Adaptation to Fungicides in *Monilinia fructicola* Isolates with Different Fungicide Resistance Phenotypes

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Accepted for publication 25 September 2007.

ABSTRACT

Luo, C.-X., and Schnabel, G. 2008. Adaptation to fungicides in *Monilinia fructicola* isolates with different fungicide resistance phenotypes. *Phytopathology* 98:230-238.

The ability to develop fungicide resistance was assessed in *Monilinia fructicola* isolates with different fungicide sensitivity phenotypes by adapting mycelium and conidia to increasing concentrations of selective fungicides and UV mutagenesis. Results showed that adaptation to Quinone outside inhibitor (QoI) fungicide azoxystrobin and sterol demethylation inhibitor (DMI) fungicide propiconazole was more effective in conidial-transfer experiments compared to mycelial-transfer experiments. DMI-resistant (DMI-R) isolates adapted to significantly higher doses of azoxystrobin in both, mycelial- and conidial-transfer experiments compared to benzimidazole-resistant (BZI-R) and sensitive (S) isolates. Adaptation to propiconazole in conidial-transfer experiments was accelerated in BZI-R isolates when a stable, nonlethal dose of 50 µg/ml thiophanate-methyl was added to the selection medium. One of two azoxystrobin-

resistant mutants from DMI-R isolates did not show any fitness penalties; the other isolate expired before further tests could be carried out. The viable mutant caused larger lesions on detached peach fruit sprayed with azoxystrobin compared to the parental isolate. The azoxystrobin sensitivity of the viable mutant returned to baseline levels after the mutant was transferred to unamended medium. However, azoxystrobin resistance recovered quicker in the mutant compared to the corresponding parental isolate after renewed subculturing on medium amended with 0.2 and 1 µg/ml azoxystrobin; only the mutant but not the parental isolate was able to adapt to 5 µg/ml azoxystrobin. In UV mutagenesis experiments, the DMI-R isolates produced significantly more mutants compared to S isolates. All of the UV-induced mutants showed stable fungicide resistance with little fitness penalty. This study indicates the potential for QoI fungicide resistance development in *M. fructicola* in the absence of a mutagen and provides evidence for increased mutability and predisposition to accelerated adaptation to azoxystrobin in *M. fructicola* isolates resistant to DMI fungicides.

Brown rot blossom blight and brown rot of peach are caused by the ascomycete fungus *Monilinia fructicola* (G. Wint) Honey. In commercial peach production, these two phases of the disease can only be controlled with fungicide applications but fungicide resistance development in the causal organism is a growing concern. The pathogen has developed resistance to dicarboxamides (3,16,21), benzimidazoles (BZIs) (24), and more recently, sterol demethylation inhibitor (DMI) fungicides (17,23). Among fungicides to which *M. fructicola* has not yet developed resistance, quinone outside inhibitors (QoI) are the only fungicides that are as effective as DMI fungicides for brown rot control (17) under southeastern growing conditions. Consequently, current management recommendations rely heavily on this group of fungicides (17).

As part of an effort to manage fungicide resistance in commercial peach orchards, QoI and DMI fungicides are currently alternated for preharvest brown rot control in the southeastern United States. Benzimidazole fungicide resistance is believed to be stable and scattered throughout the Southeast (24), but some growers still use them up to one time per season for brown rot management. No information is available concerning the risk of resistance development to QoI fungicides in *M. fructicola*. The existence of BZI- and DMI-resistant populations may give rise to accelerated development of resistance to fungicides of another chemical class (7). Isolates of *Venturia inaequalis* qualifying as resistant to dodine or benomyl, for example, accelerated the selection toward benomyl or dodine resistance, respectively. Simi-

larly, the speed of selection by dodine or DMI fungicide fenarimol was accelerated for isolates already resistant to the other fungicide (7).

The main objective of this study was to assess the risk of fungicide resistance development, including the risk of azoxystrobin (QoI) resistance development in *M. fructicola* isolates with different fungicide sensitivity backgrounds. The ability to adapt to fungicides was assessed by transferring mycelium and conidia to increasing doses of selective fungicides and standard UV-mutagenesis. A second objective was to characterize the fungicide-adapted and UV-generated mutants in regard to stability of resistance and fitness.

MATERIALS AND METHODS

Isolate characteristics and maintenance. Only *M. fructicola* isolates were used in this study since the other species causing brown rot in North America, *M. laxa*, has not been reported from the Southeastern United States. Furthermore, only conidial isolates were used since the teleomorphic stage of this fungus is rarely found in southeastern commercial orchards. The origin and other characteristics, such as different resistance phenotypes, of the isolates used in this study are listed in Table 1. Three isolates (Bmpc7, -10, and -13 from Georgia) were resistant to DMI fungicides and were designated DMI-R; three isolates (139, 190, and 213 from California) were resistant to BZI fungicides and were designated BZI-R; and three isolates (99.A3 and 99.A4 from South Carolina and Kac-18 from California) were sensitive to both of these fungicide classes and were designated S. All of the isolates have had little, if any, exposure to QoI fungicides prior to isolation. BZI-R isolates from California were used in this study because no BZI-resistant isolates from the Southeast were

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doi:10.1094/PHYTO-98-2-0230

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part of our laboratory collection at that time. The resistance phenotype is indicated in superscript following the isolate name. For example, DMI-resistant isolate Bmpc7 is denoted by Bmpc7^{DMI-R}. The isolates, as well as all mutants created in this study were allowed to grow on filter paper disks placed on potato dextrose agar (PDA; EMD Chemicals, Gibbstown, NJ) and after 7 days, disks with mycelium were removed, desiccated, and stored at -25°C.

Determination of fungicide sensitivity. The sensitivity of parental isolates and corresponding mutants to the QoI fungicide azoxystrobin, the DMI fungicide propiconazole, and the BZI fungicide thiophanate-methyl was determined. Mycelial inhibition was determined by calculating the mycelial growth on fungicide-amended PDA medium relative to the growth on unamended medium. PDA was amended with azoxystrobin (Abound 2.08F; Syngenta Crop Protection, Greensboro, NC) or propiconazole (PropiMax EC; Syngenta) at 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 µg a.i./ml, or with thiophanate-methyl (Topsin M; Ceraxagri, King of Prussia, PA) at 0, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 50, 100, and 500 µg a.i./ml. Additional concentrations of 30, 100, and 300 µg a.i./ml were used to determine azoxystrobin sensitivity of azoxystrobin-resistant mutants. Salicylhydroxamic acid (SHAM) was added to the azoxystrobin amended PDA at 100 µg/ml to suppress the alternative oxidase pathway. Corresponding control dishes contained SHAM without the azoxystrobin. Three replicates of each fungicide concentration were used for each isolate. For inoculations, mycelial plugs (5 mm in diameter) were removed from the periphery of a 4-day-old colony of each isolate using a cork borer and placed upside down onto unamended and fungicide-amended PDA. Dishes were incubated at 22°C for 4 days in the dark. The average colony diameter of three replicates was determined by measuring each colony in two perpendicular directions in three replicates. A linear regression analysis was performed to calculate the effective

concentration to inhibit mycelium growth by 50, 75, and 99.99% (EC₅₀, EC₇₅, and EC_{99.99}, respectively).

Selection for fungicide resistance. Mycelial- and conidial-transfer protocols were largely as described previously (19), except that growth medium was amended with increasing concentrations of a selective fungicide. The experimental setup and a schematic of the experimental procedure are shown in Table 2 and Figure 1, respectively. DMI-R isolates were grown either on unamended medium or on medium amended with 0.1 µg/ml propiconazole, and adapted for azoxystrobin or thiophanate-methyl resistance. BZI-R isolates were grown either on unamended medium or on medium amended with 50 µg/ml thiophanate-methyl, and adapted for azoxystrobin or propiconazole resistance. S isolates were adapted to azoxystrobin, propiconazole, and thiophanate-methyl (Table 2). V8 medium (200 ml of V8 juice and 20 g of agar per liter) was chosen over PDA in selection experiments because all isolates used in this study sporulated more readily on V8 medium.

For mycelial-transfer experiments, approximately 2 by 2 mm square pieces of agar with actively growing mycelium were transferred to V8 medium in petri dishes (90 mm diameter) amended with the EC₇₅ dose of a selective fungicide. Four pieces were placed in a square about 3 cm apart in a single dish. Three dishes were used for each isolate, totaling 12 replicates per isolate. After 1 week of incubation, similar sized pieces (2 by 2 mm) of mycelium-containing medium from the most actively growing part among all of 12 colonies of each isolate were transferred to medium amended with the EC₇₅ dose, and the twofold (2×) EC₇₅ dose of the same selective fungicide (Fig. 1). This transfer was repeated weekly until the relative mycelial growth (relative to the growth of the parental isolate, which was simultaneously transferred at each transfer event to unamended V8 medium) of the colony on the higher dose (2 × EC₇₅) exceeded 20%. Then the colony was maintained on the 2 × EC₇₅

TABLE 1. Characteristics and fungicide sensitivity of *Monilinia fructicola* isolates used in this study

Isolate	Origin	Host	Year isolated	Fungicide sensitivity phenotype ^a	EC ₅₀ (µg/ml)		
					Azoxystrobin	Propiconazole	Thiophanate-methyl
Bmpc7	Experimental Farm, Byron, GA	Peach	2006	DMI-R	0.05	0.1	0.16
Bmpc10	Experimental Farm, Byron, GA	Peach	2006	DMI-R	0.09	0.13	0.15
Bmpc13	Experimental Farm, Byron, GA	Peach	2006	DMI-R	0.08	0.1	0.16
139	Commercial orchard, Fresno, CA	Peach	2005	BZI-R	0.14	0.006	>50
190	Commercial orchard, Fresno, CA	Peach	2005	BZI-R	0.1	0.006	>50
213	Commercial orchard, Fresno, CA	Peach	2005	BZI-R	0.1	0.005	>50
99.A3	Commercial orchard, Edgefield, SC	Peach	2004	S	0.13	0.013	0.11
99.A4	Commercial orchard, Edgefield, SC	Peach	2004	S	0.08	0.003	0.14
Kac-18	Commercial orchard, Fresno, CA	Prune	2005	S	0.13	0.007	0.12

^a DMI-R: sterol demethylation inhibitor fungicide-resistant (an isolate was considered DMI-R when the relative mycelial growth was equal or greater than 50% at the discriminatory dose of 0.1 µg/ml propiconazole); BZI-R: benzimidazole fungicide-resistant (an isolate was considered BZI-R when it was able to grow uninhibited at 50 µg/ml thiophanate-methyl); S: sensitive to DMI, BZI, and QoI (quinone outside inhibitor) fungicides.

TABLE 2. Combinations of fungicide sensitivity phenotype of *Monilinia fructicola*, V8-media amendments, and selective fungicide used in mycelial- and conidial-transfer experiments and in UV-mutagenesis experiments

Fungicide resistance phenotype	V8-media supplement ^a	Selective fungicide ^b		
		Azoxystrobin (QoI)	Propiconazole (DMI)	Thiophanate-methyl (BZI)
DMI-R	None	x		
	None			x
	Propiconazole	x		
BZI-R	Propiconazole			x
	None	x		
	None		x	
	Thiophanate-methyl	x		
S	Thiophanate-methyl		x	
	None	x	x	x

^a Propiconazole was added at 0.1 µg/ml; thiophanate-methyl was added at 50 µg/ml.

^b QoI = Quinone outside inhibitor; DMI = sterol demethylation inhibitor; BZI = benzimidazole.

dose and simultaneously started on the next higher dose ($4 \times \text{EC}_{75}$) of the selective fungicide (Fig. 1). The weekly transfers were continued for 10 weeks. Before each transfer, the diameters of the fungicide-adapted colonies and the colonies of the parental isolate were recorded.

The conidial-transfer experiments involved the transfer of conidia instead of the fastest growing mycelium of a colony (Fig. 1). To induce sporulation, isolates were grown on unamended V8 medium for one week at 25°C with alternating 12-h periods of dark and light. The conidia were subsequently harvested by pouring 5 ml of saline-tween solution (0.8 g/liter of NaCl and 0.01% Tween 80) into each dish and scraping the surface of the colony with a wrapped sterile cotton-tipped applicators (Puritan Medical Products, Guilford, ME). The suspension was then filtered through two layers of sterile gauze (CVS Pharmacy, Inc., Woonsocket, RI), and adjusted to 10^6 conidia/ml on the basis of a hemacytometer count. For each isolate, 12 5- μ l droplets of the conidia suspension were applied on a total of three dishes, with four drops per dish. The drops were applied approximately 3 cm apart in a square on V8 medium amended with selective fungicide. After 1 week of incubation, the conidia from the newly formed colonies were harvested, filtered, adjusted to 10^6 conidia/ml, and 5- μ l droplets were again transferred to a new set of V8 medium amended with the selective fungicide (Fig. 1). Conidia were allowed to germinate and if the relative mycelial growth exceeded 20%, transfer to the next higher concentrations was initiated (Fig. 1).

Mutagenesis by UV-irradiation. Conidia were obtained as previously described. They were evenly spread on the surface of V8 medium (100 μ l of a 10^7 conidia/ml suspension per dish) and irradiated with a portable UV-lamp at a wavelength of 254 nm at a distance of 20 cm for 30 s. Afterwards, dishes were kept for 30 min in the dark to minimize photo repair of radiation damage (11). The irradiated conidia were overlaid with V8 media containing selective fungicide and 50 μ g/ml ampicillin. Selective fungicide doses were 8.0 μ g/ml azoxystrobin, 3.0 μ g/ml propiconazole, or 5.0 μ g/ml thiophanate-methyl. These doses were chosen because they represented 10 times the averaged 10-fold $\text{EC}_{99.99}$ values for the QoI-sensitive, DMI-sensitive, and BZI-sensitive isolates, respectively, of this study. Overlaid dishes were incubated for one week at 22°C in the dark to allow for the development of colonies on the media surface. Three independent experiments with five replicates per isolate were carried out. Small pieces of mycelium from each emerging fungal colony were transferred to fresh V8 medium amended with the selective fungicide to confirm resistance. Mutant colonies were incubated for one week at 22°C before further analysis. The frequency of

mutant generation was calculated by dividing the number of mutants by the total number of viable spores. The latter was calculated by multiplying the total number of irradiated spores per isolate by the spore germination rate.

Stability of fungicide resistance and fitness. The stability of fungicide resistance in fungicide-adapted mutants and UV-induced mutants was assessed after five consecutive transfers on unamended V8-medium by determining EC_{50} values as described.

Mycelium growth rate, sporulation, and spore germination were evaluated as components of *M. fructicola* fitness. For each parental isolate, fungicide-adapted mutant, and UV-induced mutant, a 5-mm diameter mycelial plug was taken from the periphery of a 4-day-old colony and transferred to unamended V8 medium. The inoculated dishes were incubated at 25°C with alternating 12-h periods of dark and light. Colony diameter was determined after 5 days. Dishes were incubated for another 2 days before the number of conidia was determined. Conidia were harvested as previously described and their concentration was determined using a hemacytometer. The germination frequency was determined following the spore count. Ten microliter of each spore suspension was diluted with 90 μ l sterile saline-tween solution and spread on PDA medium. Dishes were incubated at 22°C in the dark for 12 h. The germination frequency was assessed using 200 arbitrarily selected conidia for each parental isolate, fungicide-adapted mutant and UV-induced mutant. Each isolate was tested in triplicate.

Effect of azoxystrobin treatment of detached peach fruit on brown rot development caused by the azoxystrobin-resistant mutant and its parent. The sensitivity of the azoxystrobin-resistant mutant and its parental isolate Bmpc7^{DMI-R} to azoxystrobin was determined on detached peach fruit. Commercially mature fruit (cv. Coronet) was submerged in a 200 μ g a.i./ml azoxystrobin solution or sterile water for 5 s and allowed to dry in carbon-fiber trays (4 $\times$ 6 pockets). Fruit was inoculated using three 30- μ l drops of a spore suspension (1.0×10^6 conidial spores/ml) placed in a triangular fashion 2 cm apart on the top of each peach. Control peaches received sterile water drops lacking conidia. Five peaches per treatment were used for each the mutant and the parental isolate and treatments were randomized in the trays. The trays were covered with a plastic dome and sealed carefully with clear cardboard wrapping tape to maintain high humidity. Peaches were incubated at $25 \pm 2^\circ\text{C}$ for 4 days before lesion diameters were measured. Control (%) was determined with the formula $(1 - A/B) \times 100$, where A and B are the average lesion diameters measured on azoxystrobin-treated and water-treated fruit, respectively.

Statistical analyses. The pairwise Student's *t* test was used to compare treatment means. Statistical analyses were performed using SigmaStat for Windows Version 3.00 (Systat Software, Inc., San Jose, CA).

RESULTS

Sensitivity of parental isolates to azoxystrobin, propiconazole, and thiophanate-methyl. All isolates used in this study were sensitive to azoxystrobin with EC_{50} values ranging from 0.05 to 0.14 μ g/ml. For isolates Bmpc7^{DMI-R}, Bmpc10^{DMI-R}, Bmpc13^{DMI-R}, 139^{BZI-R}, 190^{BZI-R}, 213^{BZI-R}, 99.A3^S, Kac-18^S and 99.A4^S, the EC_{50} values for propiconazole were 0.1, 0.13, 0.1, 0.006, 0.006, 0.005, 0.013, 0.007, and 0.003 μ g/ml, respectively (Table 1). For thiophanate-methyl, the EC_{50} values for each of the three BZI-R isolates were greater than 50 μ g/ml, whereas values for isolates Bmpc7^{DMI-R}, Bmpc10^{DMI-R}, Bmpc13^{DMI-R}, 99.A3^S, Kac-18^S, and 99.A4^S were 0.16, 0.15, 0.16, 0.11, 0.12, and 0.14 μ g/ml, respectively (Table 1).

Adaptation to fungicides using mycelial- and conidial-transfer experiments. Adaptation to azoxystrobin and propiconazole was more effective in conidial-transfer experiments compared to

Fig. 1. Schematic of the consecutive transfer procedure in the mycelial- and conidial-transfer experiments of *Monilinia fructicola*. EC_{75} , $2 \times \text{EC}_{75}$, or $4 \times \text{EC}_{75}$ indicated that the medium in petri dishes was amended with a selective fungicide at the EC_{75} , 2-fold EC_{75} , or 4-fold EC_{75} dose, respectively. Isolates were always maintained on two doses of the selective fungicide, one that allowed $>20\%$ growth and one that was double that concentration. Isolates were transferred individually to the next higher dose if the relative mycelial growth exceeded 20%. The weekly transfers were carried out for 10 weeks.

mycelial-transfer experiments (Fig. 2A through F). DMI-R isolates adapted to significantly higher doses of azoxystrobin in both mycelial- and conidial-transfer experiments compared to BZI-R and S isolates (Fig. 2A and D). Specifically, DMI-R isolates adapted to 8-fold the EC_{75} dose of azoxystrobin in mycelial-transfer experiments compared to a maximum of 2-fold the EC_{75} dose for BZI-R and S isolates. In conidial-transfer experiments, DMI-R isolates adapted to 64- and 128-fold the EC_{75} dose compared to a maximum of 8-fold the EC_{75} dose for BZI-R and S isolates. BZI-R isolates adapted the least to azoxystrobin in both selection procedures. BZI-R and S isolates adapted to 4-fold the

EC_{75} dose of propiconazole in mycelial-transfer experiments (Fig. 2B) and 16-fold the EC_{75} dose in conidial-transfer experiments (Fig. 2E) with the exception of isolate 139^{BZI-R}, which did not adapt as well to propiconazole. DMI-R and S isolates adapted up to the 4-fold the EC_{75} dose of thiophanate-methyl in both mycelial- and conidial-transfer experiments, with the exception of isolate Bmpc10^{DMI-R}, which was the only isolate able to adapt to 8-fold the EC_{75} dose of thiophanate-methyl (Fig. 2C and F).

Mycelial- and conidial-transfer experiments were also carried out under continuous thiophanate-methyl pressure for BZI-R isolates and continuous propiconazole pressure for DMI-R iso-

Mycelial-transfer

Conidial-transfer

Fig. 2. Adaptation of sterol demethylation inhibitor-resistant (DMI-R), benzimidazole-resistant (BZI-R) and sensitive (S) isolates of *Monilinia fructicola* to azoxystrobin, propiconazole, and thiophanate-methyl in mycelial (A, B, and C) and conidial (D, E, and F) transfer experiments.

lates by adding a nonlethal dose of 50 $\mu\text{g/ml}$ thiophanate-methyl and 0.1 $\mu\text{g/ml}$ propiconazole, respectively, to the V8 medium. The addition of propiconazole to the V8 medium did not enhance the ability of DMI-R isolates to adapt to either azoxystrobin (Fig. 3A and D) or thiophanate-methyl (Fig. 3C and F). Similarly, the addition of thiophanate-methyl did not increase the ability of BZI-R isolates to adapt to azoxystrobin (Fig. 3A and D). Conidia of BZI-R isolates adapted to significantly higher concentrations of propiconazole on thiophanate-methyl amended V8-medium compared to unamended V8 medium (Fig. 3E). This phenomenon was not observed for mycelium in the corresponding mycelial-transfer experiment (Fig. 3B).

Generation of fungicide-resistant mutants by UV-mutagenesis. Conidia of DMI-R, BZI-R, and S isolates were UV-treated for the generation of azoxystrobin-resistant mutants, but none of the isolates produced viable azoxystrobin-resistant mutants (Fig. 4A). In many cases, mycelium was growing through the azoxystrobin-amended medium-layer but when transferred to fresh azoxystrobin-amended V8-medium, mycelium growth did not continue to develop. Conidia of BZI-R and S isolates were

UV-treated for the generation of propiconazole-resistant mutants, but no difference was found in the ability of BZI-R and S isolates to produce mutants (Fig. 4B). When conidia of DMI-R and S isolates were UV-treated to obtain thiophanate-methyl-resistant mutants, the DMI-R isolates produced significantly more mutants (Fig. 4C). The addition of a nonlethal dose of propiconazole or thiophanate-methyl to DMI-R or BZI-R isolates, respectively, did not significantly alter their ability to produce mutants (data not shown).

Characterization of mutants. Only mutants with resistance factor (RF) values greater than 5.0 were selected for further analysis. Those included azoxystrobin-adapted mutants from DMI-R isolates (Fig. 2D) and propiconazole-adapted mutants from BZI-R isolates (Fig. 3E) obtained from conidial-transfer experiments. They also included thiophanate-methyl-resistant and propiconazole-resistant mutants from the UV mutagenesis experiments (Fig. 4B and C).

The azoxystrobin-adapted mutant from Bmpc7^{DMI-R} did not show fitness penalties with respect to mycelium growth or sporulation ability (Table 3). The RF of this mutant decreased from

Mycelial-transfer

Conidial-transfer

Fig. 3. Adaptation of sterol demethylase inhibitor-resistant (DMI-R) and benzimidazole-resistant (BZI-R) isolates of *Monilinia fructicola* to azoxystrobin, propiconazole and thiophanate-methyl in mycelial (A, B, and C) and conidial (D, E, and F) transfer experiments on V8-base media with or without a nonlethal dose of propiconazole (0.1 $\mu\text{g/ml}$) or thiophanate-methyl (50 $\mu\text{g/ml}$), respectively.

54.4 (RF1) to the parental level of 1.0 (RF2) after five consecutive transfers on unamended V8 medium (Table 3). However, azoxystrobin resistance recovered more quickly compared to the parental isolate after renewed subculturing on V8 medium amended with azoxystrobin and 100 µg/ml SHAM (Table 4). Differences in recovery between the parental and the mutant isolate were most pronounced when the medium was amended with the higher concentration of azoxystrobin (5.0 µg/ml). At this dose the parental isolate was completely inhibited and the mutant was able to adapt to an RF of 27.4 (Table 4). The azoxystrobin-adapted mutant from isolate Bmpc10^{DMI-R} adapted to 64-fold the EC₇₅ dose of azoxystrobin after 9 weeks of conidial-transfer (Fig. 2A, line 1); however, most of the resistance was lost immediately after the adaptation experiment was completed (RF1 = 8.6). The death of the mutant at the end of nine transfers prevented further analyses of this mutant (Table 3).

In contrast to azoxystrobin-resistance in mutants from DMI-R isolates, propiconazole-resistance was stable (RF2 = 158.1) in the mutant derived from BZI-R isolate 139 but not from isolate 190 (RF2 = 1.2) after consecutive transfers on unamended V8 medium (Table 5). No resistance recovery was observed for the 190^{BZI-R}-derived mutant after renewed subculturing on V8 medium amended with 0.04 or 0.3 µg/ml propiconazole using the conidial-transfer procedure. The propiconazole-adapted mutants showed decreased mycelial growth rate and sporulation ability. However, the spore germination rate was not impacted in either mutant compared to the parental isolates (Table 5).

UV-mutagenesis generated various mutants with RF1 values >5.0, including two thiophanate-methyl resistant mutants from isolate Bmpc7^{DMI-R}, three from Bmpc10^{DMI-R}, and three from Bmpc13^{DMI-R} (Table 6); and one propiconazole-resistant mutant obtained from each of isolate 190^{BZI-R} and Kac-18^S (Table 7). Thiophanate-methyl resistance was stable in mutants from DMI-R isolates after five consecutive transfers on unamended V8 medium (Table 6), but instability of propiconazole resistance was observed in a mutant derived from BZI-R isolate 190 (Table 7). The mycelial growth of the thiophanate-methyl-resistant mutants was reduced compared to the parental isolates with the exception of the mutants from Bmpc7^{DMI-R} (Table 6). Sporulation in mutants from isolates Bmpc7^{DMI-R} and Bmpc10^{DMI-R} was not reduced, but sporulation was reduced significantly in the mutants from Bmpc13^{DMI-R}. The spore germination rate was not impaired in any of the mutants (Table 6). The mycelial growth and sporulation ability of the propiconazole-resistant mutants from isolates 190^{BZI-R} and Kac-18^S were significantly reduced, but the germination rate was unaffected (Table 7).

Effect of azoxystrobin on brown rot development caused by the azoxystrobin-resistant mutant and its parent in detached fruit. Both, the parental isolate and the mutant produced lesions on detached fruit treated with azoxystrobin. However, the parental isolate Bmpc7^{DMI-R} was controlled more effectively in the azoxystrobin treatment compared to the mutant. Disease control (%) was 41.0% for the parental isolate compared to 9.4% for the azoxystrobin-resistant mutant (Table 8).

DISCUSSION

In this study, *M. fructicola* isolates resistant to DMI fungicides adapted faster to significantly higher doses of the unrelated fungicide azoxystrobin compared to DMI-sensitive isolates both by mycelial- and conidial-transfer. Evidence of predisposition of fungicide-resistant isolates of a fungal plant pathogen to preferential selection for resistance to other fungicides has been documented previously. Specifically, *V. inaequalis* phenotypes already resistant to dodine were prone to accelerated adaptations to DMI and benzimidazole fungicides (7). While that study was based on results obtained from pathogen populations, this study shows a similar phenomenon in single isolates under controlled experi-

mental conditions. This is the first report indicating the potential for the development of resistance to QoI fungicides in *M. fructicola*. The data also indicate that isolates resistant to one fungicide might not be predisposed to preferred selection to just any other

Fig. 4. Frequency of azoxystrobin (A), propiconazole (B), and thiophanate-methyl (C) resistant mutants of *Monilinia fructicola* in UV mutagenesis experiments. Same letters above bars indicate no significant difference ($P = 0.05$) in a student test. DMI-R = sterol demethylation inhibitor-resistant; BZI-R = benzimidazole-resistant; S = sensitive.

fungicide class. For example, the same DMI-R isolates that adapted significantly faster to QoI fungicide azoxystrobin compared to DMI-sensitive isolates did not have an advantage over S isolates when adapting to the benzimidazole fungicide thiofanate-methyl. Also, BZI-R isolates had no advantage over S isolates during selection for propiconazole resistance. The latter finding is in contrast to an earlier study, reporting that benzimidazole-resistant isolates of *V. inaequalis* showed predisposition to dodine resistance (7).

The mechanism by which the DMI-R isolates were able to adapt more effectively to azoxystrobin in this study compared to S isolates or BZI-R isolates was not investigated. The most common mechanisms for DMI-fungicide resistance are (i) mutations in the *CYP51* gene, encoding the target enzyme sterol 14 α -demethylase of DMI fungicides (1,2), (ii) increased expression of the *CYP51* gene (4,9,18), or (iii) the activity of multidrug efflux pumps belonging to the ATP-binding cassette transporters (5,12,13,14). Increased efflux pump activity in DMI-R isolates could have explained the superior ability to adapt to QoI fungicides, however, the mechanism of resistance to DMI fungicides in the *M. fructicola* isolates from Georgia is based on increased expression of the *CYP51* gene (data not shown). Perhaps, frequency of exposure to fungicides plays a role. The two DMI-R isolates used in this study were likely exposed to a greater number of total DMI fungicide applications compared to the S or BZI-R isolates because they came from a research farm that had experimented with DMI fungicides years before they were registered for commercial use in peach. Thus, the fungus may have had more opportunity to adapt to its selective environment by accumulating

adaptive mutations, which not only include useful mutations (15). Consequently, the altered genome of DMI-R isolates may be better prepared to adapt to fungicide-rich environments compared to isolates that have had less exposure to site-specific fungicides.

The superior ability of *M. fructicola* isolates to adapt to fungicides azoxystrobin and propiconazole by conidial-transfer compared to mycelial-transfer observed in this study is consistent with a study on *A. nidulans* (19). In that study, the transfer of a random sample of spores from haploid or diploid mycelium led to more rapid adaptation to genetic stress caused by major resistance mutations compared to the transfer of the visually 'fittest' mycelial sector (19). The authors speculated that one reason for this phenomenon may lie in the additional nuclear divisions during spore formation, which allows for more genetic variation to be generated and for more effective selection of adaptive mutations. Additionally, assuming that the fittest sectors of a mycelial colony are caused by adaptive mutations, only mutations near the advancing margin of this sector are transferred to a fresh dish. Adaptive mutations developing in slower growing sectors will have a chance to get incorporated in spores, as spore formation occurs on the whole surface of the colony.

The azoxystrobin-adapted mutant from isolate Bmpc10^{DMI-R} suffered severe fitness penalties; however, the other mutant from Bmpc7^{DMI-R} was not impaired in regard to mycelium growth, sporulation, and spore germination (Table 3). In addition, lesions were significantly larger on azoxystrobin-treated, detached peach fruit inoculated with the mutant compared to the corresponding parental isolate. Azoxystrobin resistance of the mutant was unstable, because sensitivity switched back to parental levels

TABLE 3. Characteristics of sterol demethylation inhibitor-resistant (DMI-R) isolate-derived and azoxystrobin-adapted mutants of *Monilinia fructicola*

Strain	Sensitivity to azoxystrobin before and after adaptation ^a			Fitness parameters					
				Mycelium growth (% of parent)		Sporulation (% of parent)		Spore germination (% of parent)	
	Parental EC ₅₀ (µg/ml)	RF1	RF2	Mean	SD	Mean	SD	Mean	SD
Bmpc7 ^{DMI-R} -mutant	0.09	54.4	1.0	119.3	0.6	89.4	26.0	93.1	8.5
Bmpc10 ^{DMI-R} -mutant	0.05	8.6	— ^b	—	—	—	—	—	—

^a Resistance factor (RF) 1 was calculated by dividing the EC₅₀ value of the parental isolate by the EC₅₀ value determined immediately after completion of the conidial-transfer experiment. RF2 was calculated by dividing the parental EC₅₀ value by the EC₅₀ value determined after the fungicide-adapted mutant was transferred five times on unamended V8 medium.

^b '—' indicates that the data could not be collected due to loss of viability of the mutant.

TABLE 4. Recovery of azoxystrobin resistance in the azoxystrobin-adapted mutant (mutant) from parental isolate Bmpc7^{DMI-R} of *Monilinia fructicola* after five consecutive transfers on unamended medium

Isolate/strain	Resistance factor (RF) ^a after consecutive transfers on azoxystrobin-amended medium								
	Third transfer azoxystrobin (µg/ml)			Fourth transfer azoxystrobin (µg/ml)			Fifth transfer azoxystrobin (µg/ml)		
	0.2	1.0	5.0	0.2	1.0	5.0	0.2	1.0	5.0
Bmpc7 ^{DMI-R}	0.8	0.7	— ^b	1.3	6.9	—	5.3	6.9	—
Bmpc7 ^{DMI-R} -mutant	1.8	2.6	2.3	27.6	20.0	10.7	12.4	14.4	27.4

^a RF was calculated by dividing the original parental EC₅₀ value (0.09) by the EC₅₀ value determined after the isolate was transferred on V8 medium amended with 0.2, 1.0, or 5.0 µg/ml azoxystrobin plus 100 µg/ml SHAM.

^b '—' indicates that the culture did not grow enough to collect data.

TABLE 5. Characteristics of the benzimidazole-resistant (BZI-R) isolate-derived and propiconazole-adapted mutants of *Monilinia fructicola*

Strain	Sensitivity to propiconazole before and after adaptation ^a			Fitness parameters					
				Mycelium growth (% of parent)		Sporulation (% of parent)		Spore germination (% of parent)	
	Parental EC ₅₀ (µg/ml)	RF1	RF2	Mean	SD	Mean	SD	Mean	SD
139 ^{BZI-R} -mutant	0.007	103.8	158.1	59.7	17.1	60.2	10.2	103.7	0.9
190 ^{BZI-R} -mutant	0.006	14.2	1.2	68.5	13.2	50.7	1.9	100.3	1.6

^a Resistance factor (RF) 1 was calculated by dividing the EC₅₀ value of the parental isolate by the EC₅₀ value determined immediately after completion of the conidial-transfer experiment. RF2 was calculated by dividing the parental EC₅₀ value by the EC₅₀ value determined after the fungicide-adapted mutant was transferred five times on unamended V8 medium.

when transferred to unamended medium. However, a rapid recovery of resistance was observed after the mutant was returned to selection medium. Very similar observations were made in laboratory mutants of *V. inaequalis*, *Ustilago maydis*, and *Botrytis cinerea* (10, 11,25,26). The mechanism of resistance in azoxystrobin-resistant mutants was not investigated in this study, but QoI resistance is typically conferred by point mutations in the mitochondrial cytochrome *b* gene. QoI fungicide-sensitive and resistant mitochondria can coexist in a single cell and resistant ones seem to be selected under selection pressure, whereas sensitive ones seem to multiply faster without selection pressure (25). Alternatively, it is possible that the unstable resistance observed in this study was not based on genetics but rather of physiological origin.

Whether or not this type of azoxystrobin resistance may be of practical resistance is debatable. The fact that high levels of azoxystrobin resistance were achieved without the influence of a mutagen combined with the fact that important fitness parameters were not impaired would be strong arguments for the possibility of practical significance. However, the experiments were conducted under controlled conditions. If QoI fungicides were to be applied all season long, adaptation similar to this lab study would

be possible. Anti-resistance strategies call for a restriction on the total number of QoI fungicides applied per season and for a rotation of QoI fungicides with fungicides of different modes of action after a maximum of two consecutive applications. Therefore, the type of adaptation to QoI fungicides that was revealed in this study, with its rapid reversion to sensitivity, should not become a practical problem as long as growers follow the label recommendations and implement anti-resistance disease management strategies.

Adaptation to propiconazole in conidial-transfer experiments was accelerated when BZI-R isolates were subjected to a stable, nonlethal dose of 50 µg/ml thiophanate-methyl. This phenomenon was most pronounced in isolate 139^{BZI-R}, which adapted to a 4-fold EC₇₅ dose on medium with selective fungicide only, but adapted to the 32-fold EC₇₅ dose on medium amended with both, a stable dose of thiophanate-methyl and the selective fungicide propiconazole. However, both propiconazole resistant mutants suffered severe fitness penalties with significantly reduced mycelium growth and sporulation ability, furthermore, the propiconazole resistance of mutant from 190^{BZI-R} decreased quickly after subculturing on fungicide free medium. This may indicate that the accelerated DMI resistance development in BZI isolates

TABLE 6. Characteristics of sterol demethylation inhibitor-resistant (DMI-R) isolate-derived and thiophanate-methyl-resistant (BZI-R) mutants of *Monilinia fructicola* obtained by UV-mutagenesis

					Fitness parameters					
					Mycelium growth (% of parent)		Sporulation (% of parent)		Spore germination (% of parent)	
		Sensitivity to thiophanate-methyl								
Parental isolate	Mutant(#)	Parental isolate EC ₅₀ (µg/ml)	RF1 ^a	RF2	Mean	SD	Mean	SD	Mean	SD
Bmpc7 ^{DMI-R}	1	0.16	10.1	40.0	95.0	4.4	103.3	49.4	110.5	6.9
	2		24.7	36.1	108.4	2.5	89.4	21.1	108.7	2.1
Bmpc10 ^{DMI-R}	1	0.15	49.2	43.7	87.2	4.0	120.1	20.2	102.3	0.3
	2		30.0	20.2	85.5	1.5	122.9	34.3	95.3	8.5
	3		12.0	14.2	89.5	2.3	169.8	35.6	98.9	0.4
Bmpc13 ^{DMI-R}	1	0.16	10.9	19.8	95.0	0.6	80.2	10.8	96.8	4.8
	2		11.0	17.8	93.1	2.0	46.4	1.0	97.0	5.1
	3		11.0	12.5	95.3	0.9	61.5	12.5	97.6	2.2

^a Resistance factor (RF) 1 was calculated by dividing the EC₅₀ value of the parental isolate by the EC₅₀ value determined immediately after completion of the UV mutagenesis experiment. RF2 was calculated by dividing the parental EC₅₀ value by the EC₅₀ value determined after the mutant was transferred five times on unamended V8 medium.

TABLE 7. Sensitivity of parental isolates 190^{BZI-R} and Kac-18^S of *Monilinia fructicola* to propiconazole and resistance factors as well as fitness parameters of derived mutants obtained by UV-mutagenesis

Parental isolate	Mutant	Sensitivity to propiconazole			Fitness parameters					
					Mycelium growth (% of parent)		Sporulation (% of parent)		Spore germination (% of parent)	
		Parent EC ₅₀ (µg/ml)	RF1 ^a	RF2	Mean	SD	Mean	SD	Mean	SD
190 ^{BZI-R}	1	0.006	70.9	13.4	90.5	2.7	74.7	5.0	101.1	1.7
Kac-18 ^S	1	0.007	20.7	62.0	77.9	5.4	21.2	11.4	101.2	3.4

^a Resistance factor (RF) 1 was calculated by dividing the EC₅₀ value of the parental isolate by the EC₅₀ value determined immediately after completion of the UV mutagenesis experiment. RF2 was calculated by dividing the parental EC₅₀ value by the EC₅₀ value determined after the mutant was transferred five times on unamended V8 medium.

TABLE 8. Effect of azoxystrobin treatments on brown rot development of detached peach fruit caused by isolate Bmpc7^{DMI-R} and its azoxystrobin-resistant mutant of *Monilinia fructicola*

Isolate	Lesion diameter (cm) ^a				Control (%) ^b
	Water		Azoxystrobin (200 µg/ml)		
	Mean	SD	Mean	SD	
Bmpc7 ^{DMI-R}	0.83	0.32	0.49	0.10	41.0
Bmpc7 ^{DMI-R} -mutant	0.62	0.28	0.56	0.20	9.4

^a Each mean and corresponding standard deviation (SD) were calculated from 15 lesions on five fruit.

^b Control (%) was calculated using the formula $(1 - A/B) \times 100$, where A and B are the average lesion diameters measured on azoxystrobin-treated and water-treated fruit, respectively.

developed only under permanent thiophanate-methyl exposure may not be of practical significance.

In this study, no azoxystrobin-resistant mutants were obtained in the UV-mutagenesis experiments (Fig. 4A). Difficulties in obtaining QoI fungicide-resistant mutants under similar experimental conditions were described for *V. inaequalis* previously (25). Mutants were obtained with the low frequency of 4.4×10^{-8} . The frequency of generating propiconazole resistant mutants in this study were 0.2×10^{-7} and 0.1×10^{-7} , for BZI-R and S isolates, respectively (Fig. 4B), which was lower than frequencies observed in UV-mutagenesis experiments for the generation of DMI fungicide-resistant mutants in *A. nidulans* (20) and *Pseudocercospora herpotrichoides* (6,8). Thiophanate-methyl resistant mutants were isolated at a frequency of 3.2×10^{-7} in DMI-R isolates but no mutants were recovered from S isolates (Fig. 4C), indicating that DMI-R isolates may be inclined to becoming more readily mutated due to UV irradiation. Still, the frequency of thiophanate-methyl-resistant mutant generation in DMI-R isolates was lower compared to findings by Yan and Dickman (22), who report a frequency of 10^{-6} for the generation of benomyl-resistant *Fusarium moniliforme* mutants. The relatively low frequency of mutants in this study might have been caused by the higher doses of selective fungicides used (10-fold the $EC_{99.99}$ dose for each fungicide). This higher concentration could have prevented more unstable mutants from developing. In a preliminary experiment, using the $1 \times EC_{99.99}$ dose of the selective fungicide, the frequency of mutants obtained was higher (up to 10^{-3}), but most mutants were not viable in subsequent transfers (data not shown).

ACKNOWLEDGMENTS

We thank P. K. Bryson and D. Baker for providing technical assistance and A. Amiri for collecting some of the isolates used in this study. This material is based upon work supported by grant no. 2004-04014 of the USDA-CSREES RAMP (Risk Avoidance and Mitigation) program. This material is based upon work supported by the CSREES/USDA, under project number SC-1000642. Technical Contribution No. 5325 of the Clemson University Experiment Station.

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