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Screening for DMI and MBC Fungicide Resistance in Monilinia fructicola and Evaluation of Biorational Products for Control of Brown Rot on Peach in the Southeast United States

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SCREENING FOR DMI AND MBC FUNGICIDE RESISTANCE IN *MONILINIA FRUCTICOLA* AND EVALUATION OF BIORATIONAL PRODUCTS FOR CONTROL OF BROWN ROT ON PEACH IN THE SOUTHEAST UNITED STATES

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by
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ABSTRACT

Monilinia fructicola (G. Winter) Honey is a fungal pathogen and is the causal agent of blossom blight, twig blight, green fruit rot, preharvest brown rot, and postharvest brown rot of peach (*Prunus persica* (L.) Batsch). Especially pre-and postharvest brown rot can have devastating economic impacts and negatively effects yield for peach growers throughout the southeastern United States. The most effective method for the control of pre- and postharvest brown rot is the application of synthetic fungicides during preharvest season. However, the consecutive use of fungicides with the same mode of action potentially give rise to resistance. This thesis focuses on the current resistance status of *M. fructicola* to methyl benzimidazole carbamate (MBC) and demethylation inhibitor (DMI) fungicides and the evaluation of polyoxin-D for brown rot control. Chapter 1 provides a snapshot of resistance to DMI fungicides for *M. fructicola* isolates collected between 2021 and 2022 from Alabama, Georgia, and South Carolina while also examining the mechanisms of resistance, namely the presence or absence of the genetic element Mona upstream Mf*CYP51*. Chapter 2 examines the same set of *M. fructicola* isolates in Chapter 1 for resistance to MBC fungicides and describes nucleotide sequence analysis of the *Tub2* gene in sensitive, low resistant and resistant isolates. Chapter 3 focuses on the preharvest application of polyoxin-D as a solo product or in combination with thyme oil or mineral oil for the control of brown rot.

DEDICATION

To my mother, father, siblings, and all those I consider to be family for encouraging me, for supporting me on a journey that was different from the life I had lived, for showing enthusiasm for the work I was, am, and will be doing for the future, and for being there when times were hard and my path was clouded.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

RE-EVALUATION OF SENSITIVITY OF MONILINIA FRUCTICOLA ISOLATES TO THE DMI FUNGICIDE PROPICONAZOLE IN THE SOUTHEASTERN UNITED STATES AND INVESTIGATION OF THE GENETIC ELEMENT MONA

ABSTRACT

Sterol demethylation inhibitor (DMI) fungicides continue to be essential components for the control of brown rot of peach caused by *Monilinia fructicola* in the United States and worldwide. In the southeastern United States, resistance to DMIs had been associated with overexpression of the cytochrome P450 14α-demethylase gene Mf*CYP51* as well as the genetic element Mona, a 65 bp in length nucleotide sequence located upstream of Mf*CYP51* in resistant isolates. About 20 years after the first survey, we re-evaluated sensitivity of *M. fructicola* from South Carolina and Georgia to propiconazole and also evaluated isolates from Alabama for the first time. A total of 238 *M. fructicola* isolates were collected from various commercial and two experimental orchards and sensitivity to propiconazole was determined based on a discriminatory dose of 0.3 µg/ml. Results indicated 16.2%, 89.2% and 72.4% of isolates from Alabama, Georgia, and South Carolina, respectively, were resistant to propiconazole. The detection of resistance in Alabama is the first report for the state. All resistant isolates contained Mona, but it was absent from most sensitive isolates. It was unclear if the resistance frequency had increased in South Carolina and Georgia. However, the resistance levels (as assessed by the isolate frequency in discriminatory dose-based relative growth categories) did not change notably, and no evidence of other resistance genotypes was found. Analysis of the upstream Mf*CYP51* gene region in the

resistant isolate CF010 revealed an insertion sequence described for the first time in this report. Our study suggests that current fungicide spray programs have been effective against increasing resistance levels in populations of *M. fructicola* and suppressing development of new resistant genotypes of the pathogen.

INTRODUCTION

Brown rot, caused by the fungal pathogen *Monilinia fructicola* (G. Winter) Honey, is a disease affecting stone fruits worldwide. Symptoms include brown lesions that expand to cause complete decay of fruit and mummy formation alongside signs of tan to grayish sporodochia observed on rotting tissue (Schnabel and Brannen 2022). Without proper disease management, especially in years and locations with extensive rainfall, rotted fruit occur in peach orchards at alarming rates and most, if not all fruit, can be rendered unmarketable. Other stages of disease associated with *M. fructicola* include blossom blight, twig canker, and green fruit rot. As orchards start to bloom, sexual ascospores are released from apothecia formed on mummies on orchard floors and asexual conidia emerge from various infected tissues such as twig cankers, fruit mummies left on the tree, and/or from neighboring wild plum and commercial host plants (Zehr 1982). These spores will infect floral tissues causing blossom blight (Rosenberger 1983; Schalagbauer and Holz 1990). Continued colonization of flowers and associated formation of twig cankers will lead to the production of additional conidia that will become secondary sources of inoculum for green and mature fruit infection (Landgraf and Zehr 1982). These infections will produce brown rot symptoms typically observed 2 to 3 weeks preharvest and postharvest.

Chemical control of blossom blight, green fruit rot, and preharvest brown rot has been essential for commercial fruit production in the southeastern United States. Demethylation

inhibitor (DMI) fungicides have been utilized since the 1980s for effective brown rot management in the Southeast. DMIs are categorized by the Fungicide Resistance Action Committee (FRAC) as group 3 of the class I sterol biosynthesis inhibitors and are divided into the chemical groups triazoles, imidazoles, pyrimidines, triazolinthiones, pyridines and piperazines (FRAC 2022). Of these groups, mainly members of the triazoles are registered in the U.S. for use in stone fruits, including difenoconazole, fenbuconazole, mefentrifluconazole, propiconazole, and tebuconazole (Blauuw et al. 2023; Adaskaveg et al. 2022). Triazoles increased in popularity as fungicides in the FRAC 1 methyl benzimidazole carbamate (MBC) class failed to control brown rot effectively, due to widespread resistance development (Zehr et al. 1991). Efficacy of DMIs is reliant on the chemical binding to the cytochrome P450 14α-demethylase enzyme inhibiting ergosterol synthesis, a key molecule required for proper fungal cell membrane integrity (Kwok and Loeffler, 1993; Parks and Casey, 1995). This specific mode of action makes DMIs less toxic to non-target organisms and more effective compared to multisite fungicides such as captan, but it also makes them more vulnerable to resistance development.

DMI resistance in *M. fructicola* has been observed in peach orchards globally. More than 20 years after the introduction of DMIs into commercial spray programs, *M. fructicola* strains with decreased sensitivity to propiconazole were first described in South Carolina experimental orchards (Zehr et al. 1999). In this study, decreased sensitivity occurred through exposing peach orchards to 29 applications of propiconazole over a 3-year time frame (Zehr et al. 1999). However, in commercial orchards propiconazole resistance would not be reported for another five years. The first case of documented DMI resistance due to in-field control failure was reported in Georgia (Schnabel et al. 2004). Other occurrences of DMI resistance continued to surface across the eastern U.S., specifically in Maryland, Michigan, New Jersey, New York, Ohio, Pennsylvania, and South

Carolina (Chen et al. 2013b; Luo et al. 2008; Parker et al. 2006; Lesniak et al. 2021; Burnett et al. 2010). Outside of the U.S., Brazil and Spain have conducted surveys and published first reports of *M. fructicola* resistance to DMIs in peach producing orchards (May De-Mio et al. 2011; Egüen et al. 2015).

Overexpression of *CYP51*, the gene responsible for encoding the cytochrome P450 14α-demethylase enzyme, is a primary resistance determinant in South Carolina and Georgia and is caused by the genetic element Mona (Luo et al. 2008). However, various isolates from New York and Michigan orchards have shown reduced sensitivity to propiconazole and fenbuconazole even when the Mona element is absent (Lesniak et al. 2021; Villani and Cox 2011), and isolates from Pennsylvania and Maryland did not have Mona but were still resistant to propiconazole (Chen et al. 2013b). These findings suggest that other potential mechanisms of resistance exist. Point mutations in *CYP51* have been identified to be the main contributor to resistance in other countries. In Brazil, the mutation G461S conferred resistance to tebuconazole (Lichtemberg et al. 2017), and lab mutants generated in China produced the amino acid change Y136F that yielded increased resistance to propiconazole (Chen et al. 2012). However, fitness penalties observed for the lab mutant alluded as to why this mutation may not be more prevalent in stone fruit orchards (Chen et al. 2012). Reduced sensitivity to DMIs may also be attributed to energy-dependent drug efflux pumps and have been previously described in other plant pathogens (Leroux and Walker 2013; Nakaune et al. 1998), but the efflux transporter Mf*ABC1*, a major efflux transport in *M. fructicola*, contributed little in overall DMI resistance (Luo and Schnabel 2008).

In response to DMI resistance in *M. fructicola* isolates collected prior to 2004 in the southeastern U.S., a region-wide resistance management strategy was implemented. This included a more tactical use of DMI fungicides and a reduction in the number of applications per season to

conventional orchards (Schnabel and Brannen 2022). The goal of this study was to conduct a follow-up survey and assess the impact of the implemented changes on the occurrence of DMI fungicide resistance and potential changes in both resistance levels and mechanisms. Specific objectives of this study were to (i) determine sensitivity of *M. fructicola* collected from three southeastern states (Alabama, Georgia, and South Carolina) to the DMI fungicide propiconazole using an in vitro discriminatory dose assay, (ii) screen for the presence or absence of Mona on isolates of varying sensitivity phenotypes, and (iii) compare relative growth values on 0.3 µg/ml propiconazole of current *M. fructicola* isolates to values published previously.

MATERIALS AND METHODS

Isolate collection of M. fructicola. A total of 238 *M. fructicola* isolates were collected from various counties around Alabama (AL, n = 62), Georgia (GA, n = 65), and South Carolina (SC, n = 111) in 2021 and 2022 (Supplementary Table). All isolates were collected from conventional farms except for 17 isolates that originated from research orchards (GA 1, GA 3, GA 6 to 9, and MRF isolates) and 14 isolates that originated from a commercial organic farm (WaF isolates). Two historical isolates, SCCC.02 and GADL_193.04, were used as reference isolates (Luo and Schnabel 2008, Schnabel et al. 2004). Isolates were obtained from commercially mature and symptomatic peach fruit. Individually wrapped sterile cotton swabs were used to collect spores from fruit in the tree or on the ground, moved to the laboratory, and stored in zip lock bags with desiccant at 3°C until needed. Single spore colonies were generated by tapping the swab over water agar (Bacto™ agar, Becton, Dickson and Company, Sparks, MD) to disperse spores along the Petri plate. After 12 h, four germinating spores were transferred to potato dextrose agar (PDA; Criterion, Hardy Diagnostics, Santa Monica, CA) plates and incubated at 25°C for 2 to 4 days. One of the

four spores was removed with a sterile scalpel and transferred to a PDA plate. For preservation, isolates were grown on PDA as described above with topical filter paper disks (5 x 5 mm in size), which upon colonization were dried and stored at -20°C with silica gel desiccant and indicator beads (Thermo Scientific, Rockford, IL).

***In vitro discriminatory dose assay for determining sensitivity to propiconazole*.** To determine sensitivity to the DMI fungicide propiconazole (PROPI-STAR® EC, Albaugh LLC, Ankeny, IA), a discriminatory dose assay (Luo et al. 2008) was used to identify sensitive and resistant phenotypes. Isolates were grown on PDA for three to five days at 25°C and four 5 mm in diameter agar plugs were collected from the periphery of actively growing cultures. For each isolate, two plugs were transferred to a single 90 mm Petri dish containing PDA amended with 0.3 µg/ml propiconazole (each plug placed was placed equidistantly from the plate center and edge), and two plugs were transferred to a non-amended PDA plate, all maintained at 25°C. The discriminatory dose of 0.3 µg/ml propiconazole was chosen based on previous studies (Zehr et al. 1999; Cox et al. 2007). When mycelia of two plugs almost touched or after five days of incubation (whichever came first), mycelial growth of an isolate was determined by calculating the average diameter of each of the plugs (each measured twice crosswise using a digital caliper) for each isolate grown on amended and non-amended medium. Isolates were considered resistant to propiconazole if the relative growth (mycelial growth on fungicide-amended plate x 100% / mycelial growth on unamended plate) was greater than or equal to 20% (Luo et al. 2008). This assay was repeated once with 40 randomly selected sensitive and resistant isolates of roughly equal proportion and a Pearson correlation coefficient of $r = 0.925$ confirmed low variability and consistency of the relative growth rate determination for each isolate.

***Extraction of M. fructicola DNA*.** Isolates were grown on sterile cellophane atop PDA for four to six days at 25°C and DNA was extracted as previously described (Chi et al. 2009). Approximately 10 mm^2 of mycelial tissue was transferred from the cellophane with a sterile scalpel to a 1.5 ml microcentrifuge tube containing 0.5 ml extraction buffer (1M KCl, 100 mM Tris-HCl, 10mM EDTA). Tissue was pulverized for 1 to 2 s using an electric grinder (Dremel®, Racine, WA) with a pestle tip followed by centrifuging for 10 min at 5,000 rpm. The pestle tip was decontaminated by running the Dremel in 70% ethanol followed by a sterile water rinse for 2 s. In a few instances, tissue of some isolates was grinded with a pipette tip for 40 s instead . Centrifuged supernatant was decanted to a 1.5 ml microcentrifuge tube containing 0.3 ml of 2-propanol and centrifuged for 10 min at 12,000 rpm. Supernatant was discarded and tubes were left to dry for 5 to 12 h to let any remaining 2-isopropanol evaporate. The DNA pellet was resuspended in 50 µl sterile deionized water.

***Detection and sequence analysis of the Mona element and various insertions upstream MfCYP51*.** Polymerase chain reaction (PCR) was applied to amplified DNA from *M. fructicola* isolates to detect the presence or absence of the genetic element Mona located upstream Mf*CYP51* (Luo et al. 2008). Primer pair *INS65-F* and *INS65-R* (Luo et al. 2008) amplified either a 376 bp (Mona present) or a 311 bp fragment (Mona absent) depending on genotype. PCR reaction mixture consisted of 1 µl extracted DNA, 1 µl each of *INS65-F* and *INS65-R* primers (10 µM), 5 µl Accupower® HotStart PCR Premix (Bioneer, Oakland, CA) and 17 µl of deionized water for a 25 µl reaction volume. Amplification and PCR protocols were as previously published (Luo et al. 2008). PCR products were separated on 2.0% agarose (Fisher Scientific) gel in 1 x TAE buffer at 90 V for 45 min. Gels were imaged on a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories Inc., Hercules, CA). PCR products from isolates CF010 and MD147 were purified using the DNA

Clean & Concentrator™-5 purification kit (Zymo Research Corporation, Irvine, CA) and samples were sequenced by Eurofins Scientific for further investigation of insertion sequences upstream Mf*CYP51*. Analysis of sequence was performed on DNASTAR Version 16.0.0 (DNASTAR, Inc, Madison, Wisconsin).

RESULTS

***In vitro sensitivity of M. fructicola isolates to propiconazole*.** Of the 238 isolates collected from experimental farms, organic farms, and conventional farms subjected to the discriminatory dose of 0.3 µg/ml propiconazole, 16.2%, 72.4%, and 89.2% of Alabama, South Carolina, and Georgia isolates were considered resistant to propiconazole, respectively (Fig 1A). For conventional farms only, the percent isolates collected from Alabama, South Carolina, and Georgia and resistant to propiconazole was 16.2%, 88.7% and 88.1%, respectively (Fig 1B). Relative growth (RG) values at 0.3 µg/ml propiconazole ranged from 0 to 90.0% for individual isolates tested (Table 1). Highest average and median RG values were found in Peach County (53.2 and 43.4, respectively), Georgia and Spartanburg County (48.7 and 41.3, respectively), South Carolina. The lowest average and median RG values were found in counties in Alabama (ranging from 2.7 to 15.1 and 0 to 7.3, respectively) and Oconee County (0 and 3.7, respectively), South Carolina. A comparison of relative growth values published by Luo et al. (2008) and this study revealed similar grouping in relative growth categories. Most isolates were in the 20 to 29.9%, 30 to 39.9%, and 40 to 49.9% categories. Only about 10% of isolates from both studies were in relative growth categories 60 to 89.9 (Fig. 2). Analysis of variance confirmed no statistical difference between relative growth values ($P = 0.1216$).

***Detection of the Mona element in M. fructicola isolates*.** Primer set INS65-F and INS65-R amplified a 376 bp fragment from all resistant isolates (n = 148) with one exception. Isolate CF010 was also considered resistant to propiconazole based on 45.6% RG at a discriminatory dose of 0.3 mg/ml propiconazole but revealed an 834 bp amplicon (Fig. 3D; Table 2). Further sequencing analysis revealed a 448 bp insertion, Insert S, located 307 bp upstream Mf*CYP51*. Investigation of the previously published isolate MD147 (Chen et al. 2013a) also uncovered a 785 bp insertion located 304 bp upstream Mf*CYP51*. This isolate was further investigated as the molecular basis of the insertion sequence found upstream MF*CYP51* had not been elaborated on in the past. Most isolates considered sensitive to propiconazole (85 out of 90 total) revealed a 311 bp fragment. DNA from the 5 remaining sensitive isolates yielded the 376 bp fragment containing Mona (Table 2). These isolates had RG values ranging from 0 to 17.7.

DISCUSSION

In response to the detection of *M. fructicola* isolates from Georgia resistant to propiconazole in 2004 (Schnabel et al. 2004), a regionwide resistance management program was implemented. Prior to 2004, spraying DMI fungicides during bloom for the control of blossom blight and for preharvest brown rot control was common among peach farmers in South Carolina and Georgia and often included two spray applications of propiconazole at 14 and 7 days preharvest (Brannen et al. 2006; Schnabel et al. 2004). Recommendations were set in place to substitute DMIs in bloom sprays with anilinopyrimidines or dicarboxamides, to use DMIs only once during the preharvest timeframe, and to increase the dose rate of DMIs in orchards with documented or suspected reduced sensitivity (Blaauw et al. 2023; Schnabel and Brannen 2022). The current regional spray guide for the Southeast recommends DMIs to be sprayed only once for

brown rot control as a preharvest application (Blaauw et al. 2023) in strategic alternation with other effective fungicides of different FRAC codes (Schnabel and Brannen 2022). Only if anthracnose caused by *Colletotrichum* spp. is a problem, which is rather rare in southeastern orchards, propiconazole in combination with difenoconazole is recommended to be used in cover sprays prior to the preharvest fungicide applications (Blaauw et al. 2023).

In this study, we determined that resistance in *M. fructicola* to DMI fungicides remains prevalent in South Carolina and Georgia with no obvious change of RG values or additional resistance mechanisms. All conventional orchards surveyed in this study from South Carolina and Georgia exhibited isolates resistant to propiconazole. This is in contrast with a previous study showing three out of six conventional farms in South Carolina exhibiting no resistant isolates (Chen et al. 2013b). This either indicates an increase of resistance occurring in South Carolina farms or simply a result of the limited number of locations included in the Chen et al. (2013b) study. Across all resistant isolates of *M. fructicola* from conventional farms in this study, RG values remained in similar relative growth categories with those reported previously in South Carolina and Georgia (Luo et al., 2008) suggesting no significant shift in the resistance level. MRF isolates from an experimental farm in South Carolina did not exhibit resistance despite previous reports of reduced sensitivity at that location (Zehr et al. 1999). At this farm, strict resistance management guidelines had been implemented in the year 2000. In contrast, GA1-GA9 isolates from Peach County, GA, all of which we confirmed were resistant, were collected from another research farm with a long history of documented DMI resistance (Schnabel et al. 2004; Brannen et al. 2006). With few exceptions, WaF isolates from an organic farm in South Carolina were sensitive to propiconazole. This farm transitioned from conventional to organic production in 2005, and since then had not sprayed DMI or any other conventional fungicides. The isolates with

resistance to propiconazole may either have survived without selection pressure or may have been wind disseminated from a conventional orchard less than 200 meters away (Lichtemberg et al. 2021).

This study represents the first survey of DMI fungicide resistance in *M. fructicola* from Alabama orchards. There were over 293 farms with over 728 hectares of peaches in the state in 2017 (NASS USDA. 2022; NASS USDA. 2017). With such a significant production of peaches, our interest in determining the resistance profile for the state was evident. Fungicide resistance management has been promoted in Alabama since the mid 1990's and most growers follow the same basic fungicide spray schedule outlined for growers in Georgia and South Carolina.

In general, *M. fructicola* populations in Alabama were sensitive to propiconazole with resistance only occurring in 16.2% of isolates collected. This is a 4.5-fold decrease in the number of resistant isolates compared to South Carolina and Georgia. This may be partially explained by the location of the farms used for the survey in Alabama as there was a significant difference in sensitivity to isolates collected from Geneva and Mobile counties as compared to those collected from Chilton and Barbour counties. The fruit from Geneva and Mobile counties were collected from relatively small, well managed farms in the southeast (Geneva) and southwest (Mobile) corners of Alabama. These farms are geographically isolated from any other peach orchard by a minimum of 50 miles. This distance likely prevented the introduction of a DMI-resistant *M. fructicola* isolates from a neighboring orchard and may account for the relatively low percentage of resistant isolates detected at these sites. The Chilton County area produces the majority of peaches in Alabama with production dating back to the early 1900's. Growers in the Chilton County region follow the spray recommendations outlined, and updated annually, in the Southeastern Peach, Nectarine, and Plum Pest Management and Culture Guide (Blaauw et al.

2023) which is also used by growers in South Carolina and Georgia. Results from the two survey sites from Chilton County are similar to those from Barbour County, Alabama, both with higher percentages of DMI-resistant *M. fructicola* isolates compared to the Geneva and Mobile County locations. Though fruit from Barbour County were also collected from a relatively small and isolated farm, such as those in Geneva and Mobile counties, it was known that this orchard was poorly managed for long periods of time over the last 40 years suggesting DMI resistance may have developed as in other production areas described in this study (Edward Sikora, personal communication).

This relatively low level of DMI resistance observed in Alabama may also be explained due to the more aggressive chemical approach by peach farmers in South Carolina and Georgia, where control of brown rot during both pre and postharvest periods is vital for farms shipping fruit out of state. This would include up to four preharvest applications of site-specific fungicides, sometimes including two DMI sprays, to protect fruit during the preharvest window when weather conditions often turn more suitable for disease development, and harvest can stretch out over two to three weeks. This approach may be justified in South Carolina and Georgia due to the existence of a zero-tolerance limit of rot in shipped fruit. Under those circumstances an increase in selection pressure for resistance is expected (Zehr et al. 1999; Schnabel et al. 2004). This zero-tolerance limit is typically not a factor in Alabama since most fruit are sold at local, in-state markets.

M. fructicola resistance to the DMI fungicide propiconazole has been examined in orchards outside the southeastern United States. Consistent with our findings, isolates from Michigan had comparable resistance distribution frequencies with 80.7% of isolates being resistance at 0.3 µg/ml propiconazole using 30% RG as a threshold (Lesniak et al. 2021). In Brazil only 7.7% of isolates displayed resistance at a discriminatory dose of 0.3 µg/ml propiconazole using 20% RG as a

threshold (Dutra et al. 2020). This apparent discrepancy may be explained because propiconazole had not been registered or used for disease management in stone fruits. However, another triazole, tebuconazole, had been used routinely in Brazil for over two decades and resistance frequencies for this fungicide were substantially higher based on the same discriminatory dose and RG assessments used for propiconazole. This suggests specific selection of reduced sensitivity to certain DMI fungicides based on spray history. A study from 2011 reported only 12% of New York isolates were resistant based on a discriminatory dose of 0.9 µg/ml propiconazole with 30% RG (Villani and Cox 2011). This dose was determined based on 100 times the historical baseline EC_{50} values for NY state established in 1992 (Villani and Cox 2011; Wilcox and Burr 1994). Due to the different choice of a discriminatory dose in the latter study, it is difficult to compare the New York results with those reported in this study.

The genetic element Mona remains a reliable indicator for resistance in the southeastern United States, but it is not suitable for this usage in other parts of the world. A direct association between the presence of Mona, overexpression of C*YP51* gene, and resistance to propiconazole was reported previously in isolates from South Carolina and Georgia, as resistance in these states has always correlated with the overexpression of *CYP51* and the presence of Mona (Luo et al. 2008; Chen et al. 2013a; Chen et al. 2013b). In this study, all resistant isolates and five sensitive isolates (BS004, DB008, TF2009, GA51, and AL19) possessed Mona. Except for AL19, RG values for each isolate were within 5% of the threshold (20%) for being resistant (Table S1). Isolates may lose resistance to propiconazole in longer-term cold storage and/or continual transfer of cultures on artificial medium (Cox et al. 2007; Zhu et al. 2011) offering explanation to why some of our isolates are sensitive to propiconazole but still possess the Mona element. Further investigation on exactly why sensitivity increases due to cold storage or consecutive transfers is

needed though methylation of Mona transcriptional factors may be a possible explanation for the phenomenon (Zhu et al. 2011) Our results are consistent with some observations reported in a previous study of isolates published previously from New York (Villani et al. 2011). While resistance to DMI fungicides was largely linked to Mona, some sensitive isolates also possessed the genetic element upstream Mf*CYP51*. A strong correlation between DMI fungicide resistance and the presence of Mona was also reported in isolates from New Jersey and Ohio (Luo et al. 2008; Burnett et al. 2010). Other studies report no link between the presence or absence of Mona in isolates from Michigan, Pennsylvania, and Maryland (Lesniak et al. 2021; Chen et al. 2013b). Moreover, relative expression of the Mf*CYP51* gene in isolates resistant to DMIs was not elevated, suggesting a different mechanism of resistance (Lesniak et al. 2021; Chen et al. 2013b). In some isolates, the expression of Mf*CYP51* was not elevated even in isolates containing Mona (Lesniak et al. 2021). In Spain, the mechanism of resistance is currently unknown, though it is suspected to be caused by a mutation (Egüen et al. 2015), while in Brazil resistance to the DMIs was caused by the G461S mutation (Lichtemberg et al. 2017). These studies show that Mona is not a reliable marker for resistance to DMI fungicides outside the southeastern United States and that mechanisms other than target gene overexpression or alteration exist.

The presence of Mona in all resistant isolates indicates that overexpression of the Mf*CYP51* gene still is the prevailing resistance mechanism in *M. fructicola* isolates from the southeastern United States. The association between Mona and overexpression has clearly been established in southeastern US isolates previously (Luo and Schnabel 2008). Overexpression of Mf*CYP51* due to Mona has also been shown to trigger a quantitative rather than a qualitative response towards reduced sensitivity to DMI fungicides in *M. fructicola* populations (Villani and Cox 2011, Luo et al. 2008). This mechanism of resistance can be overcome with a higher dose rate. A previous study

conducted on peach trees with documented resistance to DMI fungicides showed that two preharvest applications of Indar 75WSP (a.i. fenbuconazole) applied at 280.21 g/ha was significantly more effective compared to Indar 75WSP applied at 140.11 g/ha (Brannen et al. 2007). Consequently, the use of higher rates of DMI fungicides are still recommended in orchards with suspected resistance to DMI fungicides (Blaauw et al. 2023). Not only is increasing the dose rate effective at controlling resistant populations, DMIs with higher intrinsic activity than propiconazole, such as fenbuconazole and tebuconazole, have been shown to significantly increase control of *M. fructicola* (Holb and Schnabel 2007).

Besides the presence or absence of Mona, other nucleotide sequence variability in the upstream region has been reported in *M. fructicola* isolates from Michigan, New York, and South Carolina (Lesniak et al. 2021; Luo et al. 2008). In this study we have located a 448 bp insertion, designated Insert S, upstream Mf*CYP51* in the isolate CF010 using the primer pair *INS65-F* and *INS65-R*. This isolate also contains the Mona element, but there is no direct evidence this insertion aids to increase resistance to propiconazole. Interestingly, a 148 bp section of this insertion shares 92% sequence homology to a section found in propiconazole-sensitive New York isolates (Luo et al. 2008) containing a 1,508 bp insertion (Fig. 3E). Another resistant isolate collected in 2012 from South Carolina revealed an approximately 1,200 bp insertion with Mona present (Chen et al. 2013a). Re-investigation of this isolate in this study revealed the isolate to have a 1,161 bp amplicon with a 785 bp insertion. Again, there was no direct evidence that this insertion was beneficial in conferring resistance. Isolates from Michigan had a 171 bp insertion sequence (Insert A) in place of where the Mona element would be found (Fig. 3C), and no correlation to DMI resistance was reported (Lesniak et al. 2021). Additionally, the 601 bp insertion that was also found in Michigan isolates did not contain the Mona element despite being located in the same upstream

region (Lesniak et al. 2021) It is possible that insertions occur from transposable elements, and these are common in fungi and their movement can be triggered by fungicide exposure. For example, transposon *Mftc1* was inserted upstream of Mf*CYP51* as a result of exposure to high doses of mixed QoI and DMI fungicides (Chen et al. 2015).

It is not clear from this study if the frequency of resistant strains in South Carolina or Georgia has increased since the last survey, but this study does show that the resistance levels based on RG values at 0.3 ug/ml propiconazole have not increased and that no new genotype with resistance to propiconazole has emerged. We contribute this success of resistance management to the strategic, frugal use of DMI fungicides in commercial peach orchards. If current resistance management practices are maintained, DMI fungicides may continue to be a useful tool for brown rot management in southeastern peach orchards.

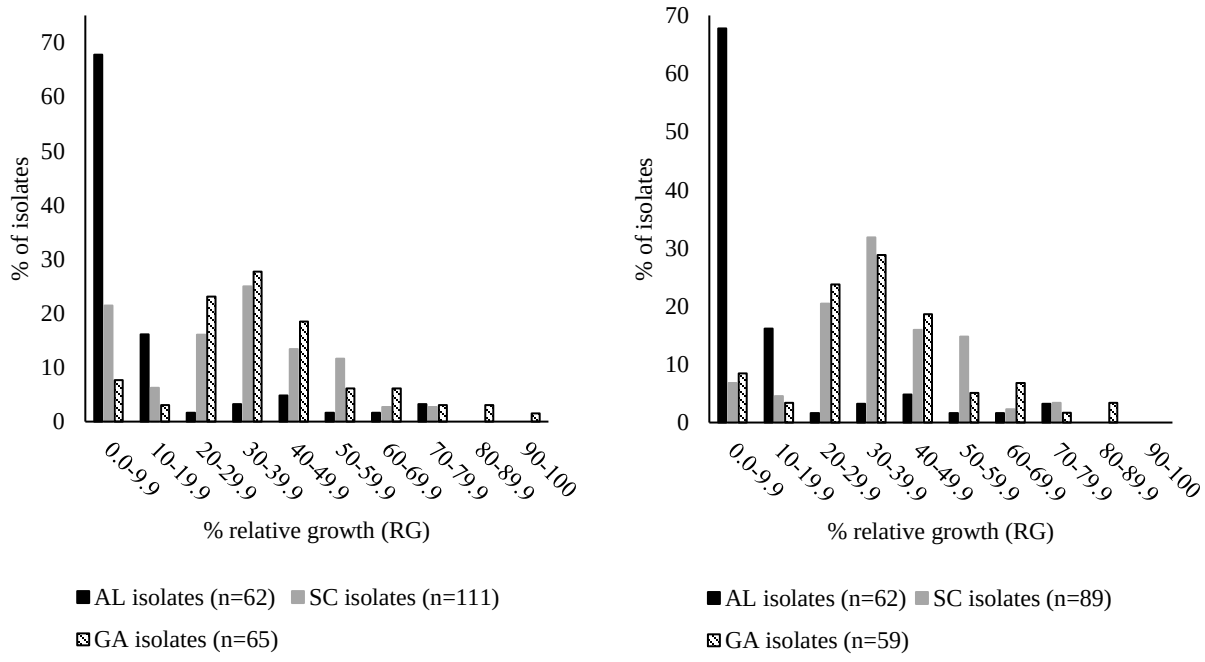

Figure 1.1 Distribution of relative growth (RG) values obtained on 0.3 µg/ml propiconazole for 238 single spore isolates of *Monilinia fructicola* collected from (A) conventional and

organic/research orchards and (B) only conventional orchards in Alabama (AL), South Carolina (SC), and Georgia (GA).

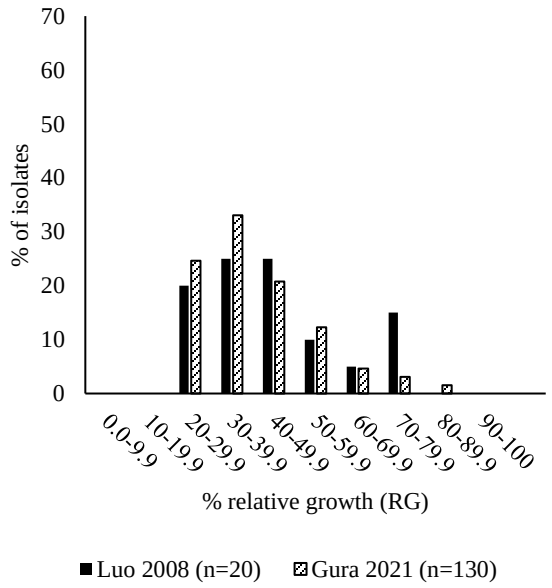

Figure 1.2 Distribution of relative growth (RG) values for 0.3 µg/ml propiconazole for 20 Mona (+) historic isolates (Luo et al. 2008) and 130 Mona (+) isolates collected in 2021 and 2022 for this study. All isolates were collected from conventional orchards in South Carolina and Georgia.

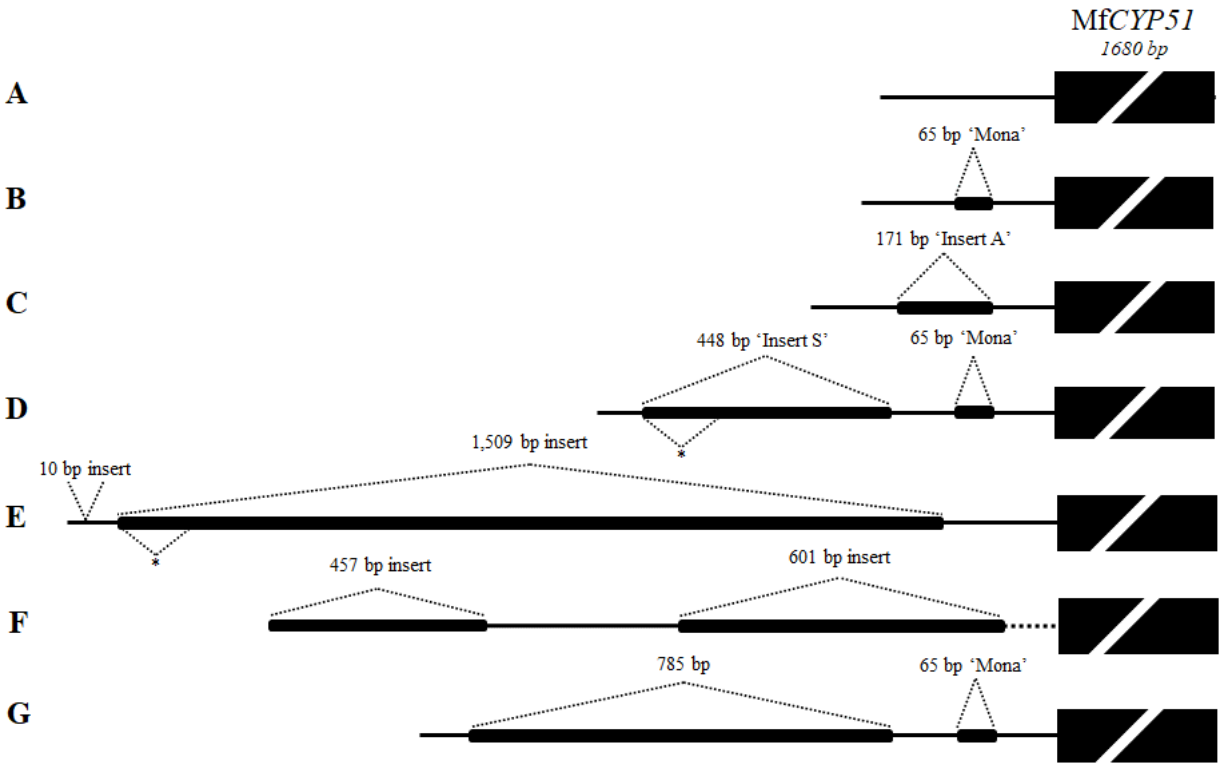

Figure 1.3 Model representation of the 14α-demethylase gene Mf*CYP51* upstream region in *Monilinia fructicola* isolates based on amplicons obtained from primers *INS65-F* and *INS65-R*. Amplicon sizes included: 311 bp (**A:** Partial sequence of GenBank accession number EU035306, Luo and Schnabel (2008)), 376 bp (**B:** Partial sequence of GenBank number accession EU035301, Luo and Schnabel (2008)), 451 bp (**C:** GenBank accession number MT739523, Lesniak et al. (2021)), 834 bp (**D**), 1,815 bp (**E:** GenBank accession number EU257287, Luo et al. (2008)), 1,381 bp (**F:** GenBank accession number MT739522, Lesniak et al. (2021)), and 1,161 bp (**G**). Black bars represent insertions in the upstream region. Asterisks (*) indicate sequence homology. The dotted line indicated an unknown distance from the beginning of the MF*CYP51* gene.

Table 1.1 Origin, in vitro sensitivity to propiconazole, and detection of 'Mona' of *Monilinia fructicola* isolates collected from South Carolina (SC), Alabama (AL), and Georgia (GA)

Origin		Year of	Isolates	Relative growth (%)[b]		
State	County[a]	isolation	(n)	Range	Average	Median
SC	Edgefield	2021	30	0.0-68.6	31.1	35
SC	Barnwell	2021	17	24.0-70.8	40.8	33.3
SC	Aiken/Saluda	2021	51	0.0-79.9	31.2	34.6
SC	Oconee	2021	10	0.0-13.3	3.7	0
SC	Spartanburg	2022	3	28.4-76.4	48.7	41.3
AL	Chilton	2021	19	0.0-71.1	15.0	7.3
AL	Barbour	2021	14	0.0-61.0	15.1	0
AL	Mobile	2021	18	0.0-79.7	8.8	0
AL	Geneva	2021	11	0-16.8	2.7	0
GA	Peach	2021/2022	7	23.0-90.0	53.2	43.4
GA	Taylor	2021/2022	33	0-87.6	36.6	37.2
GA	Crawford	2021/2022	25	0-66.3	35.0	33.4

[a] Multiple counties are listed if isolates came from orchards stretching over county lines.

[b] Percent relative growth on potato dextrose agar (PDA) amended or nonamended with 0.3 µg/ml propiconazole.

Table 1.2 Distribution of the Mona element in *Monilinia fructicola* isolates from South Carolina, Alabama, and Georgia

			M. fructicola genotype[a]		
State	Isolates (n)	Sensitivity phenotype[b]	Mona (+)	Mona (-)	PCR fragment sizes (bp) for Mona (+)/Mona(-)
SC	31	S	3	28	376/311
	79	R	79	0	376
	1	R	-	-	834

AL	52	S	1	51	376/311
	10	R	10	0	376
GA	7	S	1	6	376/311
	58	R	58	0	376

[a] *INS65-F* and *INS65-R* primers (Luo et al., 2008) were used to determine the presence (+) or absence (-) of 'Mona'.

[b] Isolates were considered resistant (R) to propiconazole when relative colony growth was ≥ 20 % at a discriminatory dose of 0.3 µg/ml propiconazole. All isolates with relative colony growth < 20% were considered sensitive (S).

Supplementary Table 1.3 Characteristics of *Monilinia fructicola* isolates from South Carolina (SC), Alabama (AL), and Georgia (GA) showing sensitivity to propiconazole in form of percent relative growth (RG), phenotypic designation, and presence or absence of the Mona element.

Isolate	**Origin (county, state)**	**Year of isolation**	**RG (%)[a]**	**Sensitivity phenotype[b]**	**PCR fragment size (bp)[c]**
BS001	Edgefield, SC	2021	0.0	**S**	311
BS002	Edgefield, SC	2021	35.1	**R**	376
BS003	Edgefield, SC	2021	68.6	**R**	376
BS004	Edgefield, SC	2021	17.7	**S**	376
BS005	Edgefield, SC	2021	50.4	**R**	376
BS006	Edgefield, SC	2021	29.3	**R**	376
BS007	Edgefield, SC	2021	0.0	**S**	311
BS008	Edgefield, SC	2021	27.0	**R**	376
BS009	Edgefield, SC	2021	27.8	**R**	376
BS010	Edgefield, SC	2021	30.3	**R**	376
BS011	Edgefield, SC	2021	34.9	**R**	376
BS012	Edgefield, SC	2021	24.8	**R**	376
CF001	Barnwell, SC	2021	56.9	**R**	376

CF002	Barnwell, SC	2021	29.9	**R**	376
CF003	Barnwell, SC	2021	40.7	**R**	376
CF004	Barnwell, SC	2021	62.7	**R**	376
CF005	Barnwell, SC	2021	54.9	**R**	376
CF006	Barnwell, SC	2021	29.1	**R**	376
CF007	Barnwell, SC	2021	70.8	**R**	376
CF008	Barnwell, SC	2021	35.4	**R**	376
CF009	Barnwell, SC	2021	54.4	**R**	376
CF010	Barnwell, SC	2021	45.6	**R**	834
CF011	Barnwell, SC	2021	26.7	**R**	376
CF012	Barnwell, SC	2021	30.5	**R**	376
CF013	Barnwell, SC	2021	33.3	**R**	376
CF014	Barnwell, SC	2021	32.3	**R**	376
CF015	Barnwell, SC	2021	33.1	**R**	376
CF016	Barnwell, SC	2021	24.0	**R**	376
CF017	Barnwell, SC	2021	32.6	**R**	376
DB001	Aiken, SC	2021	79.9	**R**	376
DB002	Aiken, SC	2021	41.9	**R**	376
DB003	Aiken, SC	2021	57.1	**R**	376
DB004	Aiken, SC	2021	48.9	**R**	376
DB005	Aiken, SC	2021	25.8	**R**	376
DB006	Aiken, SC	2021	36.3	**R**	376
DB007	Aiken, SC	2021	31.6	**R**	376
DB008	Aiken, SC	2021	17.0	**S**	376
DB009	Aiken, SC	2021	27.7	**R**	376
DB010	Aiken, SC	2021	54.7	**R**	376
DB011	Aiken, SC	2021	30.3	**R**	376
DB012	Aiken, SC	2021	34.2	**R**	376
DB013	Aiken, SC	2021	21.1	**R**	376
DB014	Aiken, SC	2021	52.5	**R**	376
DB016	Aiken, SC	2021	48.4	**R**	376

DB017	Aiken, SC	2021	48.0	**R**	376
DB018	Aiken, SC	2021	38.5	**R**	376
JC4	Spartanburg, SC	2022	76.4	**R**	376
JC5	Spartanburg, SC	2022	28.4	**R**	376
JC7	Spartanburg, SC	2022	41.3	**R**	376
TF001	Saluda, SC	2021	28.1	**R**	376
TF002	Saluda, SC	2021	35.3	**R**	376
TF003	Saluda, SC	2021	38.7	**R**	376
TF004	Saluda, SC	2021	38.9	**R**	376
TF005	Saluda, SC	2021	44.1	**R**	376
TF006	Saluda, SC	2021	54.8	**R**	376
TF007	Saluda, SC	2021	55.6	**R**	376
TF008	Saluda, SC	2021	53.9	**R**	376
TF009	Saluda, SC	2021	40.0	**R**	376
TF010	Saluda, SC	2021	36.5	**R**	376
TF2001	Saluda, SC	2021	49.3	**R**	376
TF2002	Saluda, SC	2021	24.3	**R**	376
TF2003	Saluda, SC	2021	39.9	**R**	376
TF2004	Saluda, SC	2021	57.7	**R**	376
TF2005	Saluda, SC	2021	34.6	**R**	376
TF2006	Saluda, SC	2021	28.9	**R**	376
TF2007	Saluda, SC	2021	33.1	**R**	376
TF2008	Saluda, SC	2021	53.4	**R**	376
TF2009	Saluda, SC	2021	15.1	**S**	376
TF2010	Saluda, SC	2021	0.0	**S**	311
TFJ1	Edgefield, SC	2022	21.5	**R**	376
TFJ2	Edgefield, SC	2022	0.0	**S**	311
TFJ3	Edgefield, SC	2022	39.0	**R**	376
TFJ4	Edgefield, SC	2022	45.9	**R**	376
TFJ5	Edgefield, SC	2022	0.0	**S**	311
TFJ6	Edgefield, SC	2022	31.3	**R**	376

TFJ7	Edgefield, SC	2022	47.0	**R**	376
TFJ8	Edgefield, SC	2022	35.5	**R**	376
TFJ10	Edgefield, SC	2022	0.0	**S**	311
TrF001	Edgefield, SC	2021	44.4	**R**	376
TrF002	Edgefield, SC	2021	38.4	**R**	376
TrF003	Edgefield, SC	2021	45.0	**R**	376
TrF004	Edgefield, SC	2021	37.6	**R**	376
TrF006	Edgefield, SC	2021	41.5	**R**	376
TrF007	Edgefield, SC	2021	25.3	**R**	376
TrF008	Edgefield, SC	2021	36.7	**R**	376
TrF009	Edgefield, SC	2021	40.0	**R**	376
TrF010	Edgefield, SC	2021	57.9	**R**	376
MRF001	Oconee, SC	2021	0.0	**S**	311
MRF002	Oconee, SC	2021	0.0	**S**	311
MRF003	Oconee, SC	2021	0.0	**S**	311
MRF004	Oconee, SC	2021	0.0	**S**	311
MRF005	Oconee, SC	2021	13.2	**S**	311
MRF006	Oconee, SC	2021	13.3	**S**	311
MRF007	Oconee, SC	2021	0.0	**S**	311
MRF008	Oconee, SC	2021	0.0	**S**	311
MRF009	Oconee, SC	2021	0.0	**S**	311
MRF010	Oconee, SC	2021	10.4	**S**	311
WaF001	Aiken/Saluda, SC	2021	0.0	**S**	311
WaF002	Aiken/Saluda, SC	2021	5.9	**S**	311
WaF003	Aiken/Saluda, SC	2021	15.1	**S**	311
WaF005	Aiken/Saluda, SC	2021	0.0	**S**	311

WaF006	Aiken/Saluda, SC	2021	67.3	**R**	376
WaF007	Aiken/Saluda, SC	2021	40.6	**R**	376
WaF009	Aiken/Saluda, SC	2021	0.0	**S**	311
WaF010	Aiken/Saluda, SC	2021	0.0	**S**	311
WaF011	Aiken/Saluda, SC	2021	0.0	**S**	311
WaF012	Aiken/Saluda, SC	2021	0.0	**S**	311
WaF014	Aiken/Saluda, SC	2021	0.0	**S**	311
WaF015	Aiken/Saluda, SC	2021	6.9	**S**	311
WaF016	Aiken/Saluda, SC	2021	0.0	**S**	311
WaF017	Aiken/Saluda, SC	2021	0.0	**S**	311
AL1	Chilton, AL	2021	8.6	**S**	311
AL2	Chilton, AL	2021	0.0	**S**	311
AL3	Chilton, AL	2021	18.7	**S**	311
AL4	Chilton, AL	2021	0.0	**S**	311
AL5	Chilton, AL	2021	0.0	**S**	311
AL6	Chilton, AL	2021	33.5	**R**	376
AL7	Chilton, AL	2021	11.9	**S**	311
AL8	Chilton, AL	2021	48.8	**R**	376
AL9	Chilton, AL	2021	13.8	**S**	311
AL10	Chilton, AL	2021	37.2	**R**	376
AL11	Chilton, AL	2021	29.8	**R**	376

AL13	Chilton, AL	2021	3.8	**S**	311
AL14	Chilton, AL	2021	0.0	**S**	311
AL15	Chilton, AL	2021	0.0	**S**	311
AL16	Chilton, AL	2021	0.0	**S**	311
AL17	Chilton, AL	2021	0.0	**S**	311
AL18	Chilton, AL	2021	7.3	**S**	311
AL19	Chilton, AL	2021	0.0	**S**	376
AL20	Chilton, AL	2021	71.7	**R**	376
AL22	Barbour, AL	2021	54.7	**R**	376
AL23	Barbour, AL	2021	0.0	**S**	311
AL25	Barbour, AL	2021	0.0	**S**	311
AL26	Barbour, AL	2021	44.4	**R**	376
AL27	Barbour, AL	2021	0.0	**S**	311
AL28	Barbour, AL	2021	46.7	**R**	376
AL29	Barbour, AL	2021	61.0	**R**	376
AL30	Barbour, AL	2021	0.0	**S**	311
AL31	Barbour, AL	2021	0.0	**S**	311
AL32	Barbour, AL	2021	4.0	**S**	311
AL33	Barbour, AL	2021	0.0	**S**	311
AL34	Barbour, AL	2021	0.0	**S**	311
AL35	Barbour, AL	2021	0.0	**S**	311
AL36	Barbour, AL	2021	0.0	**S**	311
AL37	Mobile, AL	2021	11.9	**S**	311
AL38	Mobile, AL	2021	8.0	**S**	311
AL39	Mobile, AL	2021	0.0	**S**	311
AL40	Mobile, AL	2021	14.3	**S**	311
AL41	Mobile, AL	2021	11.9	**S**	311
AL42	Mobile, AL	2021	9.4	**S**	311
AL43	Mobile, AL	2021	10.6	**S**	311
AL44	Mobile, AL	2021	0.0	**S**	311
AL45	Mobile, AL	2021	0.0	**S**	311

AL46	Mobile, AL	2021	12.0	**S**	311
AL47	Mobile, AL	2021	0.0	**S**	311
AL48	Mobile, AL	2021	0.0	**S**	311
AL49	Mobile, AL	2021	0.0	**S**	311
AL50	Mobile, AL	2021	0.0	**S**	311
AL51	Mobile, AL	2021	0.0	**S**	311
AL52	Mobile, AL	2021	0.0	**S**	311
AL53	Mobile, AL	2021	79.7	**R**	376
AL54	Mobile, AL	2021	0.0	**S**	311
AL56	Geneva AL	2021	13.3	**S**	311
AL57	Geneva, AL	2021	0.0	**S**	311
AL58	Geneva, AL	2021	0.0	**S**	311
AL59	Geneva, AL	2021	0.0	**S**	311
AL60	Geneva, AL	2021	0.0	**S**	311
AL62	Geneva, AL	2021	16.8	**S**	311
AL63	Geneva, AL	2021	0.0	**S**	311
AL64	Geneva, AL	2021	0.0	**S**	311
AL65	Geneva, AL	2021	0.0	**S**	311
AL66	Geneva, AL	2021	0.0	**S**	311
AL67	Geneva, AL	2021	0.0	**S**	311
GA1	Peach, GA	2021	43.4	**R**	376
GA3	Peach, GA	2021	57.6	**R**	376
GA6	Peach, GA	2021	23.2	**R**	376
GA7	Peach, GA	2021	78.4	**R**	376
GA8	Peach, GA	2021	90.0	**R**	376
GA9	Peach, GA	2021	38.5	**R**	376
GA10	Taylor, GA	2021	23.0	**R**	376
GA12	Taylor, GA	2021	50.4	**R**	376
GA13	Taylor, GA	2021	65.2	**R**	376
GA14	Taylor, GA	2021	40.4	**R**	376
GA16	Taylor, GA	2021	39.3	**R**	376

GA17	Taylor, GA	2021	34.1	**R**	376
GA18	Taylor, GA	2021	38.3	**R**	376
GA19	Taylor, GA	2021	29.3	**R**	376
GA20	Taylor, GA	2021	45.0	**R**	376
GA21	Taylor, GA	2021	45.5	**R**	376
GA22	Taylor, GA	2021	43.1	**R**	376
GA23	Taylor, GA	2021	42.8	**R**	376
GA24	Taylor, GA	2021	37.3	**R**	376
GA25	Taylor, GA	2021	37.6	**R**	376
GA26	Taylor, GA	2021	24.7	**R**	376
GA27	Crawford, GA	2021	0.0	**S**	311
GA28	Crawford, GA	2021	63.4	**R**	376
GA29	Crawford, GA	2021	0.0	**S**	311
GA30	Crawford, GA	2021	37.7	**R**	376
GA31	Crawford, GA	2021	31.0	**R**	376
GA32	Crawford, GA	2021	37.4	**R**	376
GA33	Crawford, GA	2021	47.3	**R**	376
GA34	Crawford, GA	2021	40.7	**R**	376
GA35	Taylor, GA	2021	26.2	**R**	376
GA36	Taylor, GA	2021	80.6	**R**	376
GA37	Taylor, GA	2021	87.6	**R**	376
GA38	Taylor, GA	2021	21.6	**R**	376
GA39	Taylor, GA	2021	37.2	**R**	376
GA40	Taylor, GA	2021	34.7	**R**	376
GA41	Taylor, GA	2021	37.5	**R**	376
GA42	Taylor, GA	2021	15.8	**S**	311
GA43	Crawford, GA	2021	41.5	**R**	376
GA44	Crawford, GA	2021	52.8	**R**	376
GA45	Crawford, GA	2021	32.5	**R**	376
GA46	Crawford, GA	2021	42.9	**R**	376
GA47	Crawford, GA	2021	26.6	**R**	376

GA49	Crawford, GA	2021	56.9	**R**	376
GA50	Crawford, GA	2021	28.9	**R**	376
GA51	Crawford, GA	2022	12.5	**S**	376
GA52	Crawford, GA	2022	49.4	**R**	376
GA53	Crawford, GA	2022	30.0	**R**	376
GA54	Crawford, GA	2022	24.8	**R**	376
GA55	Crawford, GA	2022	24.7	**R**	376
GA56	Crawford, GA	2022	33.4	**R**	376
GA57	Crawford, GA	2022	34.5	**R**	376
GA58	Crawford, GA	2022	27.6	**R**	376
GA59	Crawford, GA	2022	66.3	**R**	376
GA60	Crawford, GA	2022	32.2	**R**	376
GA61	Taylor, GA	2022	0.0	**S**	311
GA62	Taylor, GA	2022	31.8	**R**	376
GA63	Taylor, GA	2022	30.5	**R**	376
GA64	Taylor, GA	2022	27.4	**R**	376
GA65	Taylor, GA	2022	60.8	**R**	376
GA66	Taylor, GA	2022	23.8	**R**	376
GA67	Taylor, GA	2022	0.0	**S**	311
GA68	Taylor, GA	2022	0.0	**S**	311
GA69	Taylor, GA	2022	71.2	**R**	376
GA70	Taylor, GA	2022	26.3	**R**	376
GA71	Peach, GA	2022	41.0	**R**	376

[a] Percent relative growth on potato dextrose agar (PDA) amended or nonamended with 0.3 µg/ml propiconazole.

[b] Isolates were considered resistant (R) when relative colony growth was ≥ 20 % at a discriminatory dose of 0.3 µg/ml propiconazole. All isolates with relative colony growth < 20% were considered sensitive (S).

[c] PCR fragments were amplified from fungal DNA to identify the presence or absence of Mona using primers *INS65-F* and *INS65-R* (Luo at al., 2008).

REFERENCES

Adaskaveg, J. E., Michailides, T., and Eskalen, A. 2022. Fungicides, bactericides, biocontrols, and natural products for deciduous tree fruit and nut, citrus, strawberry, and vine crops in California. University of California, Davis.

Blaauw, B., Brannen, P., Lockwood, D., Schnabel, G., and Ritchie, D. 2023. Southeastern peach, nectarine, and plum management and culture guide. University of Georgia Extension.

Brannen, P. M., Hotchkiss, M., Reilly, C., and Amiri, A. 2007. Evaluation of fungicide programs to manage DMI-resistant *Monilinia fructicola* in a late-ripening peach, 2006. PDMR. 1:STF003.

Brannen, P. M., Hotchkiss, M., Reilly, C., and Schnabel, G. 2006. Evaluation of fungicide programs to manage a DMI-resistant *Monilinia fructicola* population in a Georgia peach research block, 2005. F&N Tests. 61:STF001.

Burnett, A., Lalancette, N., and McFarland, K. 2010. First report of the peach brown rot fungus *Monilinia fructicola* resistant to demethylation inhibitor fungicides in New Jersey. Plant Dis. 94:126-126.

Chen, F., Everhart, S. E., Bryson, P. K., Luo, C., Song, X., Liu, X., and Schnabel, G. 2015. Fungicide-induced transposon movement in *Monilinia fructicola*. Fungal Genet. Biol. 85:38-44.

Chen, F., Liu, X., Chen, S., Schnabel, E., and Schnabel, G. 2013a. Characterization of *Monilinia fructicola* strains resistant to both propiconazole and boscalid. Plant Dis. 97:645-651.

Chen, F., Liu, X., and Schnabel, G. 2013b. Field Strains of *Monilinia fructicola* resistant to both MBC and DMI fungicides isolated from stone fruit orchards in the Eastern United States. Plant Dis. 97:1063-1068.

Chen, F. P., Fan, J. R., Zhou, T., Liu, X. L., Liu, J. L., and Schnabel, G. 2012. Baseline Sensitivity of *Monilinia fructicola* from China to the DMI fungicide SYP-Z048 and analysis of DMI-resistant mutants. Plant Dis. 96:416-422.

Chi, M. H., Park, S. Y., and Lee, Y. H. 2009. A quick and safe method for fungal DNA extraction. Plant Pathol J. 25(1):108-111.

Cox, K. D., Bryson, P.K., and Schnabel, G. 2007. Instability of propiconazole resistance and fitness in *Monilinia fructicola*. Phytopathology. 97:448-453.

Dutra, P.S.S., Litchemberg, P. S. F., Martinez, M.B., and May-De Mio, L. L. 2020. Cross resistance among demethylation inhibitor fungicides with Brazilian *Monilinia fructicola* isolates as a foundation to discuss brown rot control in fruit. Plant Dis. 104: 2843-2850.

Egüen, B., Melgarejo, P., and De Cal, A. 2015. Sensitivity of *Monilinia fructicola* from Spanish peach orchards to thiophanate-methyl, iprodione, and cyproconazole: fitness analysis and competitiveness. Eur J Plant Pathol. 145:789-801.

FRAC (Fungicide Resistance Action Committee) (2023) SBI-fungicides. URL: https://www.frac.info/frac-teams/working-groups/sbi-fungicides/information.

Holb, I. J., and Schnabel, G. 2007. Differential effects of triazoles on mycelial growth and disease measurements of *Monilinia fructicola* isolates with reduced sensitivity to DMI fungicides. Crop Prot. 26:753-759.

Kwok, I. M.-Y., and Loeffler, R. T. 1993. The biochemical mode of action of some newer azole fungicides. Pestic. Sci. 39, 1-11.

Landgraf, F. A., and Zehr, E. I. 1982. Inoculum sources from *Monilinia fructicola* in South Carolina peach orchards. Phytopathology 71:246.

Leroux, P., and Walker, A. S. 2013. Activity of fungicides and modulators of membrane drug transporters in field strains of *Botrytis cinerea* displaying multidrug resistance. Eur J Plant Pathol. 135:683-693.

Lesniak, K. E., Peng, J., Proffer, T. J., Outwater, C. A., Eldred, L. I., Rothwell, N. L., and Sundin, G. W. 2021. Survey and genetic analysis of demethylation inhibitor fungicides resistance in *Monilinia fructicola* from Michigan orchards. Plant Dis. 105:958-964.

Litchtemberg, P. S. F., Luo, Y., Morales, R. G., Muehlmann-Fischer, J. M., Michailides, T. J., and May-De Mio, L. L. 2017. The point mutation G461S in the *MfCYP51* gene is associated with tebuconazole resistance in *Monilinia fructicola* populations in Brazil. Phytopathology. 107:1507-1514.

Lichtemberg, P. S. F., Moreira, L. M., Zeviani, W. M., Amorim, L., and May de Mio, L. L. 2022. Dispersal gradient of *M. fructicola* conidia from peach orchard to an open field. Eur J Plant Pathol. 162:231-236.

Luo, C.-X., Cox, K. D., Amiri, A., and Schnabel, G. 2008. Occurrence and detection of the DMI resistance-associated genetic element 'Mona' in *Monilinia fructicola*. Plant Dis. 92:1099-1103.

Luo. C.-X., and Schnabel, G. 2008. The cytochrome P450 lanosterol 14α-demethylase gene is a demethylation inhibitor fungicide resistance determinant in *Monilinia fructicola* field isolates from Georgia. Appl. Environ. Microbiol. 74:359-366.

May-De Mio, L. L., Luo, Y., and Michailides T. J. 2011. Sensitivity of *Monilinia fructicola* from Brazil to tebuconazole, azoxystrobin, and thiophanate-methyl and implication on disease management. Plant Dis. 95:821-827.

Nakaune, R., Adachi, K., Nawata, O., Tomiyama, M., Akutsu, A., and Hibi, T. 1998. A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. Appl. Environ. Microbiol. 64:3983-3988.

NASS USDA. 2017. Specified fruit and nuts by acres. Census of agriculture-state data. Alabama.

NASS USDA. 2022. State agriculture overview. Alabama. URL: https://www.nass.usda.gov/Quick_Stats/Ag_Overview/stateOverview.php?state=ALABAMA.

Parker, D. M., N. Zhang, C. D. Smart, and W. D. Koller. 2006. Polymorphism of 14 alpha-demethylase gene (CYP51) in brown rot pathogen *Monilinia fructicola* from a resistant orchard in New York State. Phytopathology 96:S90.

Parks, L. W. and W. M. Casey. 1995. Physiological implications of sterol biosynthesis in yeast. Annu. Rev. Microbiol. 49:95-116.

Rosenberger, D. A. 1983. Observations on quiescent brown rot infection in Grand Prize plums. In: Proceedings of the brown rot of stone fruit workshop. Ames, Iowa, p. 1983.

Schalagbauer, H. E. and Holz, G. 1990. Infection and colonization of plum blossoms by *Monilnia laxa*. Phytophylactica. 22:419-422.

Schnabel, G. and Brannen, P. M. 2022. Biology, epidemiology, and management of diseases of peach driving the spray program in the southeastern United States. Sci. Hortic. 295:110818.

Schnabel, G., Bryson, P. K., Bridges, W. C., and Brannen, P. M. 2004. Reduced sensitivity in *Monilinia fructicola* to propiconazole in Georgia and implications for disease management. Plant Dis. 88:1000-1004.

Villani, S. and Cox, K. D. 2011. Characterizing fenbuconazole and propiconazole sensitivity and prevalence of 'Mona' in isolates of *Monilinia fructicola* from New York. Plant Dis. 95:828-834.

Wilcox, W. F., and Burr, J. A. 1994. Baseline sensitivity of *Monilinia fructicola* to six DMI fungicides. Phytopathology 84:1078.

Zehr, E. I. 1982. Control of brown rot in peach orchards. Plant Dis. 66:1101-1105.

Zehr, E. I., Luszcz, L. A., Olien, W. C., Newall, W. C., and Toler, J. E. 1999. Reduced sensitivity in Monilinia fructicola to propiconazole following prolonged exposure in peach orchards. Plant Dis. 83:913-916.

Zehr, E. I., Toler, J. E., and Luszcz, L. A. 1991. Spread and persistence of benomyl-resistant *Monilinia fructicola* in South Carolina peach orchards. Plant Dis. 75:590-593.

Zhu, F., Bryson, P. K., and Schnabel, G. 2012. Influence of storage approaches on instability of propiconazole resistance in *Monilinia fructicola*. Pest Manag Sci. 68:1003-1009.

CHAPTER TWO

LOW FREQUENCY OF HIGH RESISTANCE TO THIOPHANATE METHYL IN MONILINIA FRUCTICOLA POPULATIONS FROM SOUTHEASTERN UNITED STATES PEACH ORCHARDS

Abstract

Methyl benzimidazole carbamate (MBC) fungicides were once widely used for brown rot (*Monilinia fructicola*) control of peach (*Prunus persica* (L.) Batsch) in the southeastern US, but their use was substantially reduced due to widespread resistance. In this study, 233 *M. fructicola* isolates were collected from major peach production areas in Alabama, Georgia, and South Carolina and sensitivity to thiophanate methyl was examined. Isolates were also collected from one organic and two experimental peach orchards. A discriminatory dose of 1 µg/ml was used to determine sensitive (S) and moderately sensitive (S-LR) versus low resistant phenotypes while 50 and 500 µg/ml thiophanate methyl were was used to determine high resistant (HR) phenotypes. Sequence analyses were performed for identification of mutations in the β-tubulin target gene and detached fruit assays were performed to determine efficacy of a commercial product against isolates representing each phenotype. Results indicated 55.7%, 63.5%, and 75.9% of isolates from Alabama, Georgia, and South Carolina, respectively, were S to thiophanate methyl; 44.3%, 36.5%, and 21.4% were S-LR; no isolates were LR; and only 3 isolates (1.3) from South Carolina were HR. No mutations in S, S-LR, and LR isolates were found, but HR isolates revealed the E198A mutation, an amino acid change of glutamic acid to alanine conferring high resistance. The high label rate of a commercial product containing thiophanate methyl controlled brown rot caused by S and S-LR isolates in detached fruit studies but was ineffective against HR isolates. These results indicate that southeastern *M. fructicola* populations may largely be S or S-LR to thiophanate

methyl and suggest a possible benefit for limited use of thiophanate methyl in future spray programs.

Introduction

Stone fruit production in the United States is affected by various diseases, including *Monilinia fructicola*, the causal agent of blossom blight, twig blight, green fruit rot, preharvest brown rot, and postharvest brown rot of peach (*Prunus persica* (L.) Batsch). *M. fructicola* causes various symptoms on different peach tissues such as cankers on twigs, necrosis of blossoms, and partial to full decay of green and mature fruit in both pre- and postharvest timeframes (Schnabel and Brannen 2022). Spread of the disease can be rampant, as wind disperses conidial spores throughout the orchard leading to the increase in new infections and secondary inoculum throughout the season (Lichtemberg et al. 2022; Landgraf and Zehr 1982). Few cultural methods are effective at controlling disease incidence and severity, though proper sanitation through pruning of infected twigs and removal of infected fruit and mummies can decrease disease pressure (Zehr 1982). The most effective strategy for decreasing disease caused by *M. fructicola* in orchards relies on the use of fungicides, including those that have single-site modes of action.

Today, several fungicides with differing modes of action are used to control blossom blight and brown rot disease. Most notably, site-specific fungicides used in modern spray programs include the FRAC 2 dicarboximides, FRAC 3 demethylation inhibitors (DMIs), FRAC 7 quinone outside inhibitors (QoIs), and FRAC 11 succinate dehydrogenase inhibitors (SDHIs). Typical spray programs recommend restricting the number of applications per season of any given FRAC code to minimize the potential for resistance development in the target pathogen, though under certain circumstances additional applications may be needed (Blaauw et al. 2023; Adaskaveg et al. 2022). Other resistance management recommendations include combining certain FRAC codes

in tank mixes and only using some specific FRAC codes during specific phenological stages of peach development.

Methyl benzimidazole carbamate (MBC) fungicides were the first site-specific fungicides used in peach production. Introduced in the early 1970s, various chemical formulations of the FRAC 1 MBCs were developed such as benzimidazoles (benomyl, carbendazim, fuberidazole, and thiabendazole) and the thiophanates (thiophanate and thiophanate-methyl) (FRAC 2023). This chemical class binds to β-tubulin, an essential sub-unit of tubulin needed for microtubule assembly, thereby preventing cellular division such as mitosis (Young 2015). Current spray guides recommend a very restricted use of MBC fungicides in peach production (Blaauw et al. 2023; Adaskaveg et al. 2022), and most producers of the southeastern United States have completely refrained from spraying any FRAC 1 products for fear of control failure due to possible resistance (Schnabel oral communications).

Resistance to MBCs was first reported in 1969 on powdery mildew of cucurbits and has since risen to over 25 fungal species (Hawkins and Fraajie 2016). Resistance in *M. fructicola* was also reported soon after MBCs were introduced to stone fruit orchards in the 1970s. Benomyl was one of the first MBC fungicides to have generated widespread decreased sensitivity in *Monilinia* species across the United States and Australia (Sonoda et al. 1983; Ma et al. 2003; Penrose et al. 1979). The first report of benomyl resistance in stone fruit was documented in California in 1977 – only five years after market introduction (Szkolnik et al. 1978). Due to US regulatory control measures, all registrations for pesticide products containing benomyl were cancelled in 2001 (EPA 2016). Thiophanate-methyl replaced benomyl as the main MBC fungicide used for brown rot control in stone fruits, and reports of resistance continued to appear in diverse international

locations such as Brazil, China, Spain and the US (Chen et al. 2013; Chen et al. 2014; May de Mio et al. 2014; Egüen et al. 2015).

Resistance to MBC fungicides is mainly a result of mutations residing in the β-tubulin gene (*Tub2*). Mutations in *Tub2* have been documented in both field and laboratory conditions at codons 6, 50, 134, 165, 167, 198, 200 and 240 in several plant pathogens (Koenraadt et al. 1992; Lehner et al. 2015; Ma et al. 2003; Ma et al. 2005; McKay et al. 1998; Orbach et al. 1986; Schmidt et al. 2006). Specifically, for *Monilinia fructicola*, notable mutations occur at codons 6, 198, and 200 (Ma et al. 2003; Chen et al. 2013). Lower levels of resistance have been associated with the H6Y mutation at codon 6 while higher levels of resistance have been predominantly associated with the E198A mutation. Other mutations such as E198K, E198Q, and F200Y also confer high resistance, though the frequency of these mutations in *M. fructicola* is less than that of E198A (Chen et al. 2013).

Extensive resistance surveys in the southeastern US have not been conducted, but MBC resistance has been documented to have occurred as early as the mid-1970s in South Carolina (Zehr et al. 1991). Further investigations concluded that benzimidazole fungicides should only be used under urgent circumstances and that surveying for resistance would be necessary if MBC fungicides were to be continually administered (Zehr et al. 1991). Nearly two decades later, the first confirmation of the resistance mutation E198A in *Tub2* was reported in South Carolina (Zhu et al. 2010). Shortly after, an eastern US survey of thiophanate-methyl sensitivity confirmed resistance and the presence of the E198A mutation in a majority of peach orchards across South Carolina (Chen et al. 2013).

Recent studies report a significant reversion toward fungicide sensitivity in *M. fructicola* populations after MBC and DMI fungicides had been discontinued for several years due to

documented widespread resistance (Fischer et al. 2023; Pereira et al. 2020). Therefore, the objectives of this study were to (i) determine the sensitivity of *M. fructicola* collected from peach orchards in three southeastern states (Alabama, Georgia, South Carolina) to the MBC fungicide thiophanate-methyl, (ii) characterize different sensitivity phenotypes for their ability to produce disease on fruit treated with label rates of a commercial product containing thiophanate methyl, and (iii) identify associated target gene mutations.

Materials and Methods

***M. fructicola* collection and isolation.** *M. fructicola* was collected from peach orchards in, Alabama (AL), Georgia (GA), and South Carolina (SC). A total of 208 isolates were collected from various conventional peach farms (Supplementary Table) and 28 isolates from research orchards in GA and SC and from a commercial organic farm in SC. Previously published isolates MDbbp6 (resistant), MDbbc3 (resistant), and SCmd17 (sensitive) were used as references (Chen et al. 2013). Spores were collected from symptomatic peach fruit at commercial maturity using a sterile cotton swab and individually wrapped to ensure sample purity. Swabs were stored at 3°C in zip lock bag with desiccant until preparation for single spore isolation. Each individual swab was placed directly over a Petri plate containing water agar (Bacto^{TM} agar, Becton, Dickson and Company, Sparks, MD) and gently tapped to distribute spores. Four actively germinating single spores of *M. fructicola* from each water agar plate were transferred to a potato dextrose agar (PDA; Criterion, Hardy Diagnostics, Santa Monica, CA) plate and incubated at 25^{o}C for 2 to 4 days. Only one growing colony would be selected for historic preservation on filter paper disk (5 x 5 mm) and dried to be stored at -20°C with silica gel desiccant and indicator beads (Thermo Scientific, Rockford, IL).

***In vitro MBC fungicide sensitivity assay*.** Isolates were subjected to three concentrations of the MBC fungicide thiophanate-methyl (Topsin® M 70WP, Cerexagri-Nisso LLC, King of Prussia, PA) to determine sensitive (S), moderately sensitive (S-LR), low resistant (LR) and highly resistant (HR) phenotypes. Two plugs, five millimeters in diameter, were taken from the periphery of 3 to 5-day old isolates grown on PDA at 25°C and placed on PDA amended with 1 µg/ml and 50 µg/ml thiophanate-methyl. No mycelial growth at 1 µg/ml indicated S, mycelial growth at 1 µg/ml but not at 50 µg/ml corresponded to the S-LR and LR and mycelial growth at 50 µg/ml and 500 µg/ml were considered HR (Ma et al. 2003). S-LR isolates have lower EC_{50} values (range 0.32-0.5) at 1 µg/ml compared to LR isolates (3.26-7.1) and lack the H6Y or any other mutation in β-tubulin (Fischer et al. 2023). The experiment was repeated with a subset of randomly selected isolates (n=30) and repeatability of RG values was confirmed by a correlation coefficient of r=0.90.

***DNA extraction and sequence analysis of Tub2*.** Mycelium of 16 isolates with varying RG values were cultured on cellophane atop PDA and incubated for 5 days at 25°C. Extraction of mycelial DNA was achieved using a previously described protocol published in Chi et al. (2009). Primer pair TubA and TubR1 were used to amplify a fragment of *Tub2* under the following polymerase chain reaction (PCR) conditions described in Ma et al. (2004). PCR reaction mixture consisted of 1 µl extracted DNA, 1 µl each of TubA and TubR1 primers, 5 µl Accupower® HotStart PCR Premix (Bioneer, Oakland, CA) and 17 µl of deionized water for a 25 µl reaction volume. Confirmation of amplified DNA was accessed using electrophoresis on 1.5% agarose gel in TAE buffer. PCR products were purified using the DNA Clean & Concentrator™-5 purification kit (Zymo Research Corporation, Irvine, CA) and samples were sequenced by Eurofins Scientific. Sequence analysis was performed with DNASTAR (DNASTAR, Inc, Madison, Wisconsin).

In vivo detached fruit assay for MBC efficacy. Two cultivars of peach, Big red and Juneprince were inoculated with isolates of differing phenotypes to evaluate their ability to cause disease on mature peach fruit treated with Topsin® M 70WP at 0.599 µg/ml (high label rate). AL16 (S), GA19 and TF2002 (both S-LR), and MRF007 (HR) were evaluated on Big Red in 2022 and AL64 (S), GA29 and MRF006 (both S-LR), and MRF007 (HR) were evaluated on Juneprince in 2023. Four fruit replicates were used for each isolate and treatment, totaling 32 peaches/isolate for each cultivar. Peaches were harvested from a research orchard and rinsed under tap water for 30 seconds to remove residual pesticides. Fruit were placed in a laminar flow hood until dry. Once dried, half of the peaches were sprayed until runoff with the fungicide. In a sealable 6.5-quart container, one control and one treated peach were placed into cups side by side, and water was poured into the bottom to achieve a relative humidity of above 95%. Before placing peaches into the containers, relative humidity above 95% was confirmed using a hygrometer. Each peach received an approximately 0.5 mm wide and 2 mm deep puncture to the epidermis using a sterile hypodermic needle to ensure infection. A 30 µl suspension of 1 x 10^5 spores/ml inoculum was applied atop each puncture. Two perpendicular lesion diameters were recorded over a 5-day incubation period at room temperature. The presence or absence of lesions (disease incidence) and growth values relative to the untreated control (disease incidence) were determined for all isolates after 5 days of growth. Experiments were repeated and data from each cultivar repetition were combined because data sets were not significantly different (P=0.7966, Big Red; P=0.7161, Juneprince).

Results

Identification of thiophanate-resistance phenotypes of M. fructicola isolates based on discriminatory dose assays and Tub2 sequence analysis. Most isolates did not grow at 1 ug/ml

thiophanate methyl and were therefore considered S (Fig. 1). The *Tub2* gene of three S isolates were sequenced and no nucleotide alterations were found. Although the *Tub2* gene was not sequenced for all S-LR isolates, isolates that grew at 1 µg/ml but not at 50 µg/ml were all considered S-LR instead of LR for two reasons. First, the *Tub2* genes of 11 S-LR isolates with the highest RG values at 1 µg/ml were sequenced and did not contain variations in the *Tub2* gene, and second S-LR isolates that were not sequenced (59 isolates in total) all had lower RG values compared to S-LR isolates that had been sequenced (see definition for S-LR and LR in M&M section). The average frequency of S isolates was 55.7%, 63.5%, and 75.9%, of S-LR isolates was 44.3%, 36.5%, and 21.4%, of LR isolates was 0%, 0%, and 0%, and of HR isolates was, 0%, 0%, and 2.7% for AL, GA, and SC, respectively (Table 1). Two of the HR isolates were from commercial orchards in SC and the third was from an experimental orchard of the same state. Two of the three HR isolates were also sequenced and revealed the E198A mutation in β-tubulin (Table 2). Among counties within each state, the ratios between S, S-LR, and LR isolates were similar (Table 1).

Evaluation of phenotypes in detached fruit assays. Preventative application of formulated thiophanate methyl (Topsin M WP) revealed complete or nearly complete control of disease by isolates except for MRF007 (Table 3). Lesion diameters of HR isolate MRF007 on treated peaches were significantly greater than those inoculated with S and S-LR isolates with an average RG value of 107.8% on Big Red peaches and 73.2% on June prince peaches. Incidence ranged from 24 to 48 hours for all untreated and treated peaches inoculated with MRF007 (Table 3).

Discussion

In this study, very few isolates from South Carolina and no isolates from Georgia and Alabama were high resistant to thiophanate-methyl. For South Carolina, this came as a surprise,

since high frequency of resistance had been documented (Zehr et al. 1991) and remained in commercial orchards (Chen et al. 2013). This suggests that selection pressure was low or non-existent at least over the last 10 years, perhaps longer, in South Carolina. This corroborates with grower communications that MBC fungicides have not been used for blossom blight or brown rot management in commercial farms in decades for fear of control failure due to historically documented resistance (Schnabel oral communication). Changing MBC resistance patterns over time were also observed in Brazil. Fisher et al. (2023) determined that historic isolates collected between 2003 and 2012 had significantly higher rates of LR and HR isolates when compared to isolates collected in 2017 from the same states. This change of resistance was supported by previous work performed in Brazil that showed that after limiting MBC fungicides to one spray application per year, an increase in sensitivity followed (May de Mio et al. 2011). Both studies suggest that the reduction of the MBC fungicide thiophanate methyl influenced the change in MBC sensitivity. Increases of sensitivity have also been noted in California. Ma et al. (2003) observed a decrease in LR isolate frequencies from 84.6% to 25.4% over an eight-year period in California. Survey data from Georgia and Alabama are not available, but one study confirmed at least the presence of resistance in the *M. fructicola* population. Specifically, one of 16 isolates from the midland region of Georgia was resistant to thiophanate methyl (Schnabel et al. 2011). No historical data is available from AL and our study suggests that resistance frequency is very low, if present at all.

Since the first characterization of the E198A mutation in California (Ma et al. 2003), high resistance to MBC fungicides in association with the E198A mutation has been frequently observed in *Monilinia spp.* from countries including Brazil (May de Mio et al. 2011; Fischer et al. 2023), China (Chen et al. 2014), Korea (Lim et al. 2006) the United States (Zhu et al. 2010) and

various countries throughout Europe (Malandrakis et al. 2012; Weger et al. 2011; Ivić et al. 2021). The mutation found in HR isolates in this study (E198A) provides complete (qualitative) resistance to thiophanate methyl and is the same reported in previous studies examining South Carolina isolates (Chen et al. 2013; Zhu et al. 2008). Other mutations at the 198 codon, including E198K and E198Q, have been observed in Maryland and Pennsylvania (Chen et al. 2013) but these have not been found in the southeastern United States. Mutations in the beta-tubulin gene other than those associated with the 198 codon confer resistance to MBC fungicides but at varying degrees. Higher levels of resistance associated with mutation F200Y were observed in Maryland and Pennsylvania (Chen et al. 2013). In Italy, both LR and HR isolates had a mutation at codon 83 conferring an amino acid change from CGA to CAA (Martini et al. 2016). However, the correlation between MBC resistance and this mutation needs confirmation. Nonetheless, these specific mutations have not been observed in our study.

Alongside E198A, H6Y is a mutation conferring resistance in *M. fructicola.* First confirmed in California, the mutation at codon 6 from histidine (CAT) to tyrosine (TAT) confers LR to MBC fungicides (Ma et al. 2003). To date, *M. fructicola* isolates from Brazil (May de Mio et al. 2011; Fischer et al. 2023) and China (Fan et al. 2009; Ke et al. 2023) have the H6Y mutation residing in the beta tubulin gene. In this study, the H6Y mutation was not observed even in isolates with growth at 1 ug/ml. Growth at 1 µg/ml with the absence of the H6Y mutation had been previously observed in Brazil and those isolates were designated S-LR (May de Mio et al. 2011; Fischer et al. 2023). As confirmed in our study, S-LR isolates can be controlled by label rates of formulated thiophanate methyl suggesting that those isolates should be considered sensitive for practical purposes. Consistent with this assessment, other published articles recommended an increase of the discriminatory dose concentration to 5 µg/ml thiophanate methyl for more accurate

distinction of LR isolates from S isolates (Chen et al. 2013; Fischer et al. 2023; Ke et al. 2023). This increase in concentration could effectively increase the percentage of S isolates that are present in this study for South Carolina, Georgia and Alabama isolates, respectively. However, growth at 1 µg/ml indicates some level of reduced sensitivity as isolates from this study have shown RG above 10% at this concentration. This suggests other mechanisms of resistance such as energy dependent drug efflux pumps found in *Botrytis cinerea* (Leroux and Walker 2013) and overexpression of the beta-tubulin gene found in *Colletotrichum acutatum* (Nakaune and Nakano 2007) may contribute to overall resistance but to a significantly lower degree, though no alternative mechanisms were determined in this study.

The evident reduction of frequency of isolates resistant to thiophanate methyl in South Carolina and perhaps Georgia and Alabama may simply be due to population dynamics rather than a consequence of fitness penalty. Fungal populations are subjected to constant selection pressure due to, for example, environmental pressures, agricultural chemistries other than MBC fungicides, gene migration and gene flow, generation of more adaptable genotypes through genetic recombination, and spontaneous mutations leading to phenotypic changes (Watson 1970; Ennos and McConnell 1995). Previous research suggests that MBC resistant *M. fructicola* display high fitness and competitiveness compared to sensitive isolates without selection pressure. Fitness of LR isolates from China with the H6Y mutation tested in vitro and in vivo revealed no significant differences in mycelial growth rate, sporulation, germination rate and lesion size on detached fruit when compared to sensitive isolates (Ke et al. 2023). In vivo competitiveness assays performed on S, LR, and HR isolates applied to untreated nectarine blossoms found no significant advantage of S isolates over LR and HR isolates in California (Yoshimura et al. 2004). An in vitro assay conducted in Spain also determined that isolates of resistant phenotypes were as competitive as

sensitive phenotypes (Egüen et al. 2015). The frequencies of resistant isolates in orchards remained unchanged over a five-year period when commercial orchards were annually treated with thiophanate methyl. While the majority of published data mainly found no fitness penalties in *M. fructicola* both in vitro and in vivo assays, Sonada et al. (1982) observed that benomyl resistant isolates grew slower on peach fruit in the absence of benomyl suggesting that *M. fructicola* sensitive to benomyl could grow faster, inherently producing more spores to outcompete the slower growing benomyl resistant strains.

Debate exists on the reintroduction of MBC fungicides as a standard preharvest application for peach production due to concern of rapid reappearance of resistant phenotypes. Reports of rapid occurrence of MBC resistance after five years of being introduced into orchards show the high risk of resistance selection associated with the chemical class (Szkolnik et al. 1978; Zehr et al. 1991). However, this rapid rise in resistance could have been a consequence of not having used anti-resistance management practices designed to limit selection of resistant individuals in a population of otherwise sensitive individuals. If MBC products such as thiophanate methyl were to be considered for reintroduction, strict guidelines would have to be developed and followed. These guidelines may include a strict limit of the number of applications per season, strategic use at certain phenological stages exhibiting tissue of limited disease susceptibility, mixtures with multisite or single site FRAC codes, and alternations with other FRAC codes. Relieving existing spray programs of an additional application of QoIs and SDHIs, for example, could prolong the efficacy of these important chemical classes. However, certain mixtures may already be doomed for failure. A previous study showed the presence of dual resistance in South Carolina isolates, specifically isolates resistant to thiophanate methyl and propiconazole (Chen et al. 2013). Thus, mixing MBCs with DMIs may quickly select for already existing, resistant phenotypes. If MBC

fungicides were to be reintroduced and strategically applied, regular resistance surveying would be imperative to detect any significant increase of resistant phenotypes and to prevent control failure.

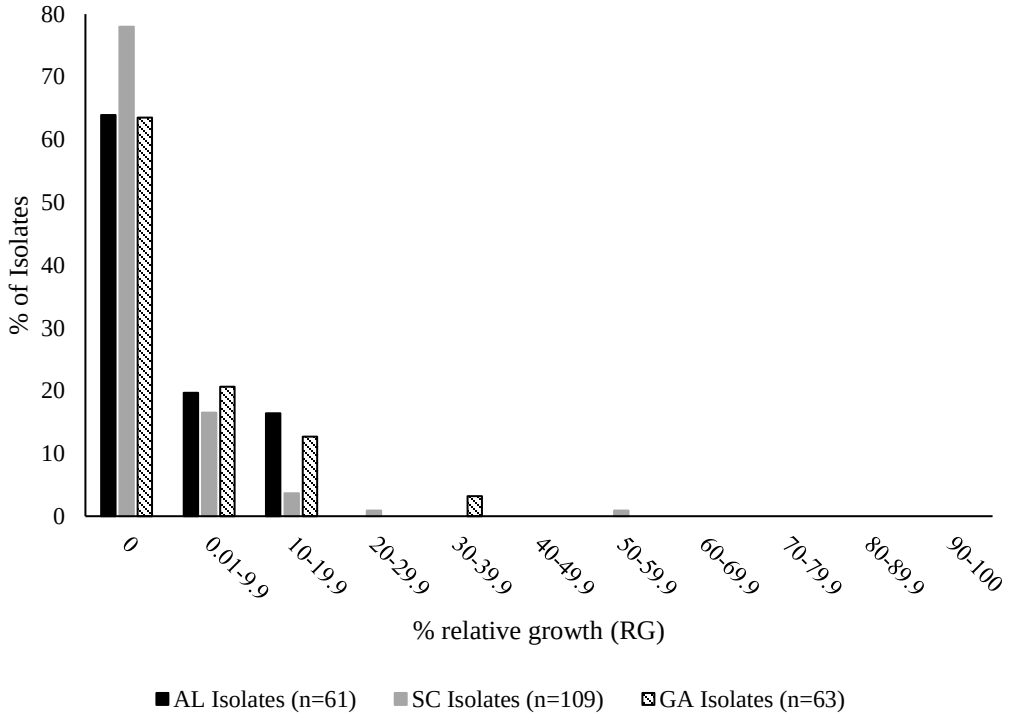

Figure 2.1. Distribution of relative growth (RG) values on PDA amended with 1 µg/ml thiophanate-methyl for 233 single spored isolates of *Monilinia fructicola* collected from conventional and organic/research orchards in Alabama (AL), South Carolina (SC), and Georgia (GA).

Table 2.1. Origin, year of isolation, thiophanate-methyl resistance phenotypes, and frequency of occurrence of Monilinia fructicola isolates collected from South Carolina (SC), Alabama (AL), and Georgia (GA).

Origin				Phenotype		
State	County[a]	Year of isolation	Isolates (n)	S / S-LR / LR / HR[b]	Frequency (%)	Mean (RG)[c]
SC	Edgefield	2021	31	25 / 6 / 0 / 0	80.6 / 19.4 / 0 / 0	1.61
SC	Barnwell	2021	17	11 / 6 / 0 / 0	64.7 / 35.3 / 0 / 0	2.77
SC	Aiken/Salud a	2021	51	40 / 11 / 0 / 0	78.4 / 21.6 / 0 / 0	2.70
SC	Oconee	2021	10	8 / 1 / 0 / 1	80 / 10 / 0 / 10	5.39[c]
SC	Spartanburg	2022	3	1 / 0 / 0 / 2	33.3 / 0 / 0 / 66.7	0.0[c]
AL	Chilton	2021	19	15 / 4 / 0 / 0	78.9 / 21.1 / 0 / 0	0.95
AL	Barbour	2021	14	12 / 2 / 0 / 0	85.7 / 14.3 / 0 / 0	0.91
AL	Mobile	2021	17	4 / 13 / 0 / 0	23.5 / 76.5 / 0 / 0	9.25
AL	Geneva	2021	11	3 / 8 / 0 / 0	27.3 / 72.7 / 0 / 0	1.53
GA	Peach	2021/2022	7	7 / 0 / 0 / 0	100 / 0 / 0 / 0	0.0
GA	Taylor	2021/2022	31	20 / 11 / 0 / 0	64.5 / 35.5 / 0 / 0	2.01
GA	Crawford	2021/2022	25	13 / 12 / 0 / 0	52.0 / 48.0 / 0 / 0	6.80

[a] Multiple counties are listed if isolates came from orchards stretching over county lines.

[b] phenotypes include sensitive (S), moderately sensitive (S-LR), low resistant (LR), and highly resistant (HR) to thiophanate-methyl.

[c] Mean relative growth (RG) on PDA amended with 1 µg/ml thiophanate-methyl. HR isolates were not included in the calculation.

Table 2.2 Sensitivity phenotype, relative growth on PDA amended with 1 µg/ml thiophanate-methyl, and identification of resistance mutations in *Tub2*.

			Amino acid mutations	
Isolates	Sensitivity phenotype[a]	RG (%)[b]	H6Y	E198A
TF010	S	0	--	--
AL16	S	0	--	--
AL64	S	0	--	--
TF2002	S-LR	16.3	--	--
TFJ8	S-LR	4.4	--	--
MRF006	S-LR	51.5	--	--
WaF010	S-LR	21.3	--	--
AL30	S-LR	5.3	--	--
GA19	S-LR	4.3	--	--
GA29	S-LR	8.2	--	--
GA33	S-LR	33.9	--	--
GA45	S-LR	34.3	--	--
GA50	S-LR	18.3	--	--
GA63	S-LR	8.7	--	--
JC7	HR	35.3[c]	--	Present
MRF007	HR	49.2[c]	--	Present

[a] Sensitivity phenotypes consisting of sensitive (S), moderately sensitive (S-LR), and highly resistant (HR) to thiophanate-methyl.

[b] RG values represent isolates grown at 1 µg/ml thiophanate-methyl.

[c] RG on potato dextrose agar (PDA) amended with 500 µg/ml thiophanate-methyl.

Table 2.3 Disease incidence and severity obtained on detached fruit assays for two peach cultivars, 'Big Red' and 'Juneprince', treated with a high label rate of thiophanate-methyl.

Isolates	Cultivar, collection year	Sensitivity phenotype[a]	Disease incidence (%)	Disease severity (%)[b]
AL16	Big Red, 2022	S	0 b[c]	0 b
GA19	Big Red, 2022	S-LR	0 b	0 b
TF2002	Big Red, 2022	S-LR	0 b	0 b
MRF007	Big Red, 2022	HR	100 a	107.8 a
AL64	Juneprince, 2023	S	0 b	0 b
GA29	Juneprince, 2023	S-LR	0 b	0 b
MRF006	Juneprince, 2023	S-LR	0 b	0 b
MRF007	Juneprince, 2023	HR	100 a	99.5 a

[a] Sensitivities to thiophanate-methyl consist of sensitive (S), moderately sensitive (S-LR), and highly resistant (HR) phenotypes.

[b] Diametric relative growth (RG) of lesions evaluated after 5 days of growth at room temperature on fruit treated with a high label rate of thiophanate-methyl (0.599 g/L).

[c] Values of the same letter within the same column are not significantly different based on Student's T analyzed with JMP 16.2.0 software.

Supplementary Table 2.4 Characteristics and thiophanate-methyl resistance phenotypes of Monilinia fructicola isolates from South Carolina (SC), Alabama (AL), and Georgia (GA).

Isolate	**Origin (county, state)**	**Year of isolation**	**RG (%)[a]**	**Sensitivity phenotype[c]**
BS001	Edgefield, SC	2021	0.0	**S**
BS002	Edgefield, SC	2021	0.0	**S**
BS003	Edgefield, SC	2021	0.0	**S**
BS004	Edgefield, SC	2021	0.0	**S**
BS005	Edgefield, SC	2021	0.0	**S**

BS006	Edgefield, SC	2021	0.0	S
BS007	Edgefield, SC	2021	0.0	**S**
BS008	Edgefield, SC	2021	0.0	**S**
BS009	Edgefield, SC	2021	0.0	**S**
BS010	Edgefield, SC	2021	0.0	**S**
BS011	Edgefield, SC	2021	7.4	**S-LR**
BS012	Edgefield, SC	2021	0.0	**S**
CF001	Barnwell, SC	2021	0.0	**S**
CF002	Barnwell, SC	2021	0.0	**S**
CF003	Barnwell, SC	2021	0.0	**S**
CF004	Barnwell, SC	2021	0.0	**S**
CF005	Barnwell, SC	2021	0.0	**S**
CF006	Barnwell, SC	2021	10.8	**S-LR**
CF007	Barnwell, SC	2021	0.0	**S**
CF008	Barnwell, SC	2021	7.9	**S-LR**
CF009	Barnwell, SC	2021	0.0	**S**
CF010	Barnwell, SC	2021	0.0	**S**
CF011	Barnwell, SC	2021	8.2	**S-LR**
CF012	Barnwell, SC	2021	6.1	**S-LR**
CF013	Barnwell, SC	2021	0.0	**S**
CF014	Barnwell, SC	2021	7.9	**S-LR**
CF015	Barnwell, SC	2021	0.0	**S**
CF016	Barnwell, SC	2021	7.1	**S-LR**
CF017	Barnwell, SC	2021	0.0	**S**
DB001	Aiken, SC	2021	4.6	**S-LR**
DB002	Aiken, SC	2021	2.7	**S-LR**
DB003	Aiken, SC	2021	0.0	**S**
DB004	Aiken, SC	2021	0.0	**S**
DB005	Aiken, SC	2021	0.0	**S**
DB006	Aiken, SC	2021	2.0	**S-LR**
DB007	Aiken, SC	2021	8.0	**S-LR**

DB008	Aiken, SC	2021	15.1	**S-LR**
DB009	Aiken, SC	2021	0.0	**S**
DB010	Aiken, SC	2021	0.0	**S**
DB011	Aiken, SC	2021	0.0	**S**
DB012	Aiken, SC	2021	0.0	**S**
DB013	Aiken, SC	2021	0.0	**S**
DB014	Aiken, SC	2021	0.0	**S**
DB016	Aiken, SC	2021	0.0	**S**
DB017	Aiken, SC	2021	0.0	**S**
DB018	Aiken, SC	2021	0.0	**S**
JC4	Spartanburg, SC	2022	0.0	**S**
JC5	Spartanburg, SC	2022	32.9[b]	**HR**
JC7	Spartanburg, SC	2022	35.3[b]	**HR***
TF001	Saluda, SC	2021	0.0	**S**
TF002	Saluda, SC	2021	0.0	**S**
TF003	Saluda, SC	2021	0.0	**S**
TF004	Saluda, SC	2021	0.0	**S**
TF005	Saluda, SC	2021	0.0	**S**
TF006	Saluda, SC	2021	0.0	**S**
TF007	Saluda, SC	2021	0.0	**S**
TF008	Saluda, SC	2021	0.0	**S**
TF009	Saluda, SC	2021	0.0	**S**
TF010	Saluda, SC	2021	0.0	**S***
TF2001	Saluda, SC	2021	0.0	**S**
TF2002	Saluda, SC	2021	16.3	**S-LR***
TF2003	Saluda, SC	2021	0.0	**S**
TF2004	Saluda, SC	2021	0.0	**S**
TF2005	Saluda, SC	2021	0.0	**S**

TF2006	Saluda, SC	2021	7.6	**S-LR**
TF2007	Saluda, SC	2021	0.0	**S**
TF2008	Saluda, SC	2021	0.0	**S**
TF2009	Saluda, SC	2021	0.0	**S**
TF2010	Saluda, SC	2021	0.0	**S**
TFJ1	Edgefield, SC	2022	0.0	**S**
TFJ2	Edgefield, SC	2022	0.0	**S**
TFJ3	Edgefield, SC	2022	0.0	**S**
TFJ4	Edgefield, SC	2022	0.0	**S**
TFJ5	Edgefield, SC	2022	0.0	**S**
TFJ6	Edgefield, SC	2022	14.8	**S-LR**
TFJ7	Edgefield, SC	2022	0.0	**S**
TFJ8	Edgefield, SC	2022	4.4	**S-LR***
TFJ10	Edgefield, SC	2022	6.6	**S-LR**
TrF001	Edgefield, SC	2021	0.0	**S**
TrF002	Edgefield, SC	2021	0.0	**S**
TrF003	Edgefield, SC	2021	0.0	**S**
TrF004	Edgefield, SC	2021	0.0	**S**
TrF005	Edgefield, SC	2021	0.0	**S**
TrF006	Edgefield, SC	2021	9.4	**S-LR**
TrF007	Edgefield, SC	2021	0.0	**S**
TrF008	Edgefield, SC	2021	0.0	**S**
TrF009	Edgefield, SC	2021	7.3	**S-LR**
TrF010	Edgefield, SC	2021	0.0	**S**
MRF001	Oconee, SC	2021	0.0	**S**
MRF002	Oconee, SC	2021	0.0	**S**
MRF003	Oconee, SC	2021	0.0	**S**
MRF004	Oconee, SC	2021	0.0	**S**
MRF005	Oconee, SC	2021	0.0	**S**
MRF006	Oconee, SC	2021	51.5	**S-LR***
MRF007	Oconee, SC	2021	49.2[b]	**HR***

MRF008	Oconee, SC	2021	0.0	**S**
MRF009	Oconee, SC	2021	0.0	**S**
MRF010	Oconee, SC	2021	0.0	**S**
WaF001	Aiken/Saluda, SC	2021	0.0	**S**
WaF002	Aiken/Saluda, SC	2021	5.9	**S-LR**
WaF003	Aiken/Saluda, SC	2021	5.1	**S-LR**
WaF005	Aiken/Saluda, SC	2021	0.0	**S**
WaF006	Aiken/Saluda, SC	2021	0.0	**S**
WaF007	Aiken/Saluda, SC	2021	11.1	**S-LR**
WaF009	Aiken/Saluda, SC	2021	0.0	**S**
WaF010	Aiken/Saluda, SC	2021	21.3	**S-LR***
WaF011	Aiken/Saluda, SC	2021	0.0	**S**
WaF012	Aiken/Saluda, SC	2021	0.0	**S**
WaF014	Aiken/Saluda, SC	2021	0.0	**S**
WaF015	Aiken/Saluda, SC	2021	0.0	**S**
WaF016	Aiken/Saluda, SC	2021	5.5	**S-LR**
WaF017	Aiken/Saluda, SC	2021	0.0	**S**

AL1	Chilton, AL	2021	0.0	**S**
AL2	Chilton, AL	2021	0.0	**S**
AL3	Chilton, AL	2021	0.0	**S**
AL4	Chilton, AL	2021	0.0	**S**
AL5	Chilton, AL	2021	0.0	**S**
AL6	Chilton, AL	2021	0.0	**S**
AL7	Chilton, AL	2021	0.0	**S**
AL8	Chilton, AL	2021	0.0	**S**
AL9	Chilton, AL	2021	0.0	**S**
AL10	Chilton, AL	2021	0.0	**S**
AL11	Chilton, AL	2021	0.0	**S**
AL13	Chilton, AL	2021	2.1	**S-LR**
AL14	Chilton, AL	2021	0.0	**S**
AL15	Chilton, AL	2021	0.0	**S**
AL16	Chilton, AL	2021	0.0	**S***
AL17	Chilton, AL	2021	9.2	**S-LR**
AL18	Chilton, AL	2021	4.6	**S-LR**
AL19	Chilton, AL	2021	2.1	**S-LR**
AL20	Chilton, AL	2021	0.0	**S**
AL22	Barbour, AL	2021	0.0	**S**
AL23	Barbour, AL	2021	0.0	**S**
AL25	Barbour, AL	2021	0.0	**S**
AL26	Barbour, AL	2021	0.0	**S**
AL27	Barbour, AL	2021	0.0	**S**
AL28	Barbour, AL	2021	0.0	**S**
AL29	Barbour, AL	2021	0.0	**S**
AL30	Barbour, AL	2021	5.3	**S-LR***
AL31	Barbour, AL	2021	0.0	**S**
AL32	Barbour, AL	2021	0.0	**S**
AL33	Barbour, AL	2021	0.0	**S**
AL34	Barbour, AL	2021	0.0	**S**

AL35	Barbour, AL	2021	7.4	**S-LR**
AL36	Barbour, AL	2021	0.0	**S**
AL37	Mobile, AL	2021	13.0	**S-LR**
AL38	Mobile, AL	2021	10.6	**S-LR**
AL39	Mobile, AL	2021	16.5	**S-LR**
AL40	Mobile, AL	2021	11.8	**S-LR**
AL41	Mobile, AL	2021	12.4	**S-LR**
AL42	Mobile, AL	2021	12.0	**S-LR**
AL43	Mobile, AL	2021	12.4	**S-LR**
AL44	Mobile, AL	2021	15.7	**S-LR**
AL45	Mobile, AL	2021	9.03	**S-LR**
AL46	Mobile, AL	2021	15.6	**S-LR**
AL47	Mobile, AL	2021	0.0	**S**
AL48	Mobile, AL	2021	0.0	**S**
AL49	Mobile, AL	2021	9.4	**S-LR**
AL51	Mobile, AL	2021	3.9	**S-LR**
AL52	Mobile, AL	2021	14.2	**S-LR**
AL53	Mobile, AL	2021	0.0	**S**
AL54	Mobile, AL	2021	0.0	**S**
AL56	Geneva, AL	2021	0.0	**S**
AL57	Geneva, AL	2021	0.0	**S**
AL58	Geneva, AL	2021	0.0	**S**
AL59	Geneva, AL	2021	0.0	**S**
AL60	Geneva, AL	2021	7.3	**S-LR**
AL62	Geneva, AL	2021	0.0	**S**
AL63	Geneva, AL	2021	7.3	**S-LR**
AL64	Geneva, AL	2021	0.0	**S***
AL65	Geneva, AL	2021	0.0	**S**
AL66	Geneva, AL	2021	0.0	**S**
AL67	Geneva, AL	2021	2.2	**S-LR**
GA1	Peach, GA	2021	0.0	**S**

GA3	Peach, GA	2021	0.0	**S**
GA6	Peach, GA	2021	0.0	**S**
GA7	Peach, GA	2021	0.0	**S**
GA8	Peach, GA	2021	0.0	**S**
GA9	Peach, GA	2021	0.0	**S**
GA12	Taylor, GA	2021	0.0	**S**
GA13	Taylor, GA	2021	0.0	**S**
GA14	Taylor, GA	2021	0.0	**S**
GA16	Taylor, GA	2021	0.0	**S**
GA17	Taylor, GA	2021	1.3	**S-LR**
GA18	Taylor, GA	2021	0.0	**S**
GA19	Taylor, GA	2021	4.3	**S-LR***
GA20	Taylor, GA	2021	0.0	**S**
GA21	Taylor, GA	2021	0.0	**S**
GA22	Taylor, GA	2021	6.5	**S-LR**
GA23	Taylor, GA	2021	1.8	**S-LR**
GA24	Taylor, GA	2021	2.1	**S-LR**
GA25	Taylor, GA	2021	11.4	**S-LR**
GA26	Taylor, GA	2021	7.9	**S-LR**
GA27	Crawford, GA	2021	0.0	**S**
GA28	Crawford, GA	2021	0.0	**S**
GA29	Crawford, GA	2021	8.2	**S-LR***
GA30	Crawford, GA	2021	9.2	**S-LR**
GA31	Crawford, GA	2021	2.2	**S-LR**
GA32	Crawford, GA	2021	14.8	**S-LR**
GA33	Crawford, GA	2021	33.9	**S-LR***
GA34	Crawford, GA	2021	0.0	**S**
GA35	Taylor, GA	2021	2.0	**S-LR**
GA36	Taylor, GA	2021	0.0	**S**
GA37	Taylor, GA	2021	0.0	**S**
GA38	Taylor, GA	2021	0.0	**S**

GA39	Taylor, GA	2021	0.0	**S**
GA41	Taylor, GA	2021	10.8	**S-LR**
GA42	Taylor, GA	2021	0.0	**S**
GA43	Crawford, GA	2021	10.6	**S-LR**
GA44	Crawford, GA	2021	0.0	**S**
GA45	Crawford, GA	2021	34.3	**S-LR***
GA46	Crawford, GA	2021	0.0	**S**
GA47	Crawford, GA	2021	13.0	**S-LR**
GA49	Crawford, GA	2021	3.7	**S-LR**
GA50	Crawford, GA	2021	18.3	**S-LR***
GA51	Crawford, GA	2022	10.6	**S-LR**
GA52	Crawford, GA	2022	0.0	**S**
GA53	Crawford, GA	2022	0.0	**S**
GA54	Crawford, GA	2022	11.1	**S-LR**
GA55	Crawford, GA	2022	0.0	**S**
GA56	Crawford, GA	2022	0.0	**S**
GA57	Crawford, GA	2022	0.0	**S**
GA58	Crawford, GA	2022	0.0	**S**
GA59	Crawford, GA	2022	0.0	**S**
GA60	Crawford, GA	2022	0.0	**S**
GA61	Taylor, GA	2022	0.0	**S**
GA62	Taylor, GA	2022	5.5	**S-LR**
GA63	Taylor, GA	2022	8.7	**S-LR***
GA64	Taylor, GA	2022	0.0	**S**
GA65	Taylor, GA	2022	0.0	**S**
GA66	Taylor, GA	2022	0.0	**S**
GA67	Taylor, GA	2022	0.0	**S**
GA68	Taylor, GA	2022	0.0	**S**
GA69	Taylor, GA	2022	0.0	**S**
GA70	Taylor, GA	2022	0.0	**S**
GA71	Peach, GA	2022	0.0	**S**

[a] Percent relative growth on potato dextrose agar (PDA) amended with 1.0 µg/ml thiophanate-methyl.

[b] Percent relative growth on potato dextrose agar (PDA) amended with 500 µg/ml thiophanate-methyl.

[c] Sensitivity phenotypes consisting of sensitive (S), moderately sensitive (S-LR), and highly resistant (HR) to thiophanate-methyl. Asterisks (*) indicate isolates that were sequenced.

References

Adaskaveg, J. E., Michailides, T., and Eskalen, A. 2022. Fungicides, bactericides, biocontrols, and natural products for deciduous tree fruit and nut, citrus, strawberry, and vine crops in California. University of California, Davis.

Amiri, A., Brannen, P. M., and Schnabel, G. 2010. Reduced sensitivity in *Monilinia fructicola* field isolates from South Carolina and Georgia to respiration inhibitor fungicides. Plant Dis. 94:737-743.

Blaauw, B., Brannen, P., Lockwood, D., Schnabel, G., and Ritchie, D. 2023. Southeastern peach, nectarine, and plum management and culture guide. University of Georgia Extension.

Brannen, P. M., Hotchkiss, M., Reilly, C., Amiri, A. 2008. Evaluation of fungicide programs to manage a DMI-resistant *Monilinia fructicola* population on peach, 2007. PDMR. 2:STF003.

Brannen, P. M., Hotchkiss, M., Reilly, C., Schnabel, G. 2006. Evaluation of fungicide programs to manage a DMI-resistant *Monilinia fructicola* population in a Georgia peach research block, 2005. F&N Tests. 61:STF001

Chen, F., Liu, X., and Schnabel, G. 2013. Field Strains of *Monilinia fructicola* resistant to both MBC and DMI fungicides isolated from stone fruit orchards in the Eastern United States. Plant Dis. 97:1063-1068.

Chen, S. N., Shang, Y., Wang, Y., Schnabel, G., Lin, Y., Yin, L. F., and Luo, C. X. 2014. Sensitivity of *Monilinia fructicola* from peach farms in China to four fungicides and characterization of isolates resistant to carbendazim and azoxystrobin. Plant Dis. 98:1555-1560.

Chi, M. H., Park, S. Y., and Lee, Y. H. 2009. A quick and safe method for fungal DNA extraction. Plant Pathol J. 25(1):108-111.

Ennos, R. A., McConnell, K. C. 1995 Using genetic markers to investigate natural selection in fungal populations. Can. J. Bot. 73:S302-S310.

EPA. 2016. Pesticides: registrations. Benomyl RED facts. URL: https://archive.epa.gov/pesticides/reregistration/web/html/benomyl_fs.html

Egüen, B., Melgarejo, P., and De Cal, A. 2015. Sensitivity of *Monilinia fructicola* from Spanish peach orchards to thiophanate-methyl, iprodione, and cyproconazole: fitness analysis and competitiveness. Eur J Plant Pathol. 145:789-801.

Fan, J., Fang, Y., and Guo, L. 2009. Sensitivity of *Monilinia fructicola* isolates to thiophanate-methyl and boscalid. Acta Phytophylacica Sinica, 36:251–256.

FRAC (Fungicide Resistance Action Committee) (2023) Background information on benzimidazoles. URL: https://www.frac.info/frac-teams/expert-fora/benzimidazoles.

Fischer, J. M. M., Dutra, P. S. S., Ebbinghaus de Araujo, H., Glienke, G., and May de Mio, L. L. 2023. Field isolates of *Monilinia fructicola* change resistance pattern to greater sensitivity to thiophanate-methyl in recent populations. Eur J Plant Pathol. 166:51-64.

Hawkins, N. J. and Fraaije, B. A. 2016. Predicting resistance by mutagenesis; lessons from 45 years of MBC resistance. Front. Microbiol. 7:1814.

Ivic, D., Fazinic, T., Novak, A., Sajbic, L., Stepanovic, M., Culjak, T. G., and Miliĉević, T. 2021. Resistance of *Monilinia fructicola* to thiophanate-methyl in Croatia. Eur. J. Plant Pathol. 159:211-217.

Koenraadt, H., Somerville, S. C., and Jones, A. L. 1992. Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. Phytopathology. 82:1348-1354.

Landgraf, F. A., and Zehr, E. I. 1982. Inoculum sources from *Monilinia fructicola* in South Carolina peach orchards. Phytopathology 71:246.

Lehner, M. S., Paula Júnior, T. J., Silva, R. A., Vieira, R. F., Carneiro, J. E. S., Schnabel, G., and Mizubuti, E. S. G. 2015. Fungicide sensitivity of *Sclerotinia sclerotiorum*: A thorough assessment using discriminatory dose, EC50, high-resolution melting analysis, and description of new point mutation associated with thiophanate methyl resistance. Plant Dis. 99:1537-1543.

Leroux, P., and Walker, A. S. 2013. Activity of fungicides and modulators of membrane drug transporters in field strains of *Botrytis cinerea* displaying multidrug resistance. Eur J Plant Pathol. 135:683-693.

Lim, T. H., Johnson, I., and Cha, B. 2006. Characterization and genetic diversity of benzimidazole-resistant and -sensitive *Monilinia fructicola* isolates in Korea. Plant Pathol. J. 22:369-374

Litchtemberg, P. S. F., Luo, Y., Morales, R. G., Muehlmann-Fischer, J. M., Michailides, T. J., and May-De Mio, L. L. 2017. The point mutation G461S in the *MfCYP51* gene is

associated with tebuconazole resistance in *Monilinia fructicola* populations in Brazil. Phytopathology. 107:1507-1514.

Ma, Z., Yoshimura, M. A., and Michailides, T. J. (2003). Identification and characterization of benzimidazole resistance in M*onilinia fructicola* from stone fruit orchards in California. Applied and Environmental Microbiology. 69:7145-7152.

Ma, Z., Yoshimura, M. A., Holtz, B. A., and Michailides, T. J. 2005. Characterization and PCR-based detection of benzimidazole-resistant isolates of *Monilinia laxa* in California. Pest Manag Sci. 61:449-457.

Malandrakis, A. A., Markoglou, A. N., and Ziogas, B. N. 2012. PCR-RFLP detection of the E198A mutation conferring resistance to benzimidazoles in field isolates of *Monilinia laxa* from Greece. Crop Protection. 39:11-17.

Martini, C., Guidarelli, M., Di Francesco, A., Ceredi, G., and Mari, M. 2016. Characterization of thiophanate methyl resistance in Italian *Monilinia fructicola* isolates. Journal of Plant Pathol 98:453-462.

May-De Mio, L. L., Luo, Y., and Michailides T. J. 2011. Sensitivity of *Monilinia fructicola* from Brazil to tebuconazole, azoxystrobin, and thiophanate-methyl and implication on disease management. Plant Dis. 95:821-827.

McKay, G. J., Egan, D., Morris, E., and Brown, A. E. (1998). Identification of benzimidazole resistance in *Cladobotryum dendroides* using a PCR-based method. Mycol Res. 102: 671-676.

Nakaune, R., & Nakano, M. (2007). Benomyl resistance of *Colletotrichum acutatum* is caused by enhanced expression of β-tubulin 1 gene regulated by putative leucine zipper protein CaBEN1. Fungal Genetics and Biology. 44:1324-1335.

Orbach, M. J., Porro, E. B., and Yanofsky, C. 1986. Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. Molecular and Cellular Biology, 6, 2452-2461.

Penrose, L. J., Davis, K.C., and Koffman, W. 1979. The distribution of *Sclerotinia fructicola* (Wint.) Rehm. in stone fruit orchards in New South Wales and comparative studies with susceptible isolates. Aust. J. Agric. Res. 30:307-319.

Pereira, W. V., Morales, R. G. F., Bauer, A. I. G., Kudlawiec, K., and May De Mio, L. L. (2020). Discontinuance of tebuconazole in the field restores sensitivity of *Monilinia fructicola* in stone fruit orchards. Plant Pathol. 69:68-76.

Schmidt, L. S., Ghosoph, J. M., Margosan, D. A., and Smilanick, J. L. (2006). Mutation at β-tubulin codon 200 indicated thiabendazole resistance in *Penicillium digitatum* collected from California citrus packinghouses. Plant Dis. 90:765-770.

Schnabel, G. and Brannen, P. M. 2022. Biology, epidemiology, and management of diseases of peach driving the spray program in the southeastern United States. Sci. Hortic. 295:110818.

Schnabel, G., Amiri, A., and Brannen, P. M. 2011. Field kit- and internet–supported fungicide resistance monitoring. Pages 116-131 in: Fungicide Resistance in Crop Protection: Risk and Management. T. S. Thind, ed. CAB International, Cambridge, MA.

Sonoda, R. M., Ogawa, J. M., Manji, B. T., Shabi, E., and Rough, D. 1983. Factors affecting control of blossom blight in a peach orchard with low level benomyl-resistant *Monilinia fructicola*. Plant Dis. 67:681-684.

Sonoda, R. M., and Ogawa, J. M. 1982 Growth rate of *Monilina fructicola* resistant and sensitive to benomyl on potato-dextrose agar and peach fruit. Plant Dis. 66:1155-1156.

Szkolnik, M., Ogawa, J. M., Manji, B. T., Frate, C. A., and Bose, E. A. 1978. Impact of benomyl treatments on populations of benomyl-tolerant *Monilinia fructicola*. (Abstr.) Phytopathol. News 12:239.

Watson, I. A. 1970. Changes in virulence and populations in plant pathogens. Annu. Rev. Phytopathol. 8:209-230.

Weger, J., Schanze, M., Hilber-Bodmer, M., Smits, T.H.M., Patocchi, A., 2011. First report of the β-tubulin E198A mutation conferring resistance to methyl benzimidazole carbamates in European isolates of *Monilinia fructicola*. Plant Dis. 95:497.

Yoshimura, M. A., Luo, Y., Ma, Z., and Michailides, T. J. 2004. Sensitivity of *Monilinia fructicola* from stone fruit to thiophanate-methyl, iprodione, and tebuconazole. Plant Dis. 88:373-378.

Young, D. H. 2015. Anti-tubulin agents. Fungicide resistance in pathogens: principles and guide to practical management. Springer. 93-103.

Zehr, E. I. 1982. Control of brown rot in peach orchards. Plant Dis. 66:1101-1105.

Zehr, E. I., Toler, J. E., and Luszcz, L. A. 1991. Spread and persistence of benomyl-resistant *Monilinia fructicola* in South Carolina peach orchards. Plant Dis. 75:590-593.

Zhu, F. X., Bryson, P. K., Amiri, A., and Schnabel, G. 2010. First report of the beta-tubulin E198A allele for fungicide resistance in *Monilinia fructicola* from South Carolina. Plant Dis. 94:1511.

CHAPTER THREE

PHYTOTOXICITY AND EFFICACY AGAINST PEACH BROWN ROT FOLLOWING PREHARVEST APPLICATIONS OF POLYOXIN-D WITH OR WITHOUT THYME OIL OR MINERAL OIL

Abstract

Polyoxin-D is a microbial fermentation product registered for use to control multiple diseases for multiple crops. However, there is limited knowledge of the effect of polyoxin-D against peach brown rot or potential efficacy improvements through combinations with essential or mineral oils. In this study, we evaluated the efficacy in a multiyear study of polyoxin-D with or without thyme oil or a mineral oil to control brown rot on peach caused by *Monilinia frucicola*. Treatments were applied in weekly intervals preharvest and included OSO 5% (polyoxin-D), OSO 5% + ThymeGuard 0.25% (polyoxin-D + thyme oil), and OSO 5% + PureSpray Green 0.5% (polyoxin-D + mineral oil). Assessment of preharvest brown rot revealed that the OSO 5% treatment had less disease incidence (9.4% on average) compared to the untreated control (20.0% on average) though no significant differences between OSO 5% + PureSpray Green (11.8% on average) and OSO 5% + ThymeGuard (11.4% on average). All three OSO 5% treatments reduced postharvest brown rot compared to the untreated control but there were no significant differences in disease incidence. Phytotoxicity was observed in the form of premature leaf drop. Greatest leaf drop was recorded for OSO 5% + PureSpray Green with 36.5% blind nodes on average. Trees treated with OSO 5% and OSO 5% + ThymeGuard revealed significantly less defoliation with 15.4% and 13.1% on average blind nodes, respectively, while the untreated control exhibited 6.6% on average blind nodes. In summary, polyoxin D applied by handgun to runoff in form of OSO 5% caused some premature leaf drop

and significantly reduced preharvest and postharvest brown rot disease but thyme oil or mineral oil did not improve its efficacy.

Introduction

Biorational products, also called biopesticides, may be useful tools to manage brown rot of peach if proven effective. These products include plant extracts, fermentation products, and biological agents (Adaskaveg et al. 2022). Biorational products possess relatively low mammalian and environmental toxicity and benefit from an overall positive public appeal (Kim and Hwang 2007; Nazzaro et al. 2017). However, few studies are available examining their usefulness to manage peach brown rot. Label-recommended usage for agricultural biopesticides for disease control of peach include bloom sprays to control blossom blight, preharvest fruit or foliar sprays to control preharvest brown rot, and postharvest applications in packinghouses to control postharvest rots. Although there are a wide variety of biorational products available, few have been shown to have efficacy either in preharvest or postharvest settings (Adaskaveg et al. 2022).

Polyoxin-D zinc salts are naturally occurring bacterial metabolites produced by *Streptomyces cacaoi* (Kim and Hwang 2007). They are considered biorationals and have shown promising results for disease management in either preharvest and/or postharvest applications (Adaskaveg et al. 2022). Polyoxin-D was first registered as a fungicide in the late 1990s and has since been recommended in other crops such as small fruits, stone fruits, pomes, vegetables, small grains and turf grasses as a FRAC 19 fungicide (FRAC 2022; USDA 2021). The fungicide inhibits the formation of chitin, an essential building block for the development of fungal cell walls, by interfering with chitin synthetase production (Endo et al. 1970). Several publications have shown that preharvest or postharvest applications of polyoxin-D on peach contributed to significantly less

brown rot when compared to an untreated control in California, New Jersey, and Virginia (Adaskaveg et al. 2022; Lanancette et al. 2017; Yoder et al. 2018).

Oils, such as essential oils and mineral oils, have been used in plant protection in mixtures with fungicides to increase their efficacy. Specifically for mineral oils, the addition has been observed increasing efficacy of fungicides by minimizing evaporation and doubling as an adhesive spreader (Erwin et al. 1974). Additionally, mineral oils can display antisporulative activity and curative action as studied on powdery mildew (Northover and Schneider 1996). Essential oils, such as thyme oil, have been shown to have some efficacy against a variety of plant pathogens on their own and in combination with other essential oils (Nikkhah and Hashemi et al. 2020). Multiple modes of action were reported for essential oils, including cell wall damage, increase of reactive oxygen species (ROS), enzyme inactivation, and DNA damage (Cao et al., 2022). Mixtures of essential oils and synthetic fungicides in pharmaceutical and agricultural settings show promising results such as the synergistic effect of mixing bay leaf oil (*Laurus nobilis* L.) and iprodione for controlling *Sclerotium cepivorum* (Camiletti et al. 2016) and mixing cinnamaldehyde and fluconazole for controlling the human pathogens *Aspergillus fumigatus* and *Trichophyton rubrum* (Khan and Ahmad 2011). Mixtures of fungicides and essential oils are also available for agricultural use such as Reveg^{TM}, a combination of tea tree oil and difenoconazole (Reuveni 2019). To the best of our knowledge, combinations of mineral oils or essential oils with polyoxin-D have not been studied.

The objectives of this study were to evaluate the efficacy of preharvest applications of polyoxin-D (OSO 5%) with or without an essential oil (ThymeGuard) or a mineral oil (PureSpray Green) on preharvest and postharvest brown rot development and to assess any potential side effects.

Materials and Methods

Plant and biorational product materials. Peach cultivar 'Juneprince' was used for the evaluation of preharvest and postharvest brown rot and phytotoxicity development between years 2021 to 2023. Trees were approximately 7 yrs-old at the start of the trials and were located at the Musser Fruit Research Center in Seneca, SC. Biorational products included OSO 5% (Certis U.S.A L.L.C, Columbia, MD, USA), PureSpray Green (Intelligro^{TM}, Mississagua, ON, CAN), and ThymeGuard (Agro Research International, Sorrento, FL, USA) at doses 13 fl oz/acre, 0.5%, and 0.25%, respectively.

Application of biorational fungicides to peach trees. Prior to the application of biorational fungicides, peach trees received fungicide and insecticide sprays of both captan and pyrethroids or phosmet in intervals of 10- to 14-days. They were applied starting 2 weeks after shuck split until four weeks before harvest. Immediately prior to experimental preharvest applications, any symptomatic fruit with brown rot was removed. Treatments were applied on four single tree replicates in a randomized design. Trees were sprayed using a handgun until the suspensions were dripping off the leaves and fruit (sprayed to runoff). Spray volume and pressure of handgun application was 2 gal/tree (200 gal/acre) and 100 psi, respectively. Trees received three preharvest treatments on 27 May, 5 June, and 11 June 2021; 1 June, 7 June, and 16 June 2022; and May 19, May 26, and June 1 2023.

Preharvest evaluation of brown rot. One day after the third preharvest application, fruit of commercial shipping maturity (firm fruit with little to no green color at stem area present) were evaluated for the percent preharvest disease incidence. Incidence was assessed by averaging the presence and absence of brown rot in the canopy on ~50 fruit in 2021 and 2022 and 100 fruit in 2023.

Postharvest evaluation of brown rot. Fifty asymptomatic fruit of commercial shipping maturity were collected from each experimental tree and placed into two 28-pocket plastic trays per tree and stored in an air-conditioned room at 70-72ºF. Percent decay was recorded after 3 and 7 days postharvest (dph). Decayed fruit were removed from trays at 3 days to prevent spread of disease through proximity.

Phytotoxicity analysis. Approximately one week after harvest, experimental trees were rated for premature leaf drop. Ten 1-year old twigs emerging from terminal branches were used per experimental tree to determine the percent premature leaf drop. For that, the number of blind nodes and the number of leaves per branch were counted to calculate percent blind nodes.

Statistical analysis. Analysis of Variance (ANOVA) was used to test for the effects of treatment, year; and treatment by year interaction on preharvest and postharvest brown rot and phytotoxicity. If effects were found to be significant, mean separation (using Student's t-test) was used to determine the nature of the effect. To ensure that ANOVA and Student's t-test were accurate we checked for outliers, normal distribution, and unequal variances and found no presiding issues. All statistical calculations were performed with JMP 16.2.0 software. P-values less than 0.05 were considered evidence of statistical significance.

Results

Preharvest evaluation of brown rot. The ANOVA test for the interaction of treatment by year was not significant (P=0.8833). This indicated that the treatment effects were very similar from year to year. Therefore, the overall treatment means (pooled over years) were used when determining the nature of the treatment effect. Incidence in brown rot was highest in the untreated control at 20.0%. All experimental treatments performed better than the untreated control but were not significantly different between each treatment (Table 1).

Postharvest evaluation of brown rot. The ANOVA test for the interaction of treatment by year was not significant at 3 dph (P= 0.1131) and 7 dph (P= 0.1821). This indicated that the treatment effects at 3 dph and 7 dph were very similar from year to year. Therefore, the overall treatment means at 3 dph and 7 dph (pooled over years) were used when determining the nature of the treatment effect. Disease pressure was extremely high resulting in brown rot incidences in the untreated control of 30.8% and 86.6% for 3 and 7 pdh, respectively. All experimental treatments significantly reduced brown rot incidence at 3 and 7 dph compared to the control but none of the treatments reduced the disease substantially. OSO 5%, OSO 5% + PureSpray Green, and OSO 5% + ThymeGuard had incidences of 18.8%, 17.6%, and 20.3%, respectively, for 3 dph and 70.7%, 64.9%, and 69.4%, respectively, for 7 dph.

Phytotoxicity analysis. The ANOVA test for the interaction of treatment by year was not significant (P= 0.1667). This indicated that the treatment effects were very similar from year to year. Therefore, the overall treatment means (pooled over years) were used when determining the nature of the treatment effect. Increased premature leaf drop was observed in all treatments, including the untreated control. OSO 5% + PureSpray Green had the greatest percentage of premature leaf drop expressed as 'blind nodes' with 36.5% (Table 2). OSO 5% and OSO 5% + ThymeGuard treatments were not significantly different and exhibited significantly less defoliation than OSO 5% + PureSpray Green , with 15.4% and 13.1% blind nodes, respectively. The untreated control had the least percentage of blind nodes at 6.6%.

Discussion

Biorational products, including polyoxin-D, have not yet been adopted for peach disease management in the United States mostly because of lack of reliable efficacy. Typically, products with polyoxin-D have shown more promise as a postharvest drench, though preharvest

applications have been studied. Adaskaveg et al. (2022) showed that polyoxin-D is moderately effective at brown rot control as a preharvest application but had excellent efficacy in controlling blossom blight when applied as a bloom spray. Polyoxin-D when combined with an adjuvant reduced brown rot at 3 and 6 dph compared to the untreated control (Lalancette et al. 2017). Use of polyoxin-D from bud swell until 3 dph provided reduced brown rot compared to the untreated control when sprayed at 100 gal/acre via single nozzle hand gun at 200-250 psi (Yoder et al. 2018). Interestingly, Yoder el al. (2018) also found that using a lower dose of polyoxin-D (3.25 fl oz) resulted in less brown rot incidence on peach when compared to a higher dose (6.5 fl oz). While our focus was on preharvest application, postharvest applications also performed significantly better than the untreated control when used as a drench (Adaskaveg et al. 2022). This has also been seen in cherry as polyoxin-D as a suspension concentrate effectively controlled brown rot and is comparable with fludioxonil (300 mg/L) when used a as a drench (Adaskaveg and Förster 2015). In our study, we also confirm that polyoxin-D has efficacy against preharvest and postharvest brown rot when applied as preharvest treatment and is consistent with the already published studies. However, the level of control may not be at the level a farmer may find acceptable.

The addition of thyme oil or a mineral oil did not improve the efficacy of OSO 5%. To the best of our knowledge this is the first study examining a polyoxin-D/thyme oil or polyoxin-D/mineral oil combination. Thyme oil, produced by *Thymus valgaris* L., contains the monoterpene constituent thymol which has known fungicidal properties and has been registered as a pesticide in the US since 1964 (EPA 1993). Antifungal properties of thyme oil have been documented and include the induction of reactive oxygen species (ROS) in spores (Shen et al. 2016) and inhibition of H^+-ATPase efflux pumps (Ahmad et al. 2013) though further research is being conducted to

clarify the overall mode of action. Inhibition of *M. fructicola* mycelial growth and spore germination inhibition has been documented in vitro (Lazar-Baker et al. 2011; Santoro et al. 2018; Elshafie et al. 2015). However, no known accounts of longstanding preharvest efficacy have been documented for thyme oil (Adaskaveg et al. 2022). Mineral oil has many uses in agriculture such as the control of insects, weeds, and disease. Along with antisporulative properties, the ability to act as a spreader, and decreased evaporative qualities (Northover and Schneider 1996), mixtures of with fungicides could potentially increase efficacy against brown rot. If two products with fungicidal efficacy are combined at least some additive effect would be expected. The lack of additive effects therefore indicated antagonistic effects. Perhaps both oils had no fungicidal efficacy. Thymol is a volatile compound that is also photosensitive (Zhang et al. 2022). Environmental effects such as UV degradation and increased volatility due to the high temperatures of South Carolina may thus have impaired the efficacy of thyme oil and perhaps that of mineral oil as well. Another explanation may be that oils capture and inactivate some of the polyoxin D salts.

In the above-mentioned studies, no phytotoxicity was reported when polyoxin-D was applied to peach trees. In fact, Yoder et al. (2018) observed that applications of OSO 5% had less defoliation than untreated trees, though the concentrations of OSO 5% in our study was greater (13 fl oz compared to 6.5 and 3.25 fl oz). This is contrary to our finding as we observed a significant increase in leaf drop compared to an untreated control. A potential explanation for the defoliation could be the mode of application. In this study treatments were applied to runoff with a handgun. Handgun applications to runoff leave more spray material on the tree and increase the exposure time in liquid form due to longer drying time. While Yoder et al. (2018) used the same method as we did, the lower concentration may have mitigated the level of phytotoxicity. In

addition, the mixture of polyoxin-D and mineral oil (0.5%) significantly increased the amount of phytotoxicity compared to all other treatments. This increase in phytotoxicity may also be linked to the increased spray material and drying time or it could be caused by a phenomenon known as membrane disruption theory. Mineral oils can induce phytotoxicity through dissolution of foliar semipermeable cell membranes (Van Overbeek and Bloudeau 1954). This dissolution could cause an influx of polyoxin-D to enter through the cell membrane and cause phytotoxicity. Further research would need to be conducted to confirm this hypothesis.

In conclusion, our study indicates that polyoxin-d has efficacy against brown rot, but the addition of oils may not improve efficacy. Phytotoxicity is possible but may be dependent on the mode of applications.

Table 3.1 Combined three-year average incidence of brown rot (%) on 'Juneprince' peaches treated with OSO 5% and combinations with oils.

		Brown rot incidence (%)[b]		
Treatment (rate/A)	Time of Application (dbh)[a]	0 dph[c]	3 dph	7 dph
OSO 5% (13 fl oz)	14, 7, 1	9.4 b	18.8 b	70.7 b
OSO 5% (13 fl oz) + PureSpray Green (0.5%)	14, 7, 1	11.8 b	17.6 b	64.9 b
OSO 5% (13 fl oz) + ThymeGuard (0.25%)	14, 7, 1	11.4 b	20.3 b	69.4 b
Untreated Control	N/A	20.0 a	30.7 a	86.6 a

[a] dbh = days before harvest. N/A = no sprays were conducted.

[b] Column numbers followed by the same letter within the same column are considered not significantly different based on Student's t-test (preharvest p = 0.0016; postharvest (3 pdh) p = 0.0113; postharvest (7 dph) p = 0.0115).

[c] 0 dph represents evaluation on the day of harvest (preharvest evaluation).

Table 3.2 Combined three-year average of premature leaf drop (expressed as 'blind nodes') on 'Juneprince' peach trees following experimental treatments.

Treatment (rate/A)	Time of Application (dbh)[a]	Blind nodes (%)[b]
OSO 5% (13 fl oz)	14, 7, 1	15.4 b
OSO 5% (13 fl oz) + PureSpray Green (0.5%)	14, 7, 1	36.5 a
OSO 5% (13 fl oz) + ThymeGuard (0.25%)	14, 7, 1	12.8 b
Untreated Control	N/A	6.6 c

[a] dbh = days before harvest. N/A = no sprays were conducted.

[b] Column numbers followed by the same letter within the same column are considered not significantly different based on Student's t-test ($p < 0.0001$).

References

Adaskaveg, J. E., Chen, D., Cary. D., and Förster H. 2022. Overview of management practices with the use of biocontrols, natural products, and conventional fungicides to combat pre- and postharvest diseases of peach and nectarine fruit. Acta Horticulturae 1352:17-26

Adaskaveg, J. E., and Förster H. 2015. New postharvest decay control treatments that are exempt from residue tolerances in the United States. Acta Hort. 1079:299-306.

Ahmad, A., Khan, A., Manzoor, N. 2013. Reversal of efflux mediated antifungal resistance underlies synergistic activity of two monoterpenes with fluconazole. Eur. J. Pharm. Sci. 48:80–86.

Camiletti, B. X., Asensio, C. M., Gadban, L. C., Pecci, M. D. L. P. G., Conles, M. Y., and Lucini, E. I. 2016. Essential oils and their combinations with iprodione fungicide as potential antifungal agents against withe rot (*Sclerotium cepivorum* Berk) in garlic (*Allium sativum* L.) crops. Industrial Crops and Products. 85:117–124.

Cao, Z., Zhou, D., Ge, X., Luo, Y., and Su, J. 2022. The role of essential oils in maintaining the postharvest quality and preservation of peach and other fruits. Journal of Food Biochemistry 46.

Elshafie, H. S., Mancini, E., Camele, I., De Martino, L., and De Feo, V. 2015. *In vivo* antifungal activity of two essential oils from Mediterranean plants against postharvest brown rot disease of peach fruit. Industrial Crops and Products 66:11-15.

Endo, A., Kakiki, K., and Misato, T. 1970. Mechanism of action of the antifungal agent polyoxin D. J. Bacteriol. 104:189-196.

EPA. 1993 Thymol. R.E.D Facts. EPA-738-F-93-010. URL: www3.epa.gov/fs_PC-080402_1-Sep-93.

Erwin, D. C., Khan, R. A., and Buchenauer, H. 1974. Effect of oil emulsions on the uptake of benomyl and thiabendazole in relation to control of Verticillium wilt of cotton. Phytopathology 64:485-489.

FRAC. 2022. FRAC code list: fungal control agents sorted by cross-resistance pattern and mode of action (including coding for FRAC groups on product labels). URL: frac.info/frac-code-list-2022-final.pdf.

Kim, B. S., and Hwang, B. K. 2007. Microbial fungicides int the control of plant diseases. J. Phytopathology 155:641-653.

Khan, M. S. A., and Ahmad, I. 2011. Antifungal activity of essential oils and their synergy with fluconazole against drug-resistant strains of *Aspergillus fumigatus* and *Trichophyton rubrum*. Appl Microbiol Biotechnol. 90:1083–1094.

Lalancette, N., Blaus, L., and Feldman, P. 2017. Peach disease management with early season biorational and late season conventional fungicide programs, 2016. PDMR 11:STF015.

Lazer-Baker, E. E., Hetherington, S. D., Ku, V. V., and Newman, S. M. Evaluation of commercial essential oil samples on the growth of postharvest pathogen *Monilinia fructicola* (G. Winter) Honey. Applied Microbio. 52:227-232.

Nazzaro, F., Fratianni, F., Coppola R., and De Feo, V. 2017. Essential oils and antifungal activities. Pharmaceuticals 10:86.

Nikkhah, M., & Hashemi, M. (2020). Boosting antifungal effect of essential oils using combination approach as an efficient strategy to control postharvest spoilage and preserving the jujube fruit quality. Postharvest Biology and Technology 164:111159.

Northover, J., and Schneider, K. E. 1996 Physical modes of action of petroleum and plant oils on powdery and downy mildews of grapevines. Plant Dis. 80:544-550.

Reuveni, M. 2019. Regev^{TM} – a new potent hybrid product for the control of plant diseases: a mini review. Outlook Pest Man. 30:33-35.

Santoro, K., Maghenzani, M., Chiabrando, V., Bosio, P., Gullino, M. L., Spadaro, D., Giacalone, G. 2018. Thyme and savory essential oil vapor treatments control brown rot and improve the storage quality of peaches and nectarines, but could favor gray mold. Foods 7:7.

Shen, Q., Zhou, W., Li, H., Hu, L., and Mo, H. 2016. ROS involves the fungicidal actions of thymol against spores of *Aspergillus flavus* via the induction of nitric oxide. PLoSONE 11(5):e0155647.

USDA. 2021. Polyoxin D zinc salt. Technical evaluation report. URL: ams.usda.gov/PolyoxinDZincSaltTR2022

Van Overbeek, J. and Blondeau, R. Mode of action of phytotoxic oils. Weeds 3:55-65.

Yoder, K. S., Cochran II, A. E., Royston, Jr., W. S., Kilmer, S. W., and Kowalski, A. L. 2018. Disease control and phytotoxicity by treatments applied to Redhaven peach, 2017. PDMR 12:PF034.

Zhang, J., Hao, Y., Lu, H., Li, P., Chen, J., Shi, Z., Xie, Y., Mo, H., Hu, L. (2022). Nano-thymol emulsion inhibits *Botrytis cinerea* to control postharvest gray mold on tomato fruit. Agronomy 12: 2973.