Cross-Resistance Among Demethylation Inhibitor Fungicides With Brazilian *Monilinia fructicola* Isolates as a Foundation to Discuss Brown Rot Control in Stone Fruit

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Abstract

Despite the resistance problems in *Monilinia fructicola*, demethylation inhibitor fungicides (DMIs) are still effective for the disease management of brown rot in commercial stone fruit orchards in Brazil. This study aims to investigate the sensitivity of *M. fructicola* isolates and efficiency of DMIs to reduce brown rot. A set of 93 isolates collected from Brazilian commercial orchards were tested for their sensitivities to tebuconazole, propiconazole, prothioconazole, and myclobutanil. The isolates were analyzed separately according to the presence or absence of the G461S mutation in *MfCYP51* gene, determined by allele-specific test. The mean EC₅₀ values for G461S mutants and wild-type isolates were respectively 8.443 and 1.13 µg/ml for myclobutanil, 0.236 and 0.026 µg/ml for propiconazole, 0.115 and 0.002 µg/ml for prothioconazole, and 1.482 and 0.096 µg/ml for tebuconazole. The density distribution curves of DMI sensitivity for both genotypes showed that myclobutanil and prothioconazole curves were mostly shifted toward resistance and sensitivity, respectively. Incomplete cross-resistance was detected among propiconazole and tebuconazole

in both wild-type ($r = 0.45$) and G461S ($r = 0.38$) populations. No cross-sensitivity was observed among wild-type isolates to prothioconazole and the others DMIs tested. Fungicide treatments on detached fruit inoculated with *M. fructicola* genotypes showed significant DMI efficacy differences when fruit were inoculated with wild-type and G461S isolates. Protective applications with prothioconazole were more effective for control of both G461S and wild-type isolates compared with tebuconazole. Curative applications with tebuconazole were most effective in reducing the incidence and lesion size of G461S isolates. Sporulation occurred only for G461S isolates treated with tebuconazole under curative and preventative treatments. The differences found among the performance of triazoles against *M. fructicola* isolates will form the basis for recommendations of rational DMI usage to control brown rot in Brazil.

Keywords: sterol biosynthesis inhibitors, triazoles, tebuconazole, prothioconazole, propiconazole, myclobutanil

Brown rot of stone fruit caused by *Monilinia fructicola* (G. Wint.) Honey is widely known around the world to reduce orchard productivity. When not properly controlled, this pathogen can infect blossoms and twigs and can cause fruit decay that significantly impacts crop yield (Keske et al. 2011; May De Mío et al. 2004). Several Rosaceae family members such as peaches, plums, cherries, nectarines, apricots, apples, and pears are reported to be infected by this pathogen (Byrde and Willetts 1977; Pereira and May De Mío 2018).

Brown rot outbreaks are highly dependent on weather conditions and inoculum density (Luo et al. 2005; Villarino et al. 2012). Warm and humid climatic conditions encountered in production areas such as the southeastern United States and southern Brazil are conducive to *M. fructicola* populations and their inoculum levels (Keske et al. 2011). Conventional stone fruit production relies on the usage of fungicide mixtures and alternations applied from bloom until preharvest (Blaauw et al. 2020; Keske et al. 2011; Moreira and May De Mío 2009). In the southeastern U.S. and California, four to six seasonal

sprays can be used to control blossom blight and brown rot (Adaskaveg et al. 2017; Blaauw et al. 2020) while in Spain, up to five applications can be recommended to control the disease (Usall et al. 2010). In Brazil, the number of sprays per season to control brown rot may vary from six to eight applications depending on the location and environmental conditions (May De Mío et al. 2011; Pereira et al. 2020; Tibola et al. 2005).

In Brazil, the registration of new site-specific fungicides to control *M. fructicola* had not occurred for over 20 years until quinone outside inhibitors (QoIs) became available (Bartlett et al. 2002). For many years, the management of *M. fructicola* populations relied on methyl benzimidazole carbamates (MBCs), demethylation inhibitors (DMIs), and QoIs (Lichtemberg et al. 2016a; May De Mío et al. 2011). Unfortunately, succinate dehydrogenase fungicides (SDHIs) are not registered for this crop in Brazil as in the United States (Chen et al. 2013).

The increase of *M. fructicola* populations resistant to MBCs observed in the last decades made DMIs some of the most important fungicides for controlling this disease (May De Mío et al. 2011; Zhu et al. 2010). The DMIs act by inhibiting the enzyme 14 α -demethylase (P450), which is a critical component in the pathway of ergosterol biosynthesis, an important structural component of cell membranes in ascomycetes (Parker et al. 2011). Classified by the Fungicide Resistance Action Committee (FRAC) as Group 3, DMIs contain the triazole chemical group that comprise the majority of their registered active ingredients for control of *M. fructicola* (Anonymous 2020). To date, the DMI fungicide active ingredients registered in the U.S. for brown rot control include single fungicide products such as tebuconazole, difenoconazole, fenbuconazole, propiconazole, and mefentrifluconazole, in addition to premixes with other QoI and SDHI fungicides (Adaskaveg et al. 2017; Walker et al. 2018).

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Table 1. Characterization of *Monilinia fructicola* sensitivity phenotype of four different demethylation inhibitor fungicides and their genotype for isolates used in this study

Lemid ^w	Origin ^x	Year	Genotype ^y	EC ₅₀ value (µg/ml) ^z			
				teb	prp	prt	myc
110	RS	2011	W.T.	0.001	0.011	0.002	0.251
646	PR	2009	W.T.	0.001	0.019	NA	0.004
1023	PR	2010	W.T.	0.003	0.014	0.001	0.12
753	SP	2009	W.T.	0.009	0.028	0.002	1.872
107	RS	2011	W.T.	0.01	0.044	0.002	0.62
106	RS	2011	W.T.	0.011	0.016	0.001	0.126
1099	SP	2010	W.T.	0.012	0.02	0.001	0.687
c3	SP	2015	W.T.	0.012	0.018	0.003	0.075
122	RS	2011	W.T.	0.014	0.021	0.005	0.234
c30	SP	2015	W.T.	0.016	0.026	0	0.513
750	SP	2009	W.T.	0.016	0.022	0	0.851
113	RS	2011	W.T.	0.017	0.025	0.002	0.312
21	PR	2011	W.T.	0.018	0.025	0.001	0.538
126	RS	2011	W.T.	0.019	0.038	0	15
c66	SP	2015	W.T.	0.019	0.021	0.001	0.193
643	PR	2009	W.T.	0.019	0.03	0.001	0.653
124	RS	2011	W.T.	0.019	0.035	0.002	0.534
115	RS	2011	W.T.	0.02	0.027	0.002	0.513
25	PR	2011	W.T.	0.021	0.031	0.002	0.553
1069	PR	2010	W.T.	0.021	0.066	0.002	1.207
c24	SP	2015	W.T.	0.022	0.005	0	0.24
142	RS	2011	W.T.	0.022	0.029	0.002	0.905
1020	RS	2010	W.T.	0.029	0.028	0.002	3.56
134	RS	2011	W.T.	0.037	0.03	0.001	0.11
199	SP	2011	W.T.	0.039	0.011	0.002	0.168
c79	SP	2015	W.T.	0.041	0.057	0.006	0.909
c23	SP	2015	G461S	0.042	0.024	0.006	0.272
190	SP	2011	W.T.	0.054	0.024	0.004	0.416
s45	SP	2015	G461S	0.075	0.017	0.001	0.308
176	SP	2011	W.T.	0.085	0.021	0.005	1.06
170	SP	2011	W.T.	0.098	0.023	0.003	0.569
777	SP	2009	G461S	0.12	0.026	0.005	0.66
945	SP	2009	G461S	0.141	0.069	0.013	1.436
c19	SP	2015	G461S	0.161	0.468	2.699	15
c10	SP	2015	G461S	0.204	0.065	0.013	NA
s48	SP	2015	G461S	0.23	0.073	0.005	0.525
c1	SP	2015	G461S	0.231	0.134	0.003	15
807	SP	2009	G461S	0.232	0.106	0.012	15
951	SP	2009	G461S	0.258	2.032	0.027	9.379
15	PR	2011	G461S	0.285	0.641	0.003	15
752	SP	2009	G461S	0.367	0.228	0.072	11.894
c44	SP	2015	G461S	0.37	0.368	0.085	3.578
802	SP	2009	G461S	0.373	0.137	0.056	15
c25	SP	2015	G461S	0.399	0.672	0.028	15
c26	SP	2015	G461S	0.429	0.177	0.019	4.506
761	SP	2009	G461S	0.468	0.133	0.028	0.421
c31	SP	2015	G461S	0.475	0.194	0.012	15
c86	SP	2015	G461S	0.475	0.119	0.048	15
158	SP	2011	G461S	0.49	0.01	0.004	0.35
s41	SP	2015	G461S	0.495	0.204	0.032	1.429
754	SP	2009	G461S	0.552	0.135	0.018	15
s43	SP	2015	G461S	0.602	0.358	0.013	15
152	SP	2011	G461S	0.689	0.034	0.005	0.74
940	SP	2009	G461S	1.001	0.19	0.027	15
c64	SP	2015	G461S	1.19	0.074	0.023	15
793	SP	2009	G461S	1.757	0.07	0.009	6.353
c33	SP	2015	G461S	4.835	0.172	0.019	1.207
c14	SP	2015	G461S	15	0.213	0.027	15
c58	SP	2015	G461S	15	0.171	0.267	8.166

^w Lemid is the isolate code used for the *Monilinia* spp. collection of the Laboratory of Epidemiology and Integrated Disease Management at the Federal University of Paraná (Brazil).

^x Origins where isolates were collected: SP = São Paulo, RS = Rio Grande do Sul, and PR = Paraná.

^y Isolate genotype where W.T. = wild-type (sensitive) and G461S = resistant mutant, according Lichtemberg et al. (2017).

^z Effective concentration of demethylation inhibitor fungicides to reduce mycelial growth by 50%. myc = myclobutanil, prp = propiconazole, prt = prothioconazole, teb = tebuconazole.

Reduced DMI sensitivity of *M. fructicola* isolates has been detected in many parts of the world, including the U.S. (Holb and Schnabel 2007; Villani and Cox 2011), China (Chen et al. 2012), Spain (Egüen et al. 2015), and different production areas in Brazil (Lichtemberg et al. 2017; May De Mio et al. 2011; Pereira et al. 2020). Advances in understanding *M. fructicola* resistance to DMI in Brazilian populations from stone fruits were made after the first sensitivity survey performed by May De Mio et al. (2011). Since then, resistance mechanisms and isolate fitness studies were conducted, revealing potential antiresistance strategies for commercial orchards and offering simple molecular methods for monitoring the buildup of resistant genotypes (Lichtemberg et al. 2017, 2019).

The reduced sensitivity of *M. fructicola* to other chemical groups including QoIs and SDHIs (Amiri et al. 2010; Chen et al. 2013) made DMIs one of the few systemic fungicide alternatives for brown rot management, even in orchards with documented reduced sensitivity to DMIs (Lichtemberg et al. 2016a). The DMIs propiconazole and tebuconazole are effective systemic fungicides commonly used to control brown rot in the U.S. (Horton et al. 2001; Schnabel and Dai 2004) and in Brazil (Silva et al. 2011), respectively. For this reason, most of the sensitivity studies were focused on these two active ingredients (Chen et al. 2013; Lichtemberg et al. 2016b; May De Mio et al. 2011; Schnabel et al. 2004). In addition to the shifts detected in the sensitivity to these DMIs, understanding cross-sensitivity relationships among other DMI fungicides for *M. fructicola* is another crucial step for selecting appropriate combinations of fungicides for consistent brown rot management and for maintaining the effectiveness of these fungicides.

Cross-resistance is defined as resistance to two or more fungicides conferred by the same resistance mechanism (Brent and Hollomon 2007). This type of resistance has been reported previously among DMI fungicides in many pathogenic fungi and has supported the elaboration of efficient spray programs around the world (Avenot et al. 2016; Chen et al. 2016; Karaoglanidis and Thanassouloupoulos 2003; Pfeufer and Ngugi 2012; Thomas et al. 2012). In the U.S., sensitivity studies for multiple DMI active ingredients have been made since the mid-1990s (Wilcox and Burr 1994). Even though some surveys have observed variable degrees of sensitivity correlation among different triazoles tested in *M. fructicola* (Chen et al. 2012; Holb and Schnabel 2007; Schnabel et al. 2003), there is still a lack of knowledge about the occurrence of cross-resistance among Brazilian isolates. In addition, understanding the relationship between fungicide efficacy and application timing in fruit is essential for elaborating strategies to optimize the management of brown rot in Brazil.

To overcome the lack of cross-sensitivity studies for *M. fructicola* population from Brazil, we aim with this study: (i) to phenotype and genotype *M. fructicola* population for DMI fungicides, comparing the sensitivity distribution curves and correlation of wild-type and G461S-mutants; and (ii) to evaluate the efficacy of tebuconazole and prothioconazole to control brown rot disease on detached fruit comparing the preventative against curative fungicide usage.

Materials and Methods

Isolate collection. In total, 93 single-spore isolates of *M. fructicola* were used in this study. Isolates were obtained from symptomatic and mummified peach fruit collected in commercial orchards in Paraná (PR), Rio Grande do Sul (RS), and São Paulo (SP) states from 2003 to 2015. The first isolate set was used to phenotype 52 isolates from the three states PR ($n = 17$), SP ($n = 26$), and RS ($n = 9$) using the discriminatory dose method (Supplementary Table). The second set of isolates, which included 59 isolates (PR = 7, SP = 41, and RS = 11), was used to calculate the effective concentration to inhibit 50% of colony growth (EC₅₀) (Table 1). Eighteen isolates were commonly present in both sets. Prior to sensitivity assays, isolates were recovered from –80°C storage in 25% glycerol and plated on V8 media for 5 days at 23°C.

Fungicides. Four DMI fungicides were used in this study: propiconazole (a.i. of Tilt, Syngenta Crop Protection, Greensboro, NC), tebuconazole (a.i. of Folicur 3.6F, Bayer CropScience, Morrisville,

NC), myclobutanil (a.i. of Rally 40WSP, Dow AgroScience, Indianapolis, IN), and prothioconazole (a.i. of Proline480SC, Bayer Crop Science, Morrisville, NC). Tebuconazole was selected because it is currently registered for managing brown rot in Brazil (Agrofit 2003). Propiconazole is an important component of brown rot control in the United States; in Brazil, it is registered for several crops but not on stone fruits (Agrofit 2003). Myclobutanil and prothioconazole represent different DMI generations; the first was launched in 1977 and the latter in 2002 (Ziogas and Malandrakis 2015) and neither were tested on brown rot isolates from Brazil.

Sensitivity assay using the discriminatory dose. In the first trial, 52 isolates (Supplementary Table) were separated according to their sensitivity phenotype by using the discriminatory dose method. For that, mycelial plugs measuring 5 mm were transferred onto potato dextrose agar (PDA) and amended with 0.3 µl/ml for each of the following fungicides: tebuconazole, propiconazole, and prothioconazole. Stock solutions of each a.i. were made with deionized sterile water at 100 mg a.i./ml. Controls without amended fungicides were provided. Each Petri plate combined four randomly chosen isolates where three replicates per isolate were prepared on distinct plates. Isolates were incubated at 23°C with a photoperiod of 12 h for 5 days. The percentage inhibition of mycelial growth (%IMG) of isolate *i* was calculated as $\%IMG_i = (C_{ck} - C_i)/C_{ck} \times 100$, where C_{ck} is the mean colony diameter of the control with no fungicide, and C_i is the mean colony diameter of the isolate *i* on the supplemented medium (Yoshimura et al. 2004). Resistant isolates were defined as those that had at least 20% of the colony growth in supplemented media versus nonamended media (Luo and Schnabel 2008). The experiment was conducted twice.

Sensitivity assay using EC₅₀ value. In this second assay, the conventional method to determine EC₅₀ was used to evaluate the sensitivity of 60 *M. fructicola* isolates (Table 1). The same above-mentioned fungicides were tested with the addition of myclobutanil. Stock solutions of each commercial fungicide in deionized sterile water were made at 10 and 100 mg a.i./ml and were added to autoclaved and acidified PDA (APDA, 2.5 ml of 25% lactic acid [v/v]; Microtech Scientific, Orange, CA) once media cooled to 45°C to produce final concentrations of 0 (control), 0.001, 0.005, 0.025, 0.125, 0.625, 3.125, and 15.625 µg/ml. For each isolate, a 5-mm plug was transferred from the edge of a 5-day-old colony to the center of a fresh plate containing APDA media supplemented with one of the above fungicide concentrations. Two replicate plates were prepared for each concentration. The incubation parameters and colony measurements were consistent with previous descriptions. For each concentration, the inhibition of colony growth (L_i) was determined by $L_i = (C_{ck} - C_i)/C_{ck} \times 100$, as previously described. The EC₅₀ values were obtained by logarithmic (log₁₀) transformation of the fungicide concentrations and then by performing linear regressions of the colony inhibition values (L) and the log₁₀ concentrations. The experiment was conducted twice.

Genomic DNA isolation and allele-specific PCR. To verify the presence of the G461S mutation at the *MfCYP51* gene, 60 *M. fructicola* isolates (Table 1) were tested by using the allele-specific assay developed by Lichtenberg et al. (2017). For that, genomic DNA was isolated from 5-day-old colony mycelium by using the FastDNA Kit (MP Biomedicals) with the FastPrep Instrument (MP Biomedicals) programmed at 40 s at a speed setting of 6.0. After adjusting the DNA concentration to 5 ng/µl, we used the allele-specific primer MfgaF1 and MfgaR1 to amplify 178 bp of isolates carrying the G461S mutation (Lichtenberg et al. 2017). PCR product was visualized with electrophoresis in 2% gel agarose stained with GelRed (Biotium, Fremont, CA). DNA ladder TrackIt (Invitrogen, Carlsbad, CA) was also used.

Sensitivity distribution and cross-resistance comparison. The distribution of the *M. fructicola* sensitivities to tebuconazole, propiconazole, prothioconazole, and myclobutanil were analyzed by plotting the densities and cumulative frequencies of the log-transformed EC₅₀ values from 59 isolates. Cross-resistance studies applied the Pearson's correlation coefficient (*r*) to determine significant linear correlations between two pairs of fungicides. The analysis includes

the results of the two sensitive genotype (G461S mutant and wild-type) separately.

Fungicide efficacy assay on detached fruits. The efficacy of Folicur 3.6 F (a.i. tebuconazole, standard product in Brazil) and Proline 480 SC (a.i. prothioconazole, best sensitivity result) as preventative and curative treatments to control brown rot was tested on three G461S mutants (C25, C26, and C64) and three wild-type (C3, C24, and C66) isolates infecting mature prunes (*Prunus domestica*) (cv. French). The experiment included 10 fruits per combination of isolates (*n* = 6), spray treatment (*n* = 3), and period of application (*n* = 2) accounting for 360 fruits used per experiment performed. The experiment was repeated twice. A fungicide concentration of 20 g of a.i. per 100 liters of deionized sterile water was used to spray fruits until run-off. Sprays were made 24 h before and after inoculation to simulate the preventative and curative control, respectively. As an inoculum source, 20 µl of spore suspension (5×10^4 spore/ml) was harvested from 6-day-old colonies cultured in APDA. Plums were harvested from an experimental orchard where fungicides had not been sprayed for the last 5 years. Fruits were surface disinfected with 70% ethanol (1 min), 0.5% sodium hypochlorite (1 min), then rinsed in deionized sterile water and dried inside a hood for 30 min. Humidified and transparent plastic containers measuring 31 × 23 × 10 cm were used to accommodate 10 fruits that were wounded before inoculation with a dissecting needle. Preventative and curative fungicide applications were made 24 h before and after wounding and inoculation, respectively. After 4 days incubation at room temperature, brown rot incidence, lesion size, and sporulation capacity were assessed.

The incidence considered the percentage of brown rot symptomatic fruit, while the lesion size estimated the two perpendicular lesion diameters. After taking measurements, spores were harvested with 5 ml of deionized sterile water by washing the entire lesion that had spore formation. Spore concentration was determined from two 10-µl aliquots of spore suspensions with a hemacytometer aid. Pathogen signs were only present from G461S mutants treated with tebuconazole and water (control) and therefore this was the lone source of recovery.

Statistical analysis. Comparison of mycelial growth and mycelial growth inhibition (MGI) values obtained from the discriminatory dose assay were compared between the DMI phenotypes using the Wilcoxon nonparametric range test (*P* < 0.05). The EC₅₀ values for each isolate were assessed by the conventional method and were calculated from the significant (*P* < 0.05) linear regression equations of the L_i values by the log₁₀ concentrations of fungicides. The arithmetic means of EC₅₀ values were calculated separately, and significant differences were verified with the Welch two-sample *t* test. Changes in sensitivity density curves were analyzed with two-sample Kolmogorov-Smirnov test, by comparing the cumulative frequency distribution of two datasets at a time (Kirkman 1996). Pearson correlation analysis was used to determine cross-resistance among the four tested demethylation fungicide inhibitors by log transforming values prior to analysis. Lesion size and sporulation were analyzed with ANOVA and means were compared using pairwise contrasts with false discovery rate (FDR) method. For all statistical inference, *P* < 0.05 was considered a nominal significance level. The statistical software R (version 3.4.3) was used for data analysis and graphical representation (R Core Team 2017).

Results

Sensitive assay using the discriminatory dose. Based on results provided by isolate set 1 (*n* = 52), tebuconazole was the a.i. with a higher frequency of resistant phenotypes (42.3%, *n* = 22), approximately six- and sevenfold the amount of resistant isolates found for propiconazole (7.7%, *n* = 4) and prothioconazole (5.8%, *n* = 3), respectively (Table 2).

Sensitivity assay using conventional method to determine EC₅₀ value. The mean EC₅₀ value of G461S mutants for myclobutanil (8.44 µg/ml) was significantly greater (*P* < 0.05) than the other three fungicides with 5.7-, 35.7-, and 73.4-fold the values obtained for tebuconazole, propiconazole, and prothioconazole, respectively

(Table 3). Among the wild-type isolates, myclobutanil presented the highest mean EC₅₀ value (1.13 µg/ml), corresponding to 43.4- and 565-fold the respective values obtained for propiconazole ($P = 0.039$) and prothioconazole ($P = 4.79 \times 10^{-11}$) (Table 4). Despite myclobutanil having 11.7-fold the EC₅₀ value of tebuconazole (0.096 µg/ml), the significance level of $P = 0.054$ does not differentiate the efficacy of the active ingredients (Table 4). Without significant differences ($P > 0.05$), both genotypes (G461S and wild-type) revealed that propiconazole resulted in mean EC₅₀ values between those found for tebuconazole and prothioconazole (Table 3 and 4). Finally, the tebuconazole EC₅₀ value for G461S isolates did not differ from prothioconazole ($P = 0.051$, Table 3) whereas for wild-type genotype, a difference was observed ($P = 0.035$) (Table 4). Once again, the difference between G461S sensitivity values for tebuconazole and prothioconazole was near the threshold of 5% of probability.

Sensitivity distribution and cross-resistance comparison. The analysis of the density distribution curves was performed separately for each genotype. The results revealed that myclobutanil has always significantly differed from the other three fungicides, with curves shifted toward less sensitivity (Fig. 1). The tebuconazole density distribution curve of G461S isolates differed from propiconazole ($P = 0.005$) and prothioconazole ($P = 3.5 \times 10^{-9}$) (Fig. 1A). For wild-type isolates, the tebuconazole density distribution curve was similar to propiconazole ($P = 0.06$) but not to prothioconazole ($P = 2.2 \times 10^{-10}$) (Fig. 1B). Finally, the prothioconazole density distribution

curve was always plotted on the most sensitive side of the graph, differing from all other fungicides (Fig. 1).

Among the G461S isolates, tebuconazole revealed a small, positive, and significant correlation with propiconazole ($r = 0.38$, $P = 0.033$) and prothioconazole ($r = 0.36$, $P = 0.046$). A moderate correlation was observed between prothioconazole and propiconazole ($r = 0.52$, $P = 0.002$). Myclobutanil was not significantly correlated with any other fungicide tested (Table 5). Among the wild-type isolates, propiconazole revealed moderate, positive, and significant correlations with myclobutanil ($r = 0.52$, $P = 0.004$) and tebuconazole ($r = 0.45$, $P = 0.017$). Prothioconazole was not significantly correlated to any other fungicides (Table 6).

G461S mutation. The presence of the G461S mutation at the *MfCYP51* gene was observed in 30 (50.8%) out of 59 tested isolates (Table 1).

Fungicide efficacy assay on detached fruits. Significant interactions between spray timing (preventative and curative) and fungicide treatment (tebuconazole, prothioconazole, and control) were observed when studying the G461S ($P = 3.5 \times 10^{-6}$) and wild-type ($P = 0.013$) genotypes separately. The best G461S inhibition was achieved on fruits preventatively sprayed with prothioconazole (0.1 mm), which corresponded to 1.7% of disease incidence (Table 7). Curative applications of prothioconazole and tebuconazole resulted in lesion sizes larger by approximately 32- and 78-fold and disease incidence greater by 18- and 28-fold versus the measurements obtained for our best treatment, the preventative usage of

Table 2. Sensitivity phenotype frequency of *Monilinia fructicola* to three different demethylation inhibitor fungicides using the discriminatory dose of 0.3 µg/ml (Cox et al. 2007)

Fungicide	Phenotype ^y	Frequency (%)	Mycelial growth (mm) ^z		MGI (%) ^{y,z}
			0 µl/ml	0.3 µl/ml	
Tebuconazole	S	57.69 ($n = 30$)	21.98 a	0.77 b	96.57 a
	R	42.31 ($n = 22$)	18.62 a	11.81 a	34.81 b
Propiconazole	S	92.31 ($n = 48$)	24.51 a	0.38 b	98.09 a
	R	7.69 ($n = 4$)	25.61 a	12.73 a	38.52 b
Prothioconazole	S	94.23 ($n = 49$)	19.64 a	0.78 b	95.80 a
	R	5.77 ($n = 3$)	17.38 a	5.90 a	66.07 b

^y Sensitive (S) and resistant (R) phenotypes based on the mycelial growth inhibition (MGI) as described by Luo and Schnabel (2008).

^z Means followed by the same letter in a column are not significantly different based on Wilcoxon nonparametric range test ($P \leq 0.05$).

Table 3. Comparison of the mean 50% effective concentration (EC₅₀) values of *Monilinia fructicola* isolates carrying the G461S mutation to four different demethylation inhibitor fungicides and significant value

Fungicide	n	Effective concentration value (µg/ml) ^y and P -value ^z				Percentiles (10% and 90%)
		Myclobutanil	Tebuconazole	Propiconazole	Prothioconazole	
Myclobutanil	31	8.443	5.89×10^{-6}	1.17×10^{-7}	8.85×10^{-8}	(0.421 – 15)
Tebuconazole	31	-	1.482	0.072	0.051	(0.068 – 1.757)
Propiconazole	31	-	-	0.236	0.273	(0.024 – 0.468)
Prothioconazole	31	-	-	-	0.115	(0.003 – 0.071)

^y Mean effective concentration values are reported in bold letters.

^z P -values were obtained with Welch t -test.

Table 4. Comparison of the mean 50% effective concentration (EC₅₀) values of *Monilinia fructicola* wild-type isolates to four different demethylation inhibitor fungicides and significant value

Fungicide	n	Effective concentration value (µg/ml) ^y and P -value ^z				Percentiles (10% and 90%)
		Myclobutanil	Tebuconazole	Propiconazole	Prothioconazole	
Myclobutanil	29	1.13	0.054	0.039	4.79×10^{-11}	0.118 – 1.34
Tebuconazole	29	-	0.096	0.259	0.035	0.008 – 0.087
Propiconazole	29	-	-	0.026	0.127	0.013 – 0.039
Prothioconazole	28	-	-	-	0.002	0.0006 – 0.004

^y Mean effective concentration values are reported in bold letters.

^z P -values were obtained with Welch t -test.

prothioconazole (Table 7). The preventive use of tebuconazole and controls resulted in G641S lesion sizes and disease incidences greater than 10 mm and 60%, respectively. Among the wild-type isolates, it was observed that preventative usage of prothioconazole and tebuconazole resulted in similar lesion size control and disease incidence below 5% of the inoculated fruits (Table 7).

At the end of the fourth experimental day, conidiation was only observed on the G641S isolates treated with tebuconazole and control, under preventative and curative applications. When treated with prothioconazole, the same resistant genotype produced a thin mycelial growth. The conidiation comparison of G641S isolates inoculated on tebuconazole-treated fruits and untreated fruits (control) revealed

Fig. 1. Sensitivity density distribution curves for the G641S mutants (**A**) and for wild-type (**B**) population of *Monilinia fructicola* in peach production areas in Brazil. Myclobutanil (solid line), propiconazole (dashed line), prothioconazole (dotted line), and tebuconazole (dashed/dotted line).

Table 5. Pearson correlation coefficient (r)^y and significance values (P)^z of *Monilinia fructicola* isolates carrying the G461S mutation to four different demethylation inhibitor fungicides

Fungicide	n	Myclobutanil	Tebuconazole	Propiconazole	Prothioconazole
Myclobutanil	31	1	0.16	0.33	0.03
Tebuconazole	31	0.389	1	0.38	0.36
Propiconazole	31	0.071	0.033	1	0.52
Prothioconazole	31	0.853	0.046	0.002	1

^y Pearson correlation coefficients (r) presented in the upper table side with two decimals.

^z Significance values (P) are represented in the lower table side with three decimals and italic; significant values are highlighted in bold letters.

no significant interaction with application timing ($P = 0.077$). Only a spray-treatment main effect was observed ($P = 0.001$), revealing a reduction of 38.8% for G461S isolates treated with tebuconazole (data not shown).

Discussion

With reduced sensitivity to DMIs and incomplete cross-resistance, this study provides evidence of differences among the performance of triazoles against *M. fructicola* isolates from Brazil. These results will form the basis for recommendations of rational DMI usage, aiming to delay the onset of fungicide resistance to control brown rot. Additionally, we provide baseline data for monitoring resistance to prothioconazole in populations of *M. fructicola* in the future.

The moderate cross-resistance among DMIs by *M. fructicola* was already documented by studies in the United States (Schnabel and Dai 2004). In this study, the authors reported correlation coefficients (r) of 0.46 between myclobutanil and propiconazole and 0.50 between myclobutanil and tebuconazole (Schnabel and Dai 2004). In our study, both genotypes (G461S and wild-type) presented similar myclobutanil and propiconazole cross-resistance levels as Schnabel and Dai (2004). The cross-resistance among triazoles was also documented in *Cercospora beticola* (Karaoglanidis and Thanassouloupoulos 2003), *Venturia inaequalis* (Hildebrand et al. 1988; Pfeufer and Ngugi 2012), *Botrytis cinerea* (Stehmann and De Waard 1996), *Didymella bryoniae* (Thomas et al. 2012), and *Alternaria* sp. (Avenot et al. 2016). We found no cross-resistance in G461S genotypes in the combinations trials that involved the myclobutanil fungicide. Several studies also documented the lack of cross-resistance among some DMIs (Leroux et al. 2000; McManus et al. 1999; Thomas et al. 2012). For instance, *Monilinia oxycocci*, causal agent of cotton ball disease of cranberry, showed no correlation between fenbuconazole and propiconazole (McManus et al. 1999).

The demonstrated efficacy of tebuconazole and prothioconazole in controlling wild-type isolates of *M. fructicola* using the preventative and curative treatment on detached fruits, strengthens the importance of this chemical group for the management of brown rot on stone fruit as previously reported by Schnabel et al. (2004), Holb and Schnabel (2007), and Villani and Cox (2011). Besides that, in our study, tebuconazole did not satisfactorily control brown rot on fruits inoculated with G461S isolates. This result was expected, since tebuconazole is the main DMI selecting for G461S mutants of *M. fructicola* in Brazil. However, prothioconazole treatments performed well, reducing the incidence and lesion size for both genotypes (wild-type and

G461S). Additionally, the G461S sporulation on fruits treated with prothioconazole revealed a delay of the pathogen reproductive stage at the time of evaluation. Within the wild-type genotype, the preventative and curative control of brown rot showed similar efficacy. Previous studies indicated that protective applications of DMI fungicides were more effective at reducing brown rot incidence on fruits than curative applications (Holb and Schnabel 2007; Villani and Cox, 2011). Indeed, preventative treatment with prothioconazole successfully controlled the disease when G461S isolates were used. In contrast, the curative treatment with tebuconazole was more effective to control resistant isolates than the preventative treatment. This agrees with an earlier study showing that tebuconazole has one of the best curative activities among the triazoles (Holb and Schnabel 2007). Schnabel et al. (2004) also found that curative applications of propiconazole were more effective than protective applications against *M. fructicola* resistant isolates.

In comparison with populations from the United States, the wild-type isolates of *M. fructicola* from Brazil had similar propiconazole sensitivity as isolates from South Carolina, with 0.026 $\mu\text{g/ml}$ and 0.025 $\mu\text{g/ml}$, respectively (Schnabel and Dai 2004). However, the wild-type sensitivity of isolates to tebuconazole in Brazil had higher mean EC_{50} values than *M. fructicola* populations from South Carolina and California (Schnabel and Dai 2004; Yoshimura et al. 2004). This difference may be attributed to the greater exposure of Brazilian isolates to tebuconazole, since more seasonal applications are made.

In Brazil, isolates resistant to tebuconazole occur with greater frequency compared with other DMI fungicides probably because tebuconazole has been used for two decades to control brown rot (May De Mio et al. 2011; Pereira et al. 2020). In contrast, it also explains the low sensitivity value to propiconazole in Brazil, considering this product is not registered for usage on stone fruits.

The populations tested for myclobutanil presented not only the highest EC_{50} values, but also the greatest curve shift toward resistance. Decreased sensitivity of *M. fructicola* to myclobutanil in comparison with other DMIs was reported to occur in South Carolina where the mean EC_{50} of the WT isolates was 0.683 $\mu\text{g/ml}$ (Schnabel and Dai 2004). Schnabel and Dai (2004) suggest that the lower sensitivity of *M. fructicola* to myclobutanil is inherent to this fungicide because it cannot effectively bind to the 14 α -demethylase. Myclobutanil is currently not recommended for use in peach orchards in the southeastern United States and in Brazil (Agrofit 2003; Blaauw et al. 2020). However, myclobutanil is the active ingredient of Rally

Table 6. Pearson correlation coefficient (r)^y and significance values (P)^z of wild-type *Monilinia fructicola* isolates to four different demethylation inhibitor fungicides

Fungicide	n	Myclobutanil	Tebuconazole	Propiconazole	Prothioconazole
Myclobutanil	29	1	0.19	0.52	-0.1
Tebuconazole	29	0.324	1	0.45	0.31
Propiconazole	29	0.004	0.017	1	0.19
Prothioconazole	29	0.597	0.109	0.323	1

^y Pearson correlation coefficients (r) presented in the upper table side with two decimals.

^z Significance values (P) are represented in the lower table side with three decimals and italic; significant values are highlighted in bold letters.

Table 7. Fungicide efficacy and timing for controlling brown rot caused by *Monilinia fructicola* in mature plum

Timing ^y and fungicide	n	Wild-type isolates		G461S mutants	
		Incidence (%)	Lesion size (SD) in mm ^z	Incidence (%)	Lesion size (SD) in mm ^z
Preventive, control	60	100	24.36 (6.39) d	100	20.91 (8.17) e
Curative, control	60	93.3	20.64 (6.64) d	80	17.67 (9.92) e
Preventive, tebuconazole	60	5	0.34 (1.53) ab	66.7	11.61 (8.95) d
Curative, tebuconazole	60	30	1.82 (3.65) c	48.3	7.84 (9.43) c
Preventive, prothioconazole	60	1.7	0.08 (0.65) a	1.7	0.1 (0.81) a
Curative, prothioconazole	60	13.3	1.11 (2.97) bc	31.7	3.23 (5.01) b

^y Application timing was made 24 hours before (preventive) and after (curative) isolate inoculation.

^z Means followed by the same letter within a column are not significantly different using pairwise contrasts with false discovery rate (FDR) method ($P \leq 0.05$).

40WSP (Dow AgroScience LLC, Indianapolis, IN), a commercial fungicide registered and recommended to control brown rot and blossom blight in California (Adaskaveg et al. 2017).

Despite the absence of information about *M. fructicola* sensitivity to prothioconazole currently available in the literature, Percival et al. (2018) demonstrated positive in vitro performance of prothioconazole to inhibit mycelial growth of Canadian isolates of *M. vaccinii-corymbosi*, with mean EC₅₀ of 8.6×10^{-4} µg/ml. Similarly, prothioconazole was able to inhibit our wild-type isolates under a very low concentration. As far as we know, this study is the first data for sensitivity of *M. fructicola* isolates to prothioconazole, and according to the ex vivo experiments, it can be an option to control brown rot in Brazil.

Tebuconazole and propiconazole EC₅₀ values were partially correlated in both G461S and wild-type populations. Since correlation implies the possible emergence of cross-resistance among these active ingredients, the increased *M. fructicola* resistance to tebuconazole would impact efficacy of propiconazole to control brown rot.

To manage a disease with high potential for resistance selection in the field, it is important to assess the sensitivity of a local population first, and then recommend the best active ingredients to be used. For more time-efficient assessment of resistance levels before making DMI recommendations, molecular methods such as allele-specific PCR developed by Lichtemberg et al. (2017) can be used. The DMI fungicides remain an important tool for managing brown rot in stone fruits in Brazil. Resistance development can occur, but it can also be managed, due to the fitness cost of resistant mutants demonstrated in vitro by Lichtemberg et al. (2019), and in practice by discontinuing the usage of DMI for two successive seasons in a commercial peach orchard (Pereira et al. 2020).

Based on our results, a spray program alternation between tebuconazole and propiconazole is not recommended because of a moderate risk of cross-resistance. Therefore, prothioconazole may represent a good DMI alternative to be included for brown rot management. However, field studies are needed to demonstrate efficacy under commercial conditions. Prothioconazole has already shown to be an effective product in the control of *Monilinia* blight in wild blueberries (Percival and Beaton 2012). In orchards with high G461S resistance frequency, tebuconazole should be discontinued to restore the wild-type population of *M. fructicola* (Pereira et al. 2020).

In addition, few fungicide options with different modes of action are available and registered for the management of brown rot of stone fruit in Brazil (Agrofit 2003). In some cases, it is recommended to use DMI alternating with QoI (Schnabel et al. 2004). However, QoI usage should be limited since *M. fructicola* isolates with reduced sensitivity to azoxystrobin have already been observed among Brazilian orchards (May De Mio et al. 2011; Pereira et al. 2017). The dicarboximide fungicide iprodione is an interesting option to alternate with DMIs. The Brazilian population of *M. fructicola* was shown to be highly sensitive to this fungicide, and solid performance of this active ingredient to control brown rot has already been demonstrated in the field (Dutra et al. 2019; Moreira and May De Mio 2009).

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