




Field isolates of *Monilinia fructicola* Change resistance pattern to greater sensitivity to thiophanate-methyl in recent populations

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Abstract The objectives of this study were to characterize *Monilinia fructicola* isolates that cause brown rot in stone fruit in Brazil according to resistance patterns to thiophanate-methyl (TM) and aggressiveness in detached fruit, comparing two sets of population. A total of 135 *M. fructicola* isolates were collected from three states in Brazil and divided in two sets: historic population (2003–2010) and 2017 population. Based on nucleotide substitutions at codon 6 and 198 in beta-tubulin gene and culture medium growth at $1 \mu\text{g mL}^{-1}$ and $500 \mu\text{g mL}^{-1}$ of thiophanate-methyl, the isolates were classified as: Sensitive (S), Low Resistant (LR) and Highly Resistant (HR). HR and LR isolates, confirmed by the presence of E198A and H6Y mutations, respectively, were found more frequently in the historical population (8.6% HR and 39.6% LR) than in 2017 (0% HR and 4% LR). Interestingly, the frequency of S isolates in 2017 increased three-fold (29 to 96%) compared to

the historic population. Thirteen isolates were classified as S-LR, with no mutation detected in the partial beta-tubulin gene. The ex vivo assays with two peach cultivars confirmed that the LR and HR isolates were equally pathogenic and aggressive in the absence of TM. TM completely prevented *M. fructicola* sporulation and effectively reduced the incidence and lesion size of brown rot either for S and S-LR isolates in both cultivars. Therefore, the possibility of re-registration of thiophanate-methyl for the management of brown rot in Brazil was discussed.

Keywords Brown rot · Methyl Benzimidazole Carbamates · Resistance management

Introduction

Peach (*Prunus persica* L. Batsch.) is the third most economically important fruit tree crop in the world within the Rosaceae family after apples (*Malus* spp.) and pears (*Pyrus* spp.), and its largest producer is China, followed by Spain, Italy and Turkey (FAOSTAT, 2020). Brazil represents about 1% of the world production of stone fruit (peach and nectarines), with an average of 201 thousand tons, mainly produced in the southern states of Paraná (PR), Santa Catarina (SC) and Rio Grande do Sul (RS), and in the southeastern state of São Paulo (SP) (FAOSTAT, 2020).

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Brown rot, caused by *Monilinia* spp., is one of the most important pre- and post-harvest diseases of stone fruits in many production areas worldwide (Agris, 2005; Usall et al., 2015). Economic losses can exceed 50% of the production depending on the climatic conditions, fruit tree crop and pathogen diversity (Keske et al., 2011; Larena et al., 2005). *M. fructicola* (G. Winter) Honey, *Monilinia laxa* (Aderhold and Ruhland) Honey and *M. fructigena* (Honey) are the most prevalent species that have been associated with brown rot in stone fruit worldwide (Van Leeuwen & Van Kesteren, 1998). Additionally, minor species such as *M. polystroma*, *M. mumeicola* and *M. yunnanensis* have been reported to cause brown rot in stone fruit in Asia and Europe (Hu et al., 2011; Martini et al., 2014; Munda, 2015). In Brazil, *M. fructicola* is the main species causing blossom blight, twig canker, and pre- and postharvest fruit rot on peaches (Fischer et al., 2017; May De Mio et al., 2011; Pereira et al., 2019). In addition to stone fruits, pears and apples can also be infected by this pathogen (Byrd & Willetts, 1977; Pereira and May De Mio, 2018; Zhu et al., 2016).

For many years, the conventional method to control brown rot in Brazilian stone fruit orchards relied on fungicide sprays, from flowering until pre-harvest, with site-specific fungicides such as Methyl Benzimidazole Carbamates (MBCs), Demethylation Inhibitors (DMIs) and Quinone Outside Inhibitor (QoIs) (Lichtemberg et al., 2016; May De Mio et al., 2011). The number of fungicide sprays per season may range from six to eight depending on the infection level and climatic conditions (May De Mio et al., 2011; Pereira et al., 2020; Tibola et al., 2005). Recently, Succinate Dehydrogenase Inhibitors (SDHIs), which are part of brown rot management in the USA (Adaskaveg et al., 2017; Blaauw et al., 2019), were registered for *M. fructicola* control in Brazil in a mixture with QoIs (AGROFIT, 2003).

MBCs were the first systemic fungicides used to control brown rot and a broad spectrum of ascomycete pathogens, and have been in the market for more than 50 years (Brent & Hollomon, 2007; Young, 2015). MBC fungicides are classified by the Fungicide Resistance Action Committee (FRAC) as Group 1 and include benomyl, carbendazim, thiabendazole, fuberidazole and thiophanate-methyl as commercial active ingredients (a.i), from the chemical groups benzimidazoles and thiophanates (FRAC, 2021).

Thiophanate-methyl (TM) and benomyl have both been widely used to control *Monilinia* spp. However, by the late 1970s, benomyl-resistant populations of *M. fructicola* had become widespread in stone fruit orchards throughout California, in the United States (US) (Ma et al., 2003; Michailides et al., 1987; Sonoda et al., 1983). Although benomyl was withdrawn from the market in 2001, growers used thiophanate-methyl as a replacement for benomyl (Yoshimura et al., 2004). Unfortunately, a reduction of the efficacy of thiophanate-methyl against *M. fructicola* isolates was reported later in the US (Chen et al., 2013; Ma et al., 2003; Yoshimura et al., 2004), Brazil (May De Mio et al., 2011), Spain (Egüen et al., 2015) and, recently, in China (Ke et al., 2023).

Fungicide target site alteration in β -tubulin was reported to be the primary resistance mechanism to MBCs and has been detected in field isolates and in laboratory mutants, associated with mutations at codons 6, 50, 134, 165, 167, 198, 200 and 240 in the beta-tubulin gene in several pathogenic fungi, such as *Aspergillus nidulans*, *Cladobotryum dendroids*, *Neurospora crassa*, *Botrytis cinerea*, *Penicillium digitatum*, *Venturia inaequalis* and *Sclerotinia sclerotiorum* (Koenraadt, et al., 1992; Lehner et al., 2015; Malandrakis et al., 2011; McKay et al., 1998; Schmidt et al., 2006; Orbach et al., 1986). In *M. fructicola*, two point mutations in the β -tubulin gene were related to different patterns of resistance to the MBCs benomyl and thiophanate-methyl. The first was located at codon 198 (E198A) and was associated with a high level of resistance (HR), whereas the second was located at codon 6 (H6Y) and associated with a low level of resistance (LR) (Ke et al. 2023; Ma et al., 2003; Malandrakis et al., 2012; Weger et al., 2011; Yoshimura et al., 2004). These observations were used to develop conventional and real-time PCR for rapid identification of the resistance patterns (Fan et al., 2014; Ma et al., 2003).

In most cases, brown rot control failure following the use of thiophanate-methyl is associated with mutations at positions 198 and 200 of the beta-tubulin gene. The E198A mutation in *Monilinia* spp. has been detected in both North and South America (Luo et al., 2007; Ma et al., 2003; May De Mio et al., 2011; Zhu et al., 2010) and in Europe (Malandrakis et al., 2012; Weger et al., 2011). In Brazil, different patterns of thiophanate methyl resistance in

2000–2008 *M. fructicola* populations were reported by May De Mio et al. (2011). Currently, the use of thiophanate-methyl has been very limited throughout Brazil (AGROFIT, 2003). However, this fungicide is still widely used to control brown rot in other countries such as the US (Adaskaveg et al., 2017; Blaauw et al., 2019), Greece (Thomidis et al., 2009) and Spain (MAPA, 2020).

MBC resistance is known to be qualitative and stable over years, quickly building up to high levels in the pathogen populations and leading to immediate loss of control (Brent & Hollomon, 2007). The high fitness and high resistance factor associated with *M. fructicola* resistant isolates (Yoshimura et al., 2004) comprises the most critical challenge in determining anti resistance strategies. Nevertheless, resistance to other fungicides associated with resistance to MBCs may influence the pathogen fitness differently. Reports of resistance to other systemic fungicide groups are frequent in *M. fructicola* isolates from Brazil (Dutra et al., 2020; Pereira et al., 2017; Primiano et al., 2017). However, Brazilian *M. fructicola* populations have not been screened for MBC resistance for over a decade. Knowledge of the current composition of resistant isolates of *M. fructicola* populations, and how their aggressiveness relates to different resistance patterns, are critical to understand whether MBC products could be reincorporated into brown rot management programmes in Brazilian stone fruit orchards.

Thus, the aim of this study was: 1) to investigate the sensitivity of Brazilian isolates of *M. fructicola* to thiophanate methyl in different primary production areas, comparing the historic populations to the population in 2017; 2) to partially sequence the beta-tubulin gene associating with different patterns of resistance to thiophanate-methyl and; 3) to compare the aggressiveness of *M. fructicola* isolates with different resistance patterns in peaches treated and untreated with thiophanate-methyl.

Materials and methods

Isolate collection

In total, 135 single-spore isolates of *M. fructicola* were selected from the Epidemiology Laboratory

for Integrated Disease Management (LEMID) collection located in the Department of Plant Sciences and Pest Management at the Federal University of Paraná (UFPR). The Brazilian isolates were collected from infected flowers, symptomatic mature fruit and mummified fruit in peach and nectarine (*Prunus persica* var. *nucipersica*) orchards located in São Paulo (SP), Paraná (PR) and Rio Grande do Sul (RS) states (Online Resource 1). The first set comprises 58 *M. fructicola* isolates (SP=22; PR=17; RS=19), collected between 2003 and 2010, which was used to study both the sensitivity and the molecular mechanisms of resistance associated with thiophanate-methyl (TM). The second set included 77 isolates (SP=27; PR=50) collected in the 2017 season, which was also used to determine *M. fructicola* sensitivity to TM, but no molecular characterization was done. Prior to the assays, isolates were recovered from Castellani's storage method (Castellani, 1963), plated on Potato Dextrose Agar (PDA) containing 2.5 ml of 25% [vol/vol] lactic acid per liter, and were incubated for seven days at 25 °C with a photoperiod of 12 h.

Survey of fungicide sensitivity

Discriminatory dose to thiophanate-methyl

Four-mm mycelial plugs obtained from 5-day old colonies of each isolate were transferred to Petri dishes with PDA medium amended with 1 µg mL⁻¹ and 500 µg mL⁻¹ of TM (Cercobin 700WP®, Ihara) and non-amended PDA. Four plugs of different isolates were placed in each plate and the cultures were incubated at 25 °C for 5-days with a 12-h photoperiod. The mycelial growth (MG) was estimated by measuring the diameter of each colony in two perpendicular directions. The isolates were classified phenotypically according to their sensitivity levels to thiophanate-methyl as follows: sensitive (S), when no mycelial growth was observed on PDA amended with 1 and 500 µg mL⁻¹ of TM; low resistant (LR), when mycelial growth was observed only at 1 µg mL⁻¹ of TM; and highly resistant (HR), when the isolates were able to grow in both 1 and 500 µg mL⁻¹ of TM (May De Mio et al., 2011). The assay was conducted twice in a completely randomized design with three replicates per concentration for each isolate.

Effective concentration (EC_{50}) to thiophanate-methyl

The effective concentration to inhibit 50% of colony growth (EC_{50}) to TM was determined using the Spiral Gradient Dilution method (SGD), described previously by Förster et al. (2004), except that cellophane strips were replaced with mycelial strips (MS). For this assay, nine isolates were selected based on the results of discriminatory dose and partial beta-tubulin gene sequencing (described below). Suspensions of 10^6 spores/ml were obtained by scraping the spores from inoculated canned peaches incubated for 7-days at room temperature. MS used on the SGD assay were produced by spreading 1 ml of spore suspensions onto PDA containing 20 g L^{-1} of agar, poured into 150-mm plates. After three days of incubation at $23 \text{ }^\circ\text{C}$ and a photoperiod of 12 h the MS were cut into 90×6 mm strips. The fungicide was amended on the surface of a 150-mm plate containing 50 mL of PDA using an Auto-plater 500 (Spiral Biotech, Inc). The fungicide was spirally applied to the PDA media at exponential concentration using stock solution of a.i. prepared at $1408.08 \text{ } \mu\text{g mL}^{-1}$ (range of $0.08\text{--}8.00 \text{ } \mu\text{g mL}^{-1}$) and $140,808.00 \text{ } \mu\text{g mL}^{-1}$ (range of $8\text{--}800 \text{ } \mu\text{g mL}^{-1}$). The ranges were selected based on the available data on *M. fructicola* sensitivity to TM. The amended and the control plates carried two isolates (two MS/isolate) and four isolates (one MS/isolate), respectively. Three replicates (plates) were prepared for each isolate and fungicide combination. The EC_{50} was determined after three days incubation at $23 \text{ }^\circ\text{C}$ using the SGE software. The experiment was conducted twice.

DNA Extraction

For DNA extraction, the isolates were cultured in PDA for 3 days at $25 \text{ }^\circ\text{C}$ with a 12 h photoperiod. The DNA extraction was performed using the Ultraclean Microbial DNA Isolation Kit, MoBio® (MO Bio, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and concentration of DNA was evaluated by 0.8% (w/v) agarose gel electrophoresis, with *Hind* III *Invitrogen*® molecular marker, stained with GelRed Nucleic Acid Gel Stain® (Biotium Inc, Hayward, CA, USA) and visualized under ultraviolet light.

Amplification and sequence analysis of partial beta-tubulin gene

To amplify the beta-tubulin gene, the following conditions were used: 10 ng DNA, $1 \times$ PCR buffer, 0.5 U Taq polymerase, $0.1 \text{ } \mu\text{M}$ TUBA and TUBR1 primers (Ma et al., 2003), 0.2 mM of each dNTP, 1.5 mM MgCl_2 , and final volume of $12.5 \text{ } \mu\text{L}$. The amplification was performed in *Mastercycler Gradient Eppendorf*® thermal cycler (Eppendorf, Hamburg, Germany). The amplification conditions used were initial denaturation at $95 \text{ }^\circ\text{C}$ for 3 min, 40 cycles of 40 s at $94 \text{ }^\circ\text{C}$, 40 s at $50 \text{ }^\circ\text{C}$, 1.5 min at $72 \text{ }^\circ\text{C}$, and final extension of 10 min at $72 \text{ }^\circ\text{C}$ (Ma et al., 2003). PCR amplification products were visualized and quantified in a 1.5% (w/v) agarose gel electrophoresis stained with GelRed Nucleic Acid Gel Stain®, (Biotium Inc, Hayward, CA, USA) with a Ladder 100 pb molecular weight marker (Ludwig Biotec®, Porto Alegre, RS, Brazil).

The PCR product was purified using FastAp® (Thermo Scientific San Jose, CA, USA) and ExoI® (Exonuclease I, Thermo Scientific, San Jose, CA, USA) according to the manufacturer's instructions. The partial sequencing of the beta-tubulin region was performed in the ABI3500® Automatic Sequencer (Applied Biosystems, Foster City, CA, USA) using the TUBA and LRR2 primers, and MFTF and MFTR primers (Ma et al., 2003). For the sequencing reaction, the BigDye Direct Cycle Kit® (Applied Biosystems, Foster City, USA) was used, according to the manufacturer's instructions.

The two fragments obtained from the beta-tubulin gene (regions of codons 6 and 198) were edited using MEGA v. 6.0 (Tamura et al., 2006) and BioEdit version 7.0 (Hall, 2013). The sequences were aligned using ClustalW (Thompson et al., 1997), available in MEGA v. 6.0. As reference for the observation, identification of codons and determination of resistance patterns, sequences from Ma et al. (2003) were used, available at GenBank (<https://www.ncbi.nlm.nih.gov/genbank>). The isolates were classified as S when presenting the nucleotides CAT and GAA in codons 6 and 198 respectively; as LR, when presented nucleotides TAT (which encodes the amino acid tyrosine) rather than CAT (coding for histidine) at codon 6 and GAA at codon 198; or as HR, when they present a mutation at codon 198, characterized by the nucleotide sequence GCA (which encodes the amino acid

alanine) instead of GAA (coding for glutamic acid), as proposed by Ma et al. (2003).

Effect of TM treatment of detached peach fruit on brown rot development

The same nine isolates used in the EC₅₀ assay were evaluated in ripe peaches of Chimarrita and Rubimel cultivars. The fruit were surface disinfested by immersion into 70% ethanol (1 min), 0.5% sodium hypochlorite (1 min) followed by three rinses with sterile distilled water. After 12 h, the fruit were immersed in Cercobin 700 WP® at the field recommended dose (0.7 g L⁻¹ of TM). After 12 h of the fungicide treatment, the fruit were wounded by a sterilized 2-mm wooden rod and placed inside humidified plastic containers. Each fruit was inoculated with 20 µL of spore suspension (1 × 10⁵ conidia mL⁻¹) of each isolate and incubated at 23 ± 2 °C with a 12 h photoperiod for 132 h. Subsequently, brown rot incidence (%), lesion diameter (mm), incubation period (IP – period between inoculation and the appearance of symptoms on at least 50% of fruit) and latent period (LP – period between inoculation and fungal sporulation on at least 50% of fruit) were obtained. The experiment was conducted in a completely randomized design and repeated twice, using three and twelve replicates per isolate in untreated and treated fruit, respectively.

Statistical analysis

The arithmetic means of EC₅₀ values were calculated with pooled data since no significant differences between experiments were verified by the Wilcoxon non-parametric range test ($P > 0.05$). Lesion diameter, disease incidence and sporulation incidence were analyzed with ANOVA and means were compared using Tukey test ($P < 0.05$).

The incubation and latent periods were modeled using survival analysis. For that, the results of each isolate were grouped according to the resistance pattern. Kaplan–Meier estimates were performed using the log-rank test to compare curves lengths, the median incubation and the median latent periods among the *M. fructicola* resistance patterns. The survival function $S(t)$ was estimated by the product of the probabilities of the fruit remaining without the event (symptoms or sporulation) until time t (survival

time) and it was recalculated after each individual event (Nesi et al., 2015). Data on asymptomatic fruit and fruit without visible sporulation by the end of the assessment period were considered censored since the IP and LP were unknown. Survival time data were analyzed using the ‘survival’ package (Therneau, 2018). The statistical software R (version 4.0.2) was used for data analysis (R Core Team 2020).

Results

Partial beta-tubulin gene sequencing and fungicide sensitivity to thiophanate-methyl

Among the 58 isolates from the historic population, 8.62% ($n=5$) were characterized as HR since the mutation at codon 198 (GAA → GCA) and growth on PDA with 500 µg mL⁻¹ of TM were observed (Fig. 1, Supplementary 1). Isolates classified as LR were present at a frequency of 39.6% ($n=23$) as nucleotides TAT were observed instead of CAT at codon 6 and GAA at codon 198. These isolates were able to grow only in the non-amended media and at 1 µg mL⁻¹ of fungicide. The highest frequency of LR isolates was found among isolates from PR (43.48%), followed by RS (34.78%) and SP (21.74%). The S isolates were present at a frequency of 29.31% ($n=17$) and did not present growth at either 1 and 500 µg mL⁻¹ of TM (Fig. 1). The S isolates had the CAT and GAA sequences in codons 6 and codon 198 respectively. Regarding S isolates, 29.41% were from PR, 35.29% from RS and 35.29% from SP. However, 22.41% isolates ($n=10$) were named as S-LR as they presented both characteristic of S and LR isolates. These isolates showed genetic pattern of S by partial beta-tubulin gene sequencing (with nucleotides CAT at codon 6 and GAA at codon 198) and also presented the characteristic of LR isolates by growing at 1 µg mL⁻¹ of TM (Online Resource 1). It was observed that 76.92% of the S-LR isolates were from the state of SP (Fig. 1).

Among the 77 isolates from 2017, no HR isolates were found by discriminatory dose analysis and only three isolates were classified as LR. The remaining isolates were classified as S since no mycelial growth was observed at 1 and 500 µg mL⁻¹ of TM (Fig. 1, Online Resource 2).

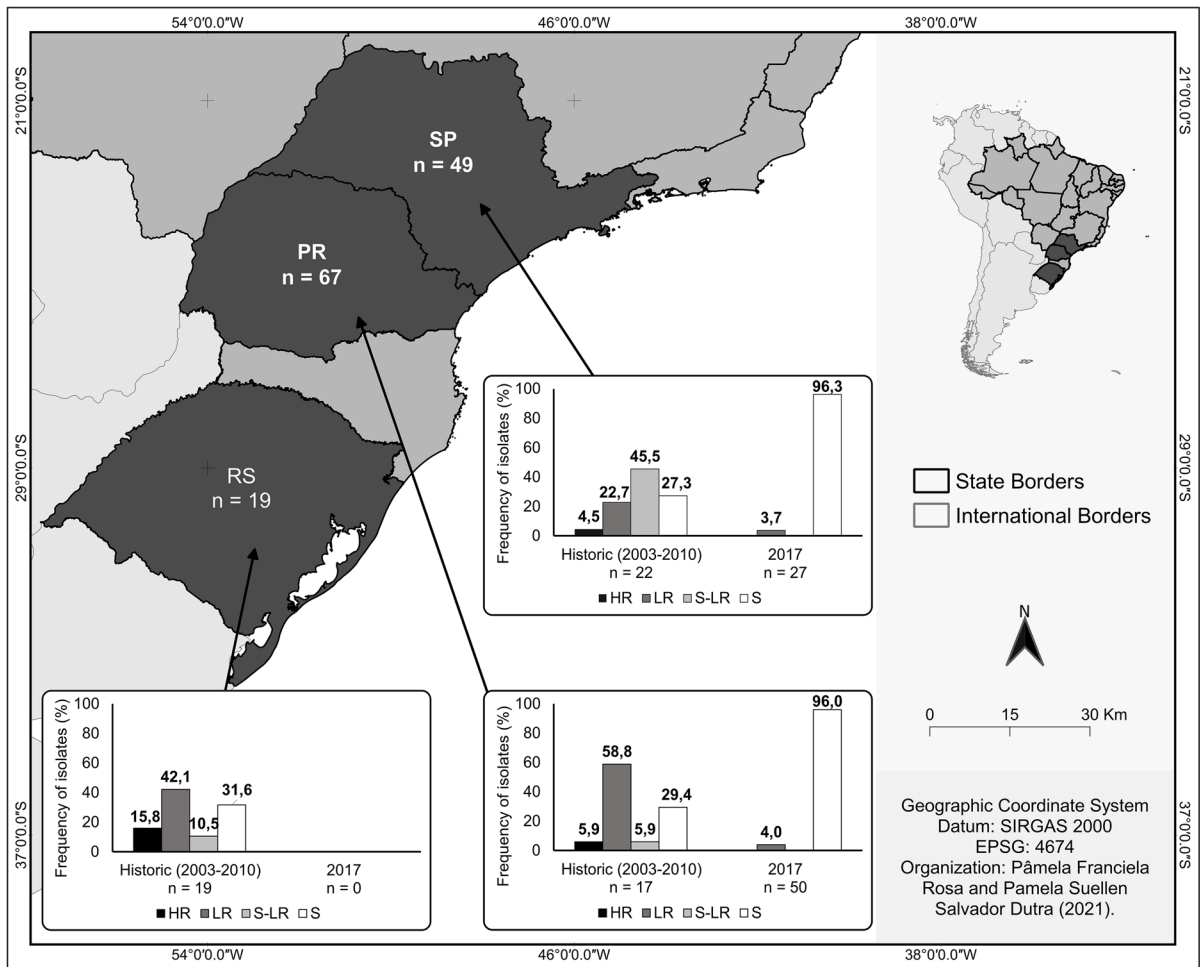


Fig. 1 Frequency of *Monilinia fructicola* resistance patterns to thiophanate-methyl from peach orchards in Paraná (PR), São Paulo (SP) and Rio Grande do Sul (RS) states. The isolates were classified as S (sensitive), S-LR (moderately sensitive),

LR (low resistant) and HR (highly resistant) based on mutations at codon 6 and 198 in beta-tubulin gene and mycelial growth at $1 \mu\text{g mL}^{-1}$ and $500 \mu\text{g mL}^{-1}$ of thiophanate-methyl

The highest EC_{50} values for thiophanate-methyl were found in all *M. fructicola* isolates with HR resistance pattern. The isolate PpMfSP07-321 (HR) had the highest EC_{50} value ($195.8 \mu\text{g mL}^{-1}$), corresponding to a 631-fold increase to the EC_{50} value of the S isolate PpMfRS03-115 (S) ($0.31 \mu\text{g mL}^{-1}$). The S-LR isolates presented EC_{50} values varying from 0.32 to $0.5 \mu\text{g mL}^{-1}$ while the LR isolates presented EC_{50} values between 3.26 and $7.103 \mu\text{g mL}^{-1}$ (Table 1).

Effect of TM treatment of detached peach fruit on brown rot development

All untreated fruit (100%) inoculated with nine *M. fructicola* isolates presented brown rot incidence and sporulation in both Chimarrita and Rubimel cultivars. The lesion diameter caused by the different *M. fructicola* resistance patterns did not differ in untreated fruit in both cultivars (Fig. 2). However, a greater reduction of brown rot incidence and lesion size was

Table 1 Nucleotides present in codon 6 and 198 in the beta-tubulin gene, mycelial growth (mm) at discriminatory doses, resistance (genotype and phenotype) patterns and effective concentration (EC₅₀) for thiophanate-methyl in *Monilinia fructicola* isolates

Isolates ^a	Sequencing β-tubulin gene				Discriminatory dose ^b				EC ₅₀ μg/ml
	Codon 6	Resistance genotype ^c	Codon 198	Resistance genotype	Control	1 μg mL ⁻¹	500 μg mL ⁻¹	Resistance phenotype ^c	
PpMfRS03-115	CAT	S	GAA	S	26.12	0	0.00	S	0.31
PpMfRS05-400	CAT	S	GAA	S	23.99	1.97	0.00	S-LR	0.50
PpMfPR09-638	CAT	S	GAA	S	31.5	1.75	0.00	S-LR	0.32
PpMfPR09-639	TAT	LR	GAA	LR	33.63	33.38	0.00	LR	7.10
PpMfRS10-960	TAT	LR	GAA	LR	36.66	26.43	0.00	LR	3.34
PpMfRS10-977	TAT	LR	GAA	LR	39.5	37.50	0.00	LR	3.26
PpMfPR07-181	CAT	HR	GCA	HR	38.13	37.25	32.40	HR	49.99
PpMfSP07-321	CAT	HR	GCA	HR	26.13	23.88	28.90	HR	195.81
PpMfRS10-996	CAT	HR	GCA	HR	35.75	29.44	30.00	HR	114.33

^a Code information: host specie (Pp=*Prunus persica*; Pn=*Prunus persica nureipersica*)+pathogen species (*Monilinia fructicola*) + State (PR=Paraná; SP=São Paulo; RS=Rio Grande do Sul) + season (2003 to 2017) – isolate code number

^b Mean colony diameters, in mm, evaluated in PDA medium non-amended (control) and amended with thiophanate-methyl, after 5 days of incubation at 25° C

^c S = sensitive; S / LR = moderately sensitive; LR = low resistant; HR = highly resistant

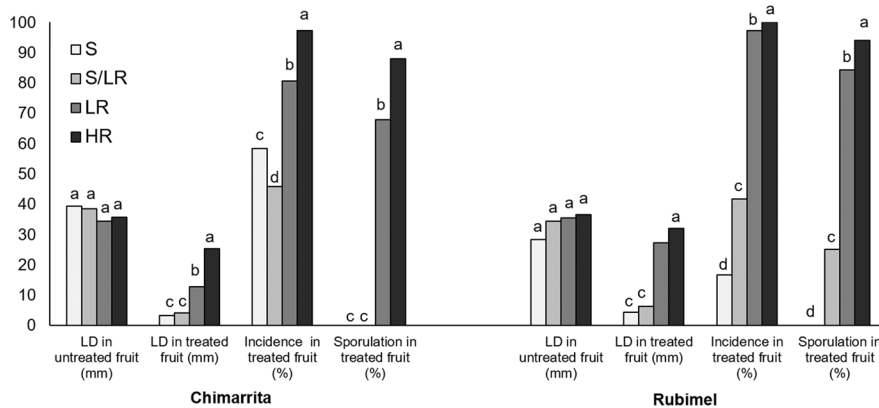


Fig. 2 Lesion diameter (LD) of brown rot comparing aggressiveness of *Monilinia fructicola* isolates from different resistance patterns to thiophanate-methyl and, disease and sporulation incidence in fruit from two cultivars (Chimarrita and Rubimel) treated with thiophanate-methyl (TM). Control

and Treatment refer to the mean lesion diameter, in mm, in untreated and treated fruit with TM, respectively. Incidence refers to the % of inoculated fruit that showed symptoms after treatment with TM. Sporulation incidence refers to the % of fruit with visible *M. fructicola* sporulation

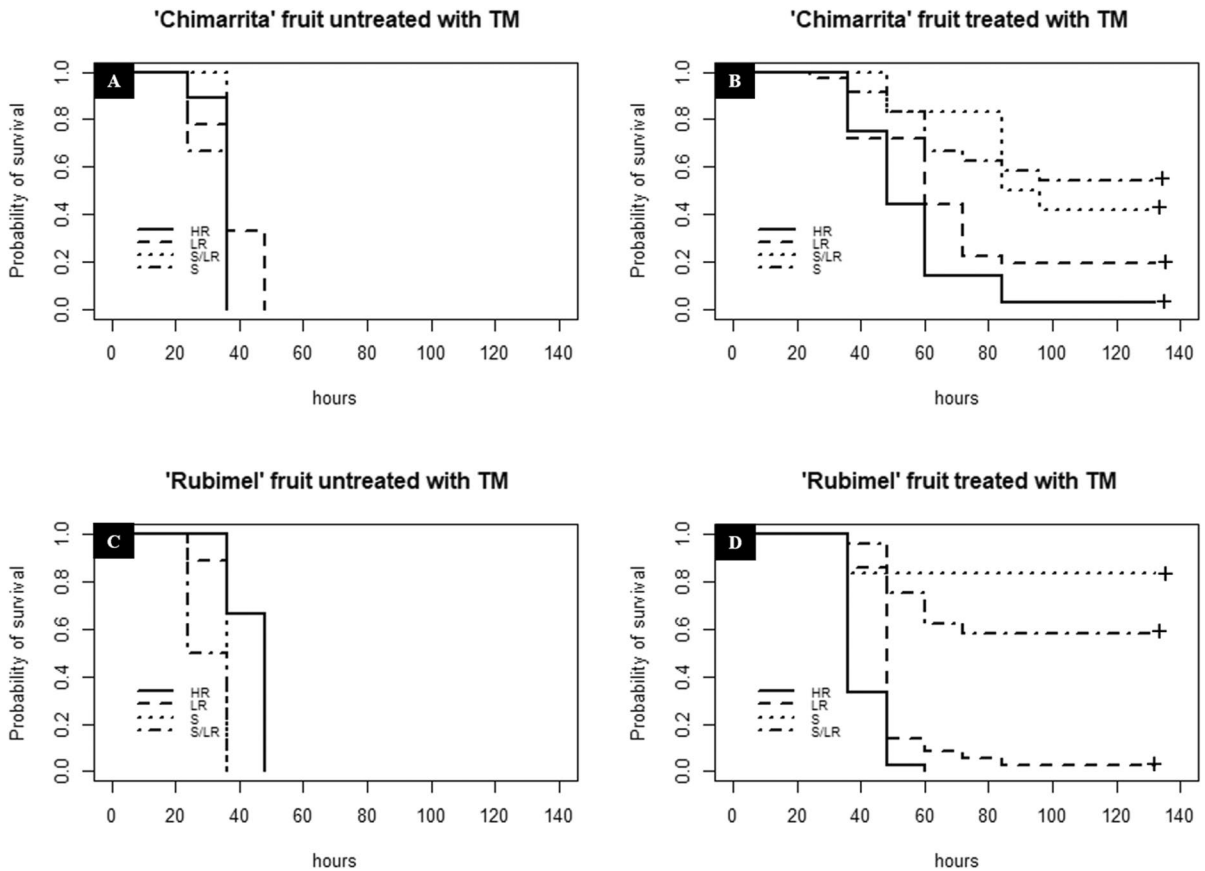
found in detached fruit treated with TM and inoculated with S and S-LR isolates. These isolates were not able to sporulate on peach fruit treated with the fungicide, therefore the latent period could not be calculated (Figs. 2 and 4).

None of the untreated fruit remained without symptoms until the end of the study (132 h) in both cultivars. The Log-Rank test indicated that survival curves of brown rot incubation period and latent

period in ‘Chimarrita’ and ‘Rubimel’ fruit treated with TM differed significantly ($P < 0.001$) among the *M. fructicola* resistance patterns (Table 2). Incubation and latent periods of S and S-LR isolates were much greater than incubation and latent periods of HR and LR isolates in both cultivars treated with TM (Figs. 3 and 4). In ‘Chimarrita’ treated fruit, the median IP for LR and HR was 60 and 48 h, while in ‘Rubimel’ the IP was 48 and 36 h, respectively (Table 2).

Table 2 Descriptive statistics, median hours of incubation period (IP) and latent period (LP) among *M. fructicola* resistance patterns in peach fruit treated and untreated with thiophanate-methyl

Resistance Patterns	Chimarrita				Rubimel			
	Untreated		Treated		Untreated		Treated	
	IP	LP	IP	LP	IP	LP	IP	LP
S	36	60	90	- ^a	36	72	-	-
S-LR	36	48	-	-	36	72	-	-
LR	36	72	60	96	48	72	48	72
HR	36	72	48	72	48	60	36	72
χ^2	3.26	8.21	31.87	65.63	15.67	2.68	76.06	40.3
<i>P</i> -value ^b	0.4 ^{ns}	0.04	6×10^{-7}	4×10^{-14}	0.001	0.4 ^{ns}	2×10^{-16}	9×10^{-9}

^a censored observation^b Median incubation and latent period of resistance patterns were compared using Log-Rank test at 95% confidence interval**Fig. 3** Kaplan–Meier of survival functions describing the incubation period of brown rot caused by *Monilinia fructicola* in peaches untreated (A, C) and treated (B, D) with thiophanate-methyl. The sign (+) indicates censored observations

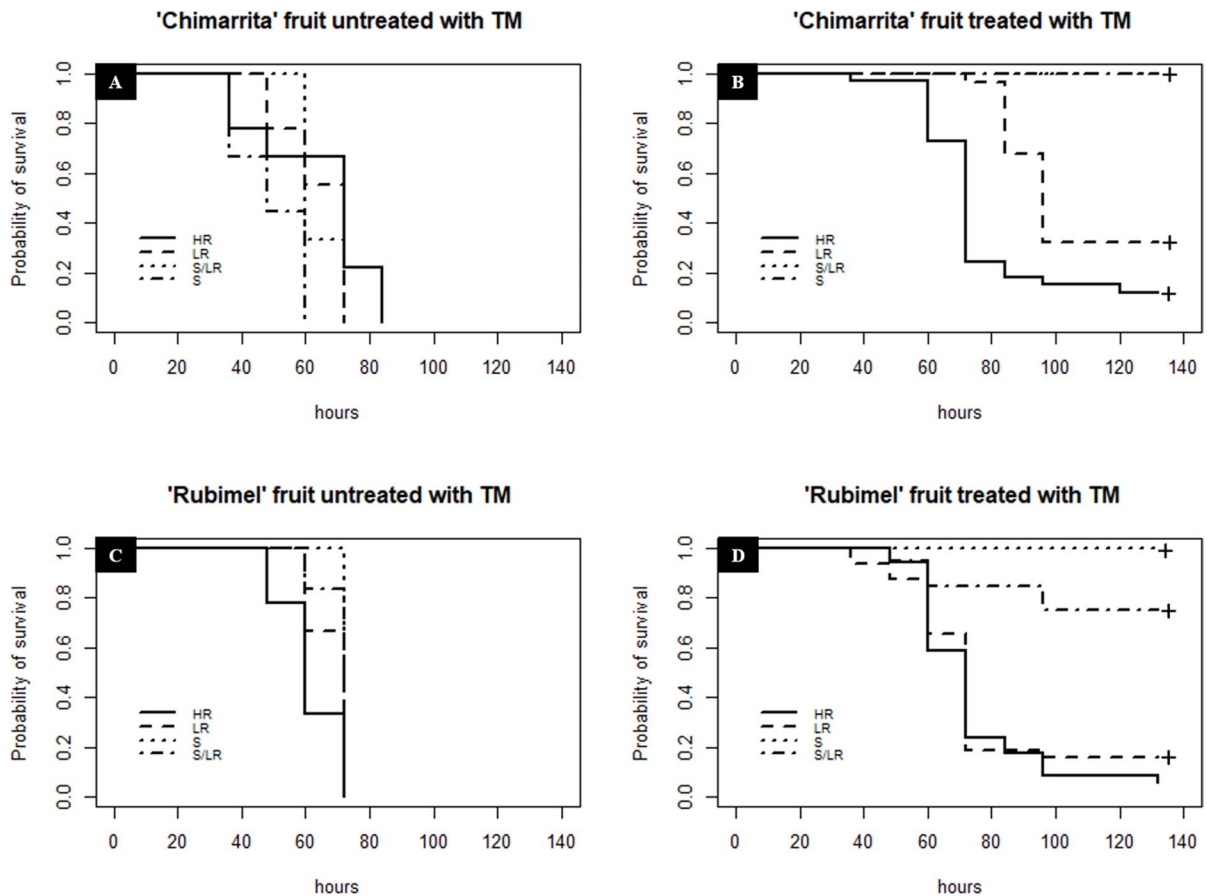


Fig. 4 Kaplan–Meier of survival functions describing the latent period of brown rot caused by *Monilinia fructicola* in peaches untreated (A, C) and treated (B, D) with thiophanate-methyl. The sign (+) indicates censored observation

Discussion

The present study revealed different patterns of resistance to the MBC fungicide thiophanate-methyl from Brazilian isolates of *Monilinia fructicola*. The partial sequencing of the beta-tubulin gene, from codons 6 and 198, demonstrated the presence of E198A and H6Y mutations that characterized *M. fructicola* isolates as high (HR) and low (LR) resistance to the fungicide, respectively. In our study, HR isolates were detected in the historic population from all three evaluated Brazilian states and the mutation in the beta-tubulin was confirmed to be the E198A mutation, which results in alanine (GCA) replacing glutamic acid (GAA). The same single base pair mutation associated with MBC resistance was detected previously in *M. fructicola* isolates from California (Ma et al., 2003), Korea (Lim et al., 2006) and China (Chen

et al., 2014; Fan et al., 2009). The H6Y mutation associated with low resistance was also observed in *M. fructicola* isolates in the historic population from all evaluated states. Ma et al. (2003) first reported the same mutation at codon 6 in *M. fructicola* field isolates from California resistant to both benomyl and thiophanate-methyl. Furthermore, *M. fructicola* isolates resistant to thiophanate-methyl were recently reported to have the H6Y mutation in the southeast of China (Ke et al., 2023).

The thirteen *M. fructicola* isolates classified as S-LR grew at the discriminatory dose of $1 \mu\text{g mL}^{-1}$ of TM but did not amplify in specific PCR to identify mutation at codon 6, using primers LRF and LRR2 as proposed by Ma et al. (2003). Previously, May De Mio et al. (2011) have also found some Brazilian LR isolates to MBCs that could not be distinguished by LRF and LRR2 primers, and the authors suggested

another unknown mechanism of resistance. In the present study, it was not possible to clarify which mechanism of resistance to MBC is associated with *M. fructicola* S-LR isolates since the beta-tubulin gene was not completely sequenced. Some isolates were evaluated in a larger genomic region and, although another point mutation was found, it does not encode different amino acids (data not shown). In addition, it should be considered that other resistance mechanisms may also affect the sensitivity to MBCs, such as beta-tubulin overexpression (Nakaune & Nakano, 2007) or the efflux mechanism involving a multidrug transporter protein (Andrade et al., 2000). Some studies have also used a discriminatory dose of $1 \mu\text{g mL}^{-1}$ to characterize *M. fructicola* isolates with low level of resistance to MBCs (e.g., Ma et al., 2003; Martini et al., 2016; May De Mio et al., 2011). Since the S-LR isolates have similar behavior to S isolates and to avoid including such isolates as LR samples, we recommend using a higher discriminatory dose of TM for phenotyping *M. fructicola* Brazilian isolates. A discriminatory dose of $5 \mu\text{g mL}^{-1}$ could distinguish *M. fructicola* resistant isolates to thiophanate-methyl with the H6Y mutation from the sensitive isolates (Ke et al., 2023). Thus, this dose can be used for future LR population monitoring.

Although the HR pattern to MBCs is primarily based on the E198A mutation, the E198K, the E198Q and the F200Y mutations in beta-tubulin have also been associated with high levels of resistance to MBCs in *M. fructicola* (Chen et al., 2013; Koenraadt et al., 1992). Martini et al. (2016) discovered Italian isolates with a point mutation at codon 83 in the beta-tubulin gene, with CAA instead of CGA. Albertini et al. (1999) reported additional point mutations at positions 198 and 200 of the beta-tubulin gene, associated with control failure due to reduced sensitivity of *Tapesia* spp. to MBCs. In *M. laxa*, a mutation at codon 240, causing substitution of CTC (leucine) by TTC (phenylamine), was associated with LR pattern (Ma et al., 2005).

Among the Brazilian isolates tested in this study, the EC_{50} values of *M. fructicola* ranged from 0.31 to $195.81 \mu\text{g mL}^{-1}$ using the SGE method. The EC_{50} values of S, LR and HR isolates obtained in this study agree with previous studies on the growth response curves of *M. fructicola* (Ma et al., 2003; Martini et al., 2016; Yoshimura et al., 2004). Yoshimura et al. (2004) reported *M. fructicola*

isolates with different resistance patterns to MBC in California, with EC_{50} values of $< 2 \mu\text{g mL}^{-1}$ for S isolates, between 2 and $30 \mu\text{g mL}^{-1}$ for LR isolates, and $> 30 \mu\text{g mL}^{-1}$ for the HR isolates.

LR isolates from the historic population were detected at high frequencies, predominantly in the PR state, in relation to the S, HR and S-LR isolates. The greater frequency of LR was also observed in the Italian population of *M. fructicola* studied by Martini et al. (2016), in which LR isolates represented 66% of the population, while HR isolates accounted for only 5%. Fortunately, the 2017 sub-population of *M. fructicola* obtained in the present study from the same orchards in SP and PR states did not show any HR phenotype and the frequency of S isolates had a threefold increase in relation to the historic population.

Resistance to MBC is referred to as ‘qualitative’ and is characterized by an abrupt loss of effectiveness. Once developed, it tends to be stable and pathogen populations can remain resistant for many years even with a decrease in the use of the fungicide or following withdrawal from the market (Brent & Hollomon, 2007). Supporting their stability, MBC-resistant and MBC-sensitive *M. fructicola* isolates are also reported to have high fitness and competitiveness in the absence of fungicide selection pressure (Egüen et al., 2015; Ke et al. 2023; Yoshimura et al., 2004). The stability of MBC resistant isolates is already documented in *M. fructicola* by Egüen et al. (2015), where the frequency of these isolates remained unchanged over a 5-year survey in Spanish orchards. However, thiophanate-methyl use is still authorized to control brown rot in Spain, restricted to one application per year (MAPA, 2020).

Contradictorily, in Brazil, an increase in the percentage of S isolates to TM was observed from 2000 to 2005 and stable frequencies from 2005 to 2008 (May De Mio et al. 2011). These observations were linked with the management of TM sprays. In the 2000 season, the number of sprays with MBC fungicides in commercial orchards in Brazil per season was 2 to 4. In 2002, the use of these fungicides was drastically reduced in some states to a maximum of 1 application per season (May De Mio et al., 2011). Interestingly, Ma et al. (2003) also noticed that the frequencies of LR isolates in California stone fruit orchards decreased from 84.6% in 1994 to 25.4% in 2002.

The occurrence of simultaneous mutations conferring resistance to other fungicide groups could imply fitness costs to the pathogen and possibly lead to a decrease in the frequency of LR field isolates. Reduced sensitivity and/or single-resistance to QoIs and DMIs, the main groups for brown rot control, was already observed in *M. fructicola* isolates from Brazilian orchards (Dutra et al., 2020; Lichtemberg et al., 2017; Pereira et al., 2017). In addition to other possible genetic mutations, genetic drift and reduced selection pressure by thiophanate-methyl applications needs to be considered to understand why the LR phenotype has decreased in the most recent *M. fructicola* population in Brazil. Lichtemberg et al. (2019) showed that the G461S mutation, related to DMI resistance, conferred fitness costs in *M. fructicola* isolates from Brazil, based on *in vitro* assays of mycelial growth rate, sporulation and germination; and aggressiveness in detached peach fruit. Dual resistance to MBC and DMI fungicides has previously been reported in *M. fructicola* isolates from the Eastern United States (Chen et al., 2013) and Spain (Egüen et al., 2015). Thus, it is possible that some isolates from our study have multiple resistance to another fungicide chemical group that may lead to a reduction in fitness components. Further studies are being performed to investigate this possibility.

The HR and LR isolates presented the lowest IP and LP and a greater incidence and diameter lesion in two peach cultivars treated with TM. Our results from fruit assays agree with studies conducted by Yoshimura et al. (2004) and Chen et al. (2013), who showed that the full rate of MBC fungicides effectively controlled the disease caused by S isolates, but not the disease caused by LR and HR of *M. fructicola* isolates. Interestingly, a higher dose (twofold the label dose) of the commercial formulation of thiophanate-methyl raised the control efficacy of LR isolates, decreasing the incidence of brown rot in peach fruits inoculated by LR isolates (Ke et al., 2023).

In the last decade, MBC fungicides, especially thiophanate-methyl, are being suspended and reintroduced in some fruits, including stone fruit in Brazil (AGROFIT, 2003). Furthermore, only 18 active ingredients registered in the Brazilian national pesticide guide website are authorized against brown rot of peach (AGROFIT, 2003). With reports of *M. fructicola* resistant or with reduced sensitivity to the site-specific

fungicide groups (May De Mio et al., 2011; Pereira et al., 2017; Lichtemberg et al., 2017), the chemical management of the disease has become more difficult and restrictive in Brazil. Although DMI-resistant isolates are present in Brazil, this fungicide is still a key component in brown rot management strategies because the discontinuous use of this fungicide group for 3 seasons was sufficient to reestablish *M. fructicola* sensitive populations (Pereira et al., 2020). The dicarboximide fungicide iprodione is another option to optimize the control of brown rot in Brazil since the *M. fructicola* population is sensitive to this fungicide (Dutra et al., 2019; Moreira & May-De Mio, 2009).

Monitoring low levels of MBC resistance in *M. fructicola* remains important because LR isolates are as aggressive as S isolates, and can cause higher disease incidence on thiophanate-methyl-treated fruits. However, considering that isolates with reduced sensitivity to other fungicides (for example, DMIs) may suffer fitness penalties and multiple resistance in the same isolate is possible, the reintroduction of TM may be considered since the frequency of these isolates has decreased over the past years. Nevertheless, a restricted number of applications, and resistance management programs interspersing fungicides from different chemical groups, should be recommended.

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Authors contributions All authors have been personally involved in this study. Juliana Marta Muehlmann Fischer: Conceptualization, Methodology, Writing—Original Draft Pamela Suellen Salvador Dutra: Data curation, Writing—Original draft preparation, software, Formal analysis. Hannah Ebbinghaus de Araujo: Visualization, Investigation. Chirlei Glienke: Supervision, Writing- Reviewing and Editing. Louise Larissa May De Mio: Validation, Writing- Reviewing and Editing, Project administration, Funding acquisition.

Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interest All authors declare that there is no conflict of interest.

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