMI fungicides of isolates of *M. fructicola* from Brazil is not known yet and measures used in other countries with temperate climates may not work. Evaluation of alternating sprays with fungicides with different active ingredient groups, such as guanidine, may provide a means for managing fungicide resistance. Moreira and May-De Mio (20) also reduced brown rot effectively by using iminocadine Tris albesilate (guanidine group) in peach orchards in southern Brazil. However, no monitoring for resistant isolates has been done under these circumstances.

Although resistance of *M. fructicola* to QoI fungicides was not discovered, some authors have observed a shift in the sensitivity of the isolates over time. Villani and Cox (29) assessed the sensitivity of 236 isolates of *M. fructicola* from various counties in New York State from 2005 to 2007 to Pristine and found a shift in the sensitivity of these isolates. Amiri et al. (2) investigated QoI resistance from 2006 to 2008 in 157 *M. fructicola* isolates from South Carolina and Georgia and observed that the mean EC₅₀ values of a number of isolates from these populations to azoxystrobin, pyraclostrobin, and boscalid were significantly higher ($P = 0.01$) than the mean EC₅₀ values of the baseline population, with a four- and threefold increases for boscalid and azoxystrobin, respectively. They concluded that there was a build-up of QoI-resistant isolates among *M. fructicola* populations in commercial peach orchards of the southeastern United States. Our results using the historic (2002 to 2004) populations of *Monilinia* isolates from Brazil are consistent with the studies mentioned above. The LSD test showed statistically significant differences ($P = 0.05$) between seven isolates from 2008 from orchards where 1 to more than 5 sprays of QoI fungicides were used per season. Luo and Schnabel (15) considered nine isolates of *M. fructicola* with EC₅₀ varying from 0.05 to 0.14 µg/ml azoxystrobin as sensitive. After mutagenesis, however, some of these isolates became resistant, with EC₅₀ values of 0.2 to 1 µg/ml. An azoxystrobin concentration of 3 µg/ml could be considered as discriminatory to distinguish resistant from sensitive isolates. Amiri

et al. (2) and Schnabel et al. (26) reported baseline data up to 1.35 µg/ml to azoxystrobin with SHAM. Using these criteria, in our study, no isolates resistant to azoxystrobin were observed. However, many Brazilian growers continue to use products containing this active ingredient or other fungicides of the same chemical class. Thus, it is necessary to monitor for resistance in the near future. Although we observed a shift of sensitivity for azoxystrobin in a sample of the *M. fructicola* population from Brazil tested in this study, using just two isolates as baseline isolates for this comparison may be too few to make strong conclusions. Future collection of isolates and population comparisons must be considered to better understand the behavior of *M. fructicola* to the QoI fungicides.

The results of PCR tests showed that the HR Brazilian population of *M. fructicola* isolates had the E198A allele conferring resistance to benzimidazole fungicides. Although LR isolates were found in populations from all states from 2000 to 2008, HR was found only in RS and SP states in 2005 and 2007, respectively. Three HR isolates came from the locations where the producers applied more than five sprays of thiophanate-methyl per season. Several reports on benomyl resistance in *M. fructicola* isolates from the United States reported concentrations between 0.5 and 4 µg/ml conferring resistance to benzimidazole (13,19,27,28,32). Among the isolates tested in this study, the EC₅₀ of insensitive isolates was >1 µg/ml and those with the HR gene could grow even at >500 µg/ml. Yoshimura et al. (31) reported HR and LR to benzimidazole among *M. fructicola* isolates in California stone fruit orchards with EC₅₀ values of <2 µg/ml for S isolates, between 2 and 30 µg/ml for LR isolates, and >30 µg/ml for the HR isolates. The E198A allele was also found in HR isolates of *M. fructicola* in California (17), South Carolina (34), and several other countries. Interestingly, some isolates with LR resistance to MBC were not distinguished by using the LRF and LRR2 primers, suggesting another new mechanism of resistance.

In peach production areas of Brazil, the mean number of sprays with benzimidazoles per season in 2000 was 2 to 4. Starting in

Fig. 5. Percent thiophanate methyl-sensitive isolates of *Monilinia fructicola* collected from 2000 to 2008. The best fit model was a second-degree polynomial ($R^2 = 0.983$; $P < 0.05$).

Table 4. Frequencies and 50% effective concentration (EC₅₀) of thiophanate-methyl-sensitive and -resistant *Monilinia fructicola* isolates collected from four states in Brazil

Resistance category ^y	Frequency of isolates (%) / state ^x				Isolates (%) (n = 118)	EC ₅₀ value (µg/ml) ^z
	SP	PR	SC	RS		
HR	6.5	0.0	0.0	3.3	2.5	162–>500
LR	6.5	53.3	58.6	26.7	35.9	1.99–12.5
S	80.5	46.7	34.5	56.7	54.8	<1
NS	3.2	0	6.9	13.3	5.8	>1

^x States of Brazil: PR = Paraná and RS = Rio Grande do Sul (30 isolates/state); SC = Santa Catarina and SP = São Paulo (29 isolates/state).

^y HR = isolates with the E198A allele coding for high resistance (EC₅₀ ≥ 30 µg/ml), LR = low-resistance isolates (EC₅₀ = 1 to 30 µg/ml), S = sensitive isolates (EC₅₀ < 1 µg/ml), and NS = nonsensitive isolates.

^z Range of EC₅₀ values of three HR and five LR isolates.

2002, the use of these fungicides was drastically reduced in some states (PR, SC, and RS), although some producers (mainly in PR and SC) continued to apply the same number of sprays and fungicides to control other diseases, such as anthracnose of peach caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (SP and RS) and peach leaf curl caused by *Taphrina deformans* (Berk.) Tul. The percentage of isolates sensitive to thiophanate-methyl increased until 2005 and remained steady from 2005 to 2008 (Fig. 5); considering 111 isolates, 4 isolates collected in 2004 and 3 in 2006 were excluded from the analyses because these 7 isolates had not come in contact with any benzimidazole sprays. Interestingly, when isolates were collected from areas where no fungicides had been used in some years, the incidence of sensitive populations increased to 90% (*data not shown*). However, in 1996, Moreira and May-De Mio assessed 210 isolates from Lapa County, PR, Brazil in PDA amended with benomyl at 1 µg/ml and observed that 87% of the isolates grew at the same rate as on PDA with no fungicide (control) (L. M. Moreira and L. L. May-De Mio, *unpublished data*). In the present study using isolates from the same region (Lapa, PR, 2003 to 2008), 80% of the isolates were LR and grew at 1 µg/ml. This could indicate stability in the population of *Monilinia* isolates resistant to benzimidazoles, which is also supported by other studies (23,24). However, special attention should be given to assessing the stability of these populations under field conditions in Brazil by considering other regions and more isolates.

An important consideration for fungicide resistance management is the amount of inoculum. Difficulties in managing brown rot in peach in Brazilian orchards located in subtropical regions, under warm and humid environmental conditions, are due not only to the high risk for developing fungicide resistance but also to the high disease inoculum levels found in the areas of peach production. The efficacy of DMI treatments decreased under high disease levels in peach orchards in Georgia, suggesting that even DMI fungicides cannot provide adequate control if disease pressure is too high (25).

To achieve acceptable brown rot control with fungicide applications, measures to reduce inoculum levels and alternation of active ingredients during the entire season (dormancy, bloom, fruit expansion, and prior to harvest) must be implemented in commercial peach orchards. This is the first report indicating a shift in the sensitivity to QoI fungicides in *Monilinia* isolates from Brazil and the first report of resistance to thiophanate-methyl and tebuconazole of this pathogen in Brazilian stone fruit orchards.

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