Field Strains of *Monilinia fructicola* Resistant to Both MBC and DMI Fungicides Isolated from Stone Fruit Orchards in the Eastern United States

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Abstract

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In 2012, significant brown rot disease was observed on stone fruit in Pennsylvania, Maryland, and South Carolina despite preharvest application of methyl benzimidazole carbamate (MBC) and demethylase inhibitor (DMI) fungicides. In total, 140 *Monilinia fructicola* isolates were collected from diseased orchards and examined for fungicide sensitivity. In addition to isolates resistant to either the DMI propiconazole or the MBC thiophanate-methyl, 22 isolates were discovered that were resistant to both fungicides, including 4 isolates from peach in South Carolina, 12 isolates from peach and sweet cherry in Maryland, and 6 isolates from sweet cherry in Pennsylvania. Analysis of MBC resistance revealed that dual-resistant isolates from South Carolina carried the β -tubulin E198A mutation, whereas isolates from Maryland and Pennsylvania carried E198 mutations not previously described in the *Monilinia* genus, E198Q or F200Y. The genetic element Mona, associated with DMI fungicide resistance in *M. fructicola*, was de-

tected in the dual-resistant isolates from South Carolina but not in the isolates from the two more northern states. An investigation into the molecular mechanism of DMI resistance in the latter isolates revealed that resistance was not based on increased expression or mutation of *MfCYP51*, which encodes the target of DMI fungicides. Label rates of formulated propiconazole or thiophanate-methyl were unable to control dual-resistant isolates on detached peach fruit, confirming field relevance of dual resistance. The same isolates were not affected by fitness penalties based on mycelial growth rate, ability to sporulate, and virulence on detached peach fruit. The emergence of *M. fructicola* strains resistant to both DMI and MBC fungicides in multiple states and multiple stone fruit crops is a significant development and needs to be considered when designing resistance management strategies in stone fruit orchards.

Monilinia fructicola (G. Winter) Honey is one of the most important causal agents of brown rot, blossom blight, and fruit brown rot of stone fruit in the United States. Although sanitation practices such as pruning, removal of fruit mummies, and elimination of wild plum are widely implemented, chemical control is still an important strategy for management of diseases caused by this pathogen. Site-specific fungicides such as the methyl benzimidazole carbamates (MBCs), targeting β -tubulin, and sterol demethylation inhibitors (DMIs), targeting 14 α -demethylase, have been used for management of *M. fructicola*-induced diseases since the 1970s and the 1980s, respectively. More recently, quinone outside inhibitors (QoIs) and succinate dehydrogenase inhibitors (SDHIs) have been rotated in to control brown rot blossom blight and fruit brown rot.

The frequent use of site-specific fungicides has the potential for fungicide resistance development. Resistance to MBCs emerged shortly after introduction in many pathogens, including *M. fructicola*, *Venturia inaequalis*, *Penicillium aurantiogriseum*, and *Botrytis cinerea* (15). Fungicide target site alteration in β -tubulin was reported to be the primary mechanism of resistance, especially at amino acid position 198. E198A and E198K genotypes were reported in *V. inaequalis*, *V. pirina*, *Penicillium* spp., *Sclerotinia homoeocarpa* (9), *Colletotrichum gloeosporioides* (7,19), and *B.*

cinerea (2). High levels of resistance to MBCs were associated with the E198A mutation in *Monilinia* spp. from California (16), South Carolina (28), Brazil (18), and Greece (17) while E198K was found in *M. fructicola* from Michigan (9). Low levels of resistance were associated with the H6Y mutation in the β -tubulin gene of strains collected in California (16). Although suspected to exist, resistance to MBCs has not yet been reported or characterized in the northeastern coastal region of the United States.

After the widespread occurrence of resistance to MBC fungicides, DMI fungicide applications increased in importance for the chemical control of brown rot in commercial orchards of the United States. For more than two decades, DMI fungicides had been used without reports of resistance development in *M. fructicola* in the United States. Resistance was first reported in 2004 in field strains from Georgia (22) and later in South Carolina, Ohio, New York, Pennsylvania (12,26), New Jersey (3), and Brazil (18). Resistant strains from the southeast were found to overexpress the *MfCYP51* gene which encodes 14 α -demethylase, the target of DMI fungicides, and to carry the Mona insertion sequence, located upstream of *MfCYP51*, which is thought to act as a promoter (13). The Mona element was also found in the *MfCYP51* promoter region of DMI-resistant strains from the northeast (26). Some field strains from New York with reduced sensitivity to propiconazole lacked Mona (26), indicating that either overexpression of the *MfCYP51* gene can be triggered by means other than Mona or other resistance mechanisms may occur. It has been shown in lab mutants that 14 α -demethylase alterations, such as F136Y, are capable of causing DMI resistance in *M. fructicola* (5), although such strains have not been isolated in the United States. Resistance to DMIs in *M. fructicola* has yet to be documented in other parts of the United States.

Despite the existence of strains resistant to either DMI or MBC fungicides, strains with resistance to both fungicides (dual resistance) have not previously been reported. Consequently, management strategies for DMI resistance involved the use of MBC fungicides to control DMI-resistant strains and vice versa. In 2012,

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heavy brown rot disease incidence was reported from several stone fruit orchards in Pennsylvania, Maryland, and South Carolina despite MBC and DMI fungicide applications. Preliminary studies indicated the presence of resistance to MBC fungicide thiophanate-methyl and DMI fungicide propiconazole in isolates collected from these orchards. The objectives of this study were to characterize the resistance phenotype of the collected *M. fructicola* isolates to thiophanate-methyl and propiconazole and to investigate the molecular mechanisms of resistance.

Materials and Methods

Isolate collection and determination of fungicide sensitivity.

As part of a resistance monitoring survey, *M. fructicola* spores from 140 symptomatic stone fruit were collected from 11 commercial orchards using cotton swabs in 2012; 10 or more isolates were collected from each location. They were from six peach orchards in South Carolina, two peach orchards and one sweet cherry orchard in Maryland, one plum orchard from Virginia, and one sweet cherry orchard in Pennsylvania (Table 1). Control failure associated with economically significant disease incidence was observed in the Maryland and Pennsylvania orchards and the three orchards in Saluda County, SC. Spores from cotton swabs were transferred with sterile toothpicks to fungicide-amended and unamended medium in 24-well plates to determine the sensitivity to thiophanate-methyl (Topsin-M; Ceraxagri), propiconazole (PropiMax EC; Dow Agro-Science LLC), azoxystrobin (Abound; Syngenta Crop Protection), iprodione (Rovral 4F; Bayer Crop Sciences), and pyraclostrobin + boscalid (Pristine, 12.8% pyraclostrobin and 25.2% boscalid; BASF), as described previously (21). Discriminatory doses were 50, 0.3, 1, 10, and 1 µg/ml for thiophanate-methyl, propiconazole, azoxystrobin, iprodione, and pyraclostrobin + boscalid, respectively. A culture was considered resistant to thiophanate-methyl, azoxystrobin, iprodione, and pyraclostrobin + boscalid if it grew at a rate of more than 50% of the control and resistant to propiconazole if growth was greater than 20% of the control (21). After incubation at 22°C for 3 days, all cultures resistant to both thiophanate-methyl and propiconazole were single spore isolated as described previously (4) and subjected to further analysis. Isolates SCDL28 and SCmd17 were collected in 2001 (22) and 2012, respectively, from a South Carolina orchard. Isolate MDbbp2 was collected in 2012 from an orchard in Maryland. Isolates Bmpc7 and Bmpc10 were collected in 2006 from a South Carolina orchard characterized previously (12) and served as DMI-resistant controls.

Extraction of genomic DNA. Genomic DNA of *M. fructicola* was extracted from mycelium as described previously (6), with slight modifications. Briefly, the mycelium with spores was scraped off the surface of a 4-day-old culture grown on potato dextrose agar (PDA; Difco Laboratories) and placed into 2-ml centrifuge tubes containing 500 µl of extraction buffer (1 M KCl, 100 mM Tris-HCl, and 10 mM EDTA) with three glass beads (425

to 600 µm; Sigma-Aldrich). The tube was vortexed for 1 min and centrifuged at 5,000 rpm for 10 min. The supernatant was collected gently and transferred to a new tube with 300 µl of 2-propanol (Sigma-Aldrich). The lysate and 2-propanol were mixed by inverting the tube several times. The mixture was then centrifuged at 12,000 rpm for 10 min before the pellet was collected, dried, and suspended in 50 µl of Tris-EDTA (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) buffer.

Amplification and sequence analysis of β-tubulin gene. Primer set TubA (5'-AAATGCGTGAGATTGTA-3' and TubR1 (5'-TGTACCAATGCAAGAAAGCCTT-3') (16) was used to amplify a fragment of the β-tubulin gene. The polymerase chain reaction (PCR) was performed in a 25-µl volume containing 1× PCR buffer, 25 ng of DNA, 0.2 µM each primer, 100 µM each dNTP, and 1.5 U of Taq DNA polymerase (New England BioLabs, Inc.). Amplifications were performed in an iCycler thermal cycler (Bio-Rad Laboratories) with the following parameters: 3 min at 95°C; followed by 32 cycles 94°C for 40 s, 50°C for 40 s, and 72°C for 1.5 min; with a final extension at 72°C for 10 min. PCR products were separated by electrophoresis in a 1% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer (40 mM Tris acetate and 1 mM EDTA, pH 8.0). PCR products were purified using the ExoSAP-IT PCR purification kit (USB Corporation) following the instructions supplied by manufacturer, and sequenced at the Clemson University Genomics Institute. The sequencing primers were TubA, TubR1, and HRF (5'-TAACAACTGGAAGGCTTCCGG-3') located at position 518 in the β-tubulin gene (16). DNA sequences were analyzed with DNAMAN7.0 software (Lynnon BioSoft).

Detection of the genetic mobile element Mona. Primer set INS65F (5'-TCGATGTTGCGTGGTCTGTCA-3') and INS65R (5'-CGGAGAGCGTCGATGTAGGTG5-3') (12) was used to detect the presence or absence of Mona upstream of *MfCYP51* in isolates resistant to propiconazole and thiophanate-methyl following published protocols. The PCR product was separated in a 1.0% agarose (Thermo Scientific) gel in 1× TAE buffer. Gels were photographed under UV light with an Electrophoresis Documentation and Analysis System (KODAK EDAS 290, Eastman Kodak Company).

RNA isolation, cDNA synthesis, and determination of relative expression of *MfCYP51* gene. Spores were scraped off the surface of a 7-day-old culture grown on tomato sauce agar (227 g of tomato sauce and 20 g of agar in 750 ml of water) and placed into 2-ml centrifuge tubes containing 1,000 µl of sterile water. The spore suspension was diluted to approximately 10⁶ spores/ml. Spore suspension (600 µl) was transferred into 150-ml flasks containing 40 ml of potato dextrose broth. The liquid cultures were incubated at room temperature at 120 rpm. After 4 days, water or propiconazole (final concentration of 0.3 µg/ml) formulated as PropiMax EC was added to the flasks. One hour later, the mycelium was collected and immediately frozen by liquid

Table 1. Origin and in vitro sensitivity to azoxystrobin (Azo), iprodione (Ipr), a mixture of pyraclostrobin + boscalid (Pyr+bos), thiophanate-methyl (Tm), and propiconazole (Pp) of *Monilinia fructicola* isolates collected from 11 stone fruit orchards in 2012

Origin					Number of resistant isolates ^y					
State	County	Orchard	Host	N ^z	Azo	Ipr	Pyr+bos	Tm	Pp	Tm & Pp
Pennsylvania	Adams	NHC	Sweet cherry	10	0	0	0	8	8	6
Maryland	Kent	BBP	Peach	10	0	0	0	8	5	5
Maryland	Kent	WMP	Peach	10	0	0	0	0	8	0
Maryland	Thurmont	BBC	Sweet cherry	10	0	0	0	7	10	7
Virginia	Oak Grove	KPL	Plum	10	0	0	0	10	0	0
South Carolina	Cherokee	ABP	Peach	10	0	0	0	8	0	0
South Carolina	Edgefield	AHP	Peach	10	0	0	0	2	0	0
South Carolina	Saluda	CCP	Peach	20	0	0	0	3	20	3
South Carolina	Saluda	YOP	Peach	20	0	0	0	1	12	1
South Carolina	Saluda	WYP	Peach	20	0	0	0	3	6	0
South Carolina	Spartanburg	GJP	Peach	10	0	0	0	7	0	0

^y Pyr+bos indicates a mixture of pyraclostrobin and boscalid; Tm & Pp indicates that the isolates were resistant to both thiophanate-methyl and propiconazole.

^z Number of isolates.

nitrogen. Total RNA was extracted from frozen mycelium using the RNeasy Plant Mini Kit (Qiagen, Inc.) following the manufacturer's instructions. The absence of genomic DNA contamination was verified by PCR using the purified RNA samples as a template. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The mRNA levels of *MfCYP51* were analyzed by reverse-transcription PCR as previously described (13). Reactions were performed in a 20- μ l volume containing 1 $\times$ iQ SYBR Green Supermix (Bio-Rad Laboratories), 0.3 μ M concentrations of each primer, and 1 μ l of cDNA. All samples were analyzed in triplicate with the following program: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 55°C for 45 s. To confirm that the expected products were amplified in these reactions, melting curve analysis was performed after amplification. Reactions were performed in an iQ5 thermal cycler (Bio-Rad Laboratories). Two biological replications were conducted. The expression of the *MfCYP51* gene was normalized to the expression of *MfActin*. The relative expression ($2^{-\Delta\Delta CT}$) was calculated by the equation (11):

$$2^{-\Delta\Delta CT} = 2^{-[(CT, MfCYP51 - CT, MfActin)x - (CT, MfCYP51 - CT, MfActin)y]}$$

where x is one of the isolate samples and y is MDbbc3, the isolate with the greatest $\Delta\Delta CT$ value and, thus, the lowest expression of *MfCYP51* compared with all other isolates.

Detached fruit assay. To assess field relevance of dual resistance, three sensitive and seven dual-resistant isolates with different point mutations in the β -tubulin gene were assessed for their ability to cause disease on fungicide-treated fruit. Fruit were purchased from a local supermarket, washed twice with tap water to remove surface pesticide residues, and placed into plastic boxes until the surface had dried. Fungicides PropiMax EC (15 ml/liter) and Topsin-M (0.6 g/liter) were sprayed to runoff onto the fruit 20 h prior to inoculation using a hand mister. Untreated fruit were sprayed with distilled sterile water. Three 4-by-2-mm (diameter by depth) plugs were removed from each fruit at three equidistant locations (2 cm apart) using a cork borer (4 mm in diameter). The plugs were replaced within 1 h by mycelium plugs of equal dimension taken from the margin of 5-day-old cultures. After inoculation, the boxes were sealed with plastic bags to keep the relative humidity around 100% and maintained at room temperature. Disease incidence and lesion diameters were measured after 3 days. The experiment was conducted twice with three replicates per experiment. Each replicate consisted of six inoculation sites on two fruit. Fitness parameters on detached fruit were investigated as described above in separate experiments, except that no fungicides were applied. Lesion diameters were recorded 3 days after inoculation.

Mycelial growth rate and conidia production in vitro. Mycelial plugs (4 mm in diameter) taken from the periphery of 5-day-old cultures grown on PDA were transferred to the center of PDA dishes. The dishes were incubated at 22°C in the dark. The colony diameter was measured after 5 days of incubation, with three replicates per isolate. Mean colony diameter divided by five was calculated for determination of average daily growth rate. The experiment was conducted twice.

To assess the ability to produce spores, dishes with tomato sauce agar (227 g of tomato sauce and 20 g of agar in 750 ml of water) were inoculated with a 4-mm mycelial plug taken from the periphery of 5-day-old cultures and incubated for 10 days at 22°C in darkness. Conidia were washed off the medium with 30 ml of distilled sterile water containing 0.1% Tween-20. The suspension was transferred to a 50-ml tube with 10 glass beads and vortexed for 1 min. The conidia concentration in the suspension was estimated using a hemocytometer. Three replicate dishes were used per isolate and three assessments for each plate. The experiment was conducted twice.

Data analysis. A statistical model was developed that related the in vitro and in vivo responses (growth rate, sporulation on tomato sauce medium, lesion size, disease incidence, control efficacy, and relative expression of *MfCYP51*) to the experimental factors of experimental replication, isolate, fungicide treatment, and their interactions. The method of least squares was used to estimate the model terms associated with the factors, and analysis of variance was used to test for a significant effect of the factors on the response means. If a factor was found to be significant, mean separation (using Student's t test) was used to further determine the nature of the effect of the factor on the responses. All calculations were performed using the statistical package JMP (version 9.0.0; SAS Institute Inc.) and all tests were performed with $P = 0.05$.

Results

Sensitivity of *M. fructicola* isolates to azoxystrobin, iprodione, pyraclostrobin + boscalid, thiophanate-methyl, and propiconazole. Of a total of 140 isolates, 57 and 69 isolates from 11 locations of four states were resistant to thiophanate-methyl and propiconazole, respectively, and 22 isolates from 5 locations of three states were resistant to both fungicides (Table 1). The dual-resistant isolates and genotypic characteristics are listed in Table 2. None of the 140 isolates were resistant to azoxystrobin, iprodione, or pyraclostrobin + boscalid.

Amino acid sequence variations in the β -tubulin gene. Nucleotide sequence analysis of the β -tubulin gene from 2 sensitive and 22 dual-resistance isolates showed that resistance to thiophanate-methyl was always associated with a point mutation. Three amino acid changes were associated with resistance. Mutation from GAA to CAA or GCA at codon 198 caused a change from glutamic acid (E) to alanine (A) in four isolates (SCyou5, SCtit1, SCtit2, and SCtit3) from South Carolina, or to glutamine (Q) in two isolates (MDbbp4 and MDbbp7) from Maryland and one (PAadc1) from Pennsylvania. Another change from TTC to TAC at codon 200 resulted in the replacement of phenylalanine (F) with Tyrosine (Y) in the remaining 15 isolates from Maryland and Pennsylvania (Fig. 1; Table 2). None of the isolates had more than one amino acid change.

Detection of the genetic mobile element Mona in dual-resistant isolates. Primer set INS65F and INS65R was used to detect Mona in DMI-resistant isolates (12). The primers amplified a 376-bp fragment for resistant isolates and a 311-bp fragment for sensitive isolates. The 376-bp amplicon was obtained for the dual-resistant isolates SCyou5 and SCtit1, -2, and -3 from South Caro-

Table 2. Presence or absence of Mona and point mutations in the β -tubulin gene in *Monilinia fructicola* isolates resistant to both propiconazole and thiophanate-methyl

State	Orchard	N ^y	Name of isolates	Mona ^z	Point mutation in β -tubulin gene	
					N ^y	Amino acid substitute
Pennsylvania	NHC	6	PAadc2, -3, -4, -6, -7	–	5	F200Y
	...	...	PAadc1	–	1	E198Q
Maryland	BBP	5	MDbbp6, -8, -9	–	3	F200Y
	...	...	MDbbp4, -7	–	2	E198Q
	BBC	7	MDbbc1, -2, -3, -5, -6, -8, -9	–	7	F200Y
South Carolina	CCP	3	SCtit1, -2, -3	+	3	E198A
	YOP	1	SCyou5	+	1	E198A

^y Number of isolates.

^z Absence (–) and presence (+) of Mona in the upstream promoter region of *MfCYP51*.

lina, as well as the DMI-resistant reference isolates Bmpc7 and Bmpc10. However, the 311-bp amplicon was obtained for all dual-resistant isolates from Maryland and Pennsylvania, as well as for DMI-sensitive isolates SCDL28, MDbbp2, and SCmd17 (Table 2).

Relative expression of *MfCYP51*. *MfCYP51* expression levels were determined using RT-qPCR in sensitive and resistant isolates (Table 3). The relative expression values in sensitive isolates of untreated mycelium ranged from 4.7 to 7.3, which was significantly lower than the value (72.2) obtained for dual-resistant isolate SCyou5 containing the Mona element. Relative expression values for dual-resistant isolates not containing Mona ranged from 1.0 to 5.0. The addition of propiconazole to the actively growing culture prior to RNA extraction of dual-resistant and DMI-sensitive isolates did not affect expression levels of the gene ($P = 0.18$ and $P = 0.98$, respectively; Table 3).

Assessment of fungicide performance on detached fruit. Most datasets were merged due to the lack of significant differences ($P > 0.05$) between the two biological replicates. There were statistical differences, however, between independent experiments for the “control efficacy” parameter in the thiophanate-methyl treatment ($P > 0.05$); therefore, this dataset was not merged (Table 4).

Isolates sensitive to both propiconazole and thiophanate-methyl were either completely controlled or largely controlled on fruit treated with Propimax or Topsin-M. In contrast, all dual-resistant isolates caused significantly higher disease incidence (more than 88%) and lower control efficacies on fruit treated with Propimax or Topsin-M, compared with sensitive isolates. The E198A genotype and with Mona present produced similar disease incidence but lower control efficacies on PropiMax-treated fruit compared with the other two genotypes (Table 4; Fig. 2).

Fitness of isolates resistant to propiconazole and thiophanate-methyl. Three fitness components were investigated for three sensitive and seven dual-resistant isolates representing all MBC genotypes. We detected no significant differences in mycelial growth rate, sporulation on PDA, and lesion size on detached fruit ($P > 0.05$ for all three fitness components; data not shown).

Discussion

Resistance to MBC fungicides in *M. fructicola* is widespread in the United States and resistance to DMI fungicides has been reported in states along the east coast. However, prior to this study, only field isolates resistant to either one or the other fungicide class had been described. This is the first report of resistance in *M. fructicola*, or any other *Monilinia* sp., to both MBC and DMI fungicides. Dual-resistant isolates were collected from one orchard in Pennsylvania, two orchards in Maryland, and two orchards in South Carolina, indicating that this new phenotype is emerging in different geographical areas. Moreover, the dual-resistant isolates revealed three different mechanisms of thiophanate-methyl resistance in the form of different point mutations in the β -tubulin gene and at least two different mechanisms of DMI fungicide resistance, indicating independent emergence of dual resistance between and within geographical locations.

In the dual-resistant isolates, thiophanate-methyl resistance was associated with point mutations at positions 198 and 200 of the β -tubulin gene, with two different amino acid changes at position 198. The E198A mutation is common in *Monilinia* spp. from the United States and Greece (16,17,28) but the E198Q and F200Y are reported here for the first time in this fungal genus. The latter mutations were found in Maryland and Pennsylvania but not in South Carolina. Both mutations are known to confer resistance to MBC fungicides in other pathogens. The E198Q mutation has been observed in field strains of *Helminthosporium solani* (20), *Tapesia yallundae* and *T. acuformis* (1), and *P. digitatum* (10). The F200Y mutation was associated with resistance to MBC fungicides in *P. aurantiogriseum*, *P. italicum*, *V. inaequalis*, *V. pirina*, and *T. yallundae* (9); *B. cinerea* (2); *C. gloeosporioides* (7); and *Fusarium asiaticum* (25). Although the alanine (A) substitution at position 198 is most common in *Monilinia* spp., this position appears to also be the most variable, with several different amino acid substitutions conferring resistance to MBC fungicides and changes to glycine (G), lysine (K), valine (V), and glutamine (Q) detected in various fungal species (9; this study). These observed point mutations cover all possible amino acid changes at codon 198 except for a

Fig. 1. Schematic of amino acid mutations associated with methyl benzimidazole carbamate resistance in the β -tubulin gene of *Monilinia* spp. Mutations reported previously are listed below the schematic of the gene, mutations reported in this study are listed above the gene. All mutations except for L240F were found in *Monilinia fructicola*. Mutations E198A and L240F were found in *M. laxa*.

Table 3. Origin, presence or absence of Mona upstream *MfCYP51*, and relative expression of the *MfCYP51* gene in *Monilinia fructicola* field isolates sensitive or resistant to propiconazole (Pp) and thiophanate-methyl (Tm)

Isolate	Origin			Mona ^y	Relative expression of <i>MfCYP51</i> ^w	
	State	Host	Phenotype (Pp/Tm) ^x		Untreated	Pp-treated ^z
MDbbp2	Maryland	Peach	S/S	–	7.3 b	4.5 b
SCDL28.01	South Carolina	Peach	S/S	–	4.8 b	6.9 b
SCmd17	South Carolina	Peach	S/S	–	4.7 b	3.0 b
MDbbc3	Maryland	Cherry	R/R ^{F200Y}	–	1.0 b	1.4 b
MDbbp6	Maryland	Peach	R/R ^{F200Y}	–	2.4 b	2.5 b
PAadc7	Pennsylvania	Cherry	R/R ^{F200Y}	–	3.0 b	1.4 b
MDbbp4	Maryland	Peach	R/R ^{E198Q}	–	3.3 b	3.6 b
MDbbp7	Maryland	Peach	R/R ^{E198Q}	–	5.0 b	6.6 b
PAadc1	Pennsylvania	Cherry	R/R ^{E198Q}	–	2.8 b	3.7 b
SCyou5	South Carolina	Peach	R/R ^{E198A}	+	72.2 a	67.5 a

^w Values followed by the same letter within a column are not significantly different according to Student's *t* test at $P < 0.05$. Mean values of two independent experiments with three runs per experiment.

^x S = sensitive; R = resistant; associated mutation in the β -tubulin gene is indicated in superscript.

^y Absence (–) and presence (+) of Mona upstream of *MfCYP51*.

^z Mycelium was treated with propiconazole at 0.3 μ g/ml 1 h prior to RNA extraction.

premature stop or conversion to aspartic acid (D). The change from phenylalanine (F) to tyrosine (Y) at codon 200 is the only reported mutation at this site for any fungal species. In several species, changes that have altered MBC effectiveness have caused measurable changes in fitness such as temperature sensitivity (16,27). Such impacts may restrict the kinds of β -tubulin mutants that emerge in field settings.

Depending on the mutations in the β -tubulin gene, strains may have different levels of resistance to thiophanate-methyl. Although mycelial growth of E198Q mutants was substantially more inhibited on thiophanate-methyl-amended PDA compared with isolates with either one of the other two point mutations, all three genotypes were equally virulent on fruit treated with Topsin M. This is different from results obtained for *T. yallundae* and *T. acutiformis* (1), which showed lower levels of resistance in F200Y mutants compared with E198A and E198Q mutants. Lower levels of resistance were also found in F200Y mutants compared with E198A mutants of *V. inaequalis* (9) and *B. cinerea* (2).

All three genotypes should be monitored in resistance surveys, because they all caused disease on fruit treated with Topsin M. A concentration of 50 mg/liter or higher has often been used to screen for MBC resistance (15,28), but our in vitro assays showed that E198Q mutants grow very slowly at this dose and, therefore, may be mistaken for a sensitive phenotype (*data not shown*). Follow up studies showed that thiophanate-methyl at 5 mg/liter allowed for uninhibited growth of all three resistant genotypes but not the sensitive genotypes (*data not shown*). This concentration is now used for routine monitoring of MBC resistance by our lab.

An investigation into the resistance mechanism of DMI resistance in our dual-resistant isolates revealed that resistance was linked to the mobile genetic element Mona in the South Carolina isolates but not in the Maryland and Pennsylvania isolates. The presence of Mona has previously been shown to be correlated with DMI fungicide resistance in isolates collected in South Carolina, Georgia, Ohio, New York (12), New Jersey (3), and Pennsylvania (26). However, some isolates from New York with low to moderate DMI-resistance lacked Mona (26). Mona contains a predicted promoter sequence and is believed to facilitate overexpression of *MfCYP51* (13). An alternate resistance mechanism has not been identified yet in field strains of *M. fructicola* but, in UV-generated laboratory mutants, resistance was attributed to point mutations in *MfCYP51* (5). No mutations were found in the *MfCYP51* gene of the dual-resistant isolates and, therefore, 14 α -demethylase mutations are not responsible for DMI fungicide resistance in the strains from Maryland and Pennsylvania. We also considered the possibility that resistance may have been caused by overexpression of

membrane-bound drug efflux proteins. Expression analysis of the *MfABC1* gene, encoding an ATP-binding cassette transporter similar to *atrB* from *Aspergillus nidulans* (23), showed that expression in DMI-resistant and -sensitive strains was the same and that exposure of mycelium to propiconazole did not induce gene expression (*data not shown*). Therefore, the molecular mechanism of resistance in field strains resistant to DMIs from Maryland and Pennsylvania remains unknown.

The emergence of *M. fructicola* field strains with resistance to multiple fungicides is significant with respect to designing anti-resistance management strategies. Control of brown rot is largely

Fig. 2. Disease development of **A**, sensitive isolate MDbbp2 and dual-resistant isolates **B**, PAadc7 representing the F200Y genotype; **C**, MDbbp7 representing the E198Q genotype; and **D**, SCyou5 representing the E198A genotype on detached fruit sprayed with water, Topsin-M, or PropiMax 24 h before inoculation.

Table 4. Disease incidence and control efficacy on detached fruit treated with propiconazole (Pp) in the form of PropiMax or thiophanate-methyl (Tm) in the form of Topsin-M for sensitive and dual-resistant *Monilinia fructicola* isolates sensitive or resistant to Pp and Tm^y

Isolate	Phenotype (Pp/Tm) ^z	Disease incidence (%)		Control efficacy (%)		
		Pp	Tm	Pp	Tm (exp. 1)	Tm (exp. 2)
MDbbp2	S/S	0.0 b	11.1 b	100.0 a	96.4 a	92.8 a
SCDL28	S/S	0.0 b	0.0 b	100.0 a	100.0 a	100.0 a
SCmd17	S/S	0.0 b	8.4 b	100.0 a	100.0 a	86.7 a
MDbbc3	R/R ^{F200Y}	83.3 a	87.5 a	52.8 b	18.9 b	-4.2 c
MDbbp6	R/R ^{F200Y}	83.3 a	100.0 a	41.2 bcd	-3.5 b	2.0 c
PAadc7	R/R ^{F200Y}	100.0 a	100.0 a	29.0 d	6.7 b	-8.9 bc
MDbbp4	R/R ^{E198Q}	100.0 a	100.0 a	47.5 bc	17.5 b	2.8 bc
MDbbp7	R/R ^{E198Q}	95.5 a	100.0 a	35.9 cd	9.9 b	-4.7 bc
PAadc1	R/R ^{E198Q}	100.0 a	100.0 a	41.1 bcd	15.8 b	-7.1 bc
SCyou5	R/R ^{E198A}	100.0 a	100.0 a	14.3 e	17.4 b	17.2 b
Mean	S/S	0.0 b	6.5 b	100.0 a	98.8 a	93.2 a
Mean	R/R ^{F200Y}	88.9 a	95.8 a	41.0 b	7.4 b	-3.7 c
Mean	R/R ^{E198Q}	98.5 a	100.0 a	41.5 b	14.4 b	-3.0 c
	R/R ^{E198A}	100.0 a	100.0 a	14.3 c	17.4 b	17.2 b

^y Disease incidence was expressed as the percentage of wounds infected compared with the total number of wounds inoculated. Control efficacy was expressed as the percentage of the mean diameter of the control minus the mean diameter of the treatment divided by the mean diameter of the control. Values followed by the same letter within a column are not significantly different according to Student's *t* test at *P* < 0.05. Data for independent experiments were merged except for control efficacy of thiophanate-methyl because of significant experiment-isolate interactions; exp. = experiment.

^z S = sensitive; R = resistant; associated mutation in the β -tubulin gene is indicated in the superscript.

dependent on the rotation or mixture of MBC, DMI, QoI, and SDHI fungicides. Resistance to multiple fungicides will complicate disease control in that such strains are susceptible to fewer chemical classes. The emergence of dual resistance to MBC and DMI fungicides follows our discovery of strains with dual resistance to SDHI and DMI fungicides (4).

QoI fungicides play an important role in the management of brown rot disease in the United States. In this study, all isolates we examined were sensitive to azoxystrobin, which is consistent with our hypothesis that the resistance risk to QoI fungicides in *M. fructicola* is low. The reason for the low resistance risk is the inability of some fungi to tolerate a point mutation in codon 143 of the gene for cytochrome *b*. Mutation at this site in many fungi has been shown to confer a high level resistance to QoI fungicides. However, the presence of an intron immediately downstream of the G143 codon in some other fungi (8,24), including *M. fructicola* (14), affects the ability of the organism to splice out the intron if the flanking sequence is altered.

In conclusion, strains with resistance to DMI and MBC fungicides are emerging in commercial peach-growing areas on the east coast of the United States. The mechanisms of resistance to both classes of fungicides vary, indicating that the fungus evolves independently in different locations to overcome selection pressure due to fungicide applications. It is still unclear how widespread dual resistance is to DMI and MBC fungicides. In this study, we acknowledge collection bias in that we only included isolates from orchards where brown rot was present at high-enough incidence to be noticed by either the grower or an extension specialist. There may have been other locations with similar brown rot incidence in stone fruit orchards which were not included in this study for a variety of reasons. Systematic sampling of orchards in South Carolina, Pennsylvania, Maryland, and other states would need to be done to determine the extent of resistance to multiple fungicides on the east coast.

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