FUNGICIDE EFFICACY AND RESISTANCE MANAGEMENT FOR *ERYSIPHE NECATOR*

by

BROOKE WARRES

(Under the Direction of Phillip M. Brannen and Marin Talbot Brewer)

ABSTRACT

Grapevine powdery mildew (GPM), caused by the fungus *Erysiphe necator*, is a devastating disease of wine grapes. *E. necator* is highly efficient at developing fungicide resistance with reports of resistance to both quinone outside inhibitors (QoIs) and demethylation inhibitors (DMIs) throughout the world. In this research, a survey was conducted to determine the frequency of QoI and DMI resistance-associated alleles in Georgia. Field trials were conducted to determine the efficacy of currently available fungicides to control GPM, if there was control synergy between sulfur and DMI mixtures, and if cross-resistance among DMIs is present in *E. necator*. Resistance to QoIs and DMIs was observed to be widespread in Georgia. There was variability in efficacy among the DMI fungicides tested, showing that cross-resistance is incomplete. Additionally, synergy between DMIs and sulfur was not observed, but sulfur can still be used in tank mixtures as an effective additive.

INDEX WORDS: Grapevine powdery mildew, *Erysiphe necator,* fungicide resistance, *Vitis vinifera*, grape

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

For grape growers worldwide, grapevine powdery mildew (GPM), caused by the fungal pathogen *Erysiphe necator* (syn. *Uncinula necator*) (Ascomycota: Erysiphaceae), is a difficult disease to manage. Many growers choose varieties of wine grapes (*Vitis vinifera* L. Vitaceae) for their flavor profiles and not their disease tolerance, so fungicide application is a commonly used disease management tool (Pirrello et al. 2019). There are many fungi and fungal-like organisms (oomycetes) in addition to *E. necator* that cause severe disease on grapes in the state of Georgia, United States, including Botrytis bunch rot (*Botrytis cinerea*), grape downy mildew (*Plasmopara viticola*), and black rot of grape (*Phyllosticta ampelicida*), making spray program development a challenge for grape growers (Wilcox et al. 2015). With the application of fungicides on at least a biweekly basis, evolution of fungicide resistance is a threat that also further complicates spray program development (Ma and Michailides 2005).

Wine Grape Production. Along with Italy, France, Spain, and China, the United States is one of the top five wine-producing countries in the world (Creasy and Creasy 2009). According to the National Association of American Wineries, grapes are the highest value fruit crop in the United States, with a production value of over $6.5 billion. The top wine producing states are California, Washington, and Oregon, but the East Coast also plays a large role with New York, North Carolina, and Virginia being in the top 10 (Wine America 2017).

While Georgia is not one of the top wine-producing states, wineries are still very important to the local economy and bring in over $24 million a year (Wolfe and Stubbs 2019). Most wineries and vineyards are found in northern counties, with the top producers being Lumpkin, White, and Habersham, but wineries and vineyards can be found scattered throughout the state. Unfortunately, it is impossible to grow *V. vinifera* grapes in the southern region of the state due to the high incidence of Pierce's disease (Hopkins and Purcell 2002), but interspecific hybrids (*Vitis vinifera* x *Vitis aestivalis*) and muscadines (*Vitis rotundifolia*) can be grown.

Grapevine Powdery Mildew. Powdery mildew is a devastating disease to the table and wine grape industries (Wilcox et al. 2015). This disease affects all green grapevine tissue; on leaves it interferes with photosynthesis and severe infections can cause fruit to split (Gadoury et al. 2007). Even a minor infection can decrease yields, cause off-flavors in wine, and make table grapes unmarketable (Calonnec et al. 2004).

E. necator is an obligate, biotrophic fungus in the phylum Ascomycota. This fungus specifically infects plants in the family Vitaceae including all *Vitis* species (Brewer and Milgroom 2010). Once a fungal spore reaches its host through wind transport, it will germinate and produce haustoria, specialized feeding hyphae that penetrate the plant's cell walls (Gadoury et al. 2011). These haustoria will absorb nutrients directly from plant cells. Eventually, cells die, resulting in necrotic patches. Most commonly, *E. necator* will reproduce asexually, producing chains of conidia that can infect any grape tissue on which they land (Willocquet and Clerjeau 1998). Once the primary inoculum is established, the GPM fungus can rapidly spread through an entire vineyard with conidia being dispersed between 12 to 24 days after colony development on the plant (Willocquet et al. 1998). Later in the season, the fungus can reproduce sexually and produce chasmothecia (formerly cleistothecia), a hardened hyphal structure containing

ascospores (Smith 1970; Gadoury 1988). In most regions, chasmothecia are produced in late autumn, but in places such as Georgia, where it is above freezing temperatures most of the year, chasmothecia can be seen from mid-summer to late fall and may cause significant secondary infections if they release their ascospores (Wilcox et al. 2015). Ascospores produced inside chasmothecia serve as the primary inoculum each spring; and the number of chasmothecia that overwinter in a vineyard heavily influences early-season disease severity (Gadoury 1988; Moyer et al. 2014). *Erysiphe necator* has two separate mating types, and they are present in a 1:1 ratio throughout the United States (Brewer et al. 2011). In order to produce chasmothecia and ascospores, both mating types of *E. necator* must be present (Smith 1970; Gadoury and Pearson 1991).

Originating from eastern North America, grapevine powdery mildew has spread to all grape-producing regions of the world (Brewer and Milgroom 2010; Pirrello et al. 2019). *Vitis vinifera*, the European wine grape, is the most susceptible species to powdery mildew. Since *E. necator* originated in North America, European wine grapes do mot have coevolved GPM resistance or tolerance (Brewer and Milgroom 2010). In 1845, the pathogen spread to Europe where it caused immense losses until it was discovered that sulfur application provided control (Tweedy 1981). Powdery mildew has been shown to infect all *Vitis* spp., including *Vitis rotundifolia* (muscadines)*;* however, *E. necator* isolates collected from muscadines are not pathogenic to other *Vitis* spp. (Gadoury and Pearson 1991; Frenkel et al. 2010).

Vitis spp. are most susceptible to GPM from bloom to three weeks post-bloom when they begin developing ontogenic, or age-related, resistance (Ficke et al. 2002; Gadoury et al. 2003). As the plant matures and the berries develop, the fruit can become completely resistant to this disease as soon as four weeks past fruit set (Ficke et al. 2002). This resistance is readily

observable in *V. vinifera* where it prevents fungal haustorium formation on the berries once they reach this stage (Gee et al. 2008). Grape leaves can also develop resistance, with a decreased infection rate observed in leaves older than 10-16 days (Doster and Schnathorst 1985; Calonnec et al. 2018).

Grapevine Powdery Mildew Management. While hybrid crosses of *V. vinifera* with American relatives, such as *Vitis aestivalis*, *Vitis rupestris*, and *Vitis riparia* are more tolerant to diseases including powdery mildew and Pierce's disease, these hybrid varieties are not as commercially popular as varieties of *V. vinifera* (Espinoza et al. 2018). Studies show "variety" is one of the most important factors to consumers when choosing a wine to purchase (Goodman 2009). Until the market shifts and encourages producers to grow more disease-tolerant plants, many vineyard owners prefer growing more susceptible varieties that can produce better-selling wine. Cultural practices are helpful for managing powdery mildew and decreasing the magnitude of epidemics, but fungicides are the main tools used to combat this disease.

Multi-site contact fungicides such as elemental sulfur, potassium bicarbonate, and paraffinic oil have been proven to provide control against powdery mildew. While sulfur is still an effective fungicide, there are many issues with its use, including the frequency of sprays required, phytotoxicity during high temperatures, and off-putting flavors in wine (Gadoury et al.1994). Unfortunately, multi-site fungicides have issues that can preclude their utility. For example, phytotoxicity is a common problem with sulfur and potassium bicarbonate (McManus et al. 2017) and contact fungicides may be rinsed off during rainfall at very susceptible time points and provide little to no disease control. Because of these issues, many growers have chosen to incorporate synthetic, single-site fungicides into their spray programs such as quinone outside inhibitors (QoIs, FRAC 11), demethylation inhibitors (DMIs, FRAC 3), and succinate

dehydrogenase inhibitors (SDHIs, FRAC 7). While these classes can provide excellent control of GPM, the Fungicide Resistance Action Committee (FRAC) lists these classes as 'medium to high risk' for resistance development in many pathogens (FRAC 2020). With resistance to all three of these classes being reported in *E. necator* in various areas of the world, growers are unsure of what to incorporate in their own spray programs (Délye et al. 1997; Gisi et al. 2002; Cherrad et al. 2018).

It has often been recommended to mix a multi-site fungicide with a systemic for added disease control and/or for resistance management (van den Bosch et al. 2014). Elemental sulfur is a common mixing component for control of a variety of fungal diseases due to its effectiveness and low risk of leading to resistance development (Tweedy 1981). When testing fungicide mixtures on *Monilinia fructicola* isolates that were less sensitive to DMIs, there was a synergistic relationship between sulfur and a DMI that resulted in better control than the individual products (Holb and Schnabel 2008). Based on these findings, it has been proposed that in areas with limited DMI sensitivity, combining a DMI and sulfur may prevent continued increases in DMI resistance. Similarly, DMI and sulfur combinations provided significantly better control of leaf spot (*Nothopassalora personata*) in peanuts (Culbreath et al. 2019). With DMI resistance becoming more widespread in *E. necator*, the combination of sulfur and various DMIs may provide an effective method to increase efficacy and reduce control failures with DMIs.

QoI Fungicide Resistance. QoI fungicides inhibit mitochondrial respiration by blocking electron flow through the electron transport chain (Bartlett et al. 2002). They became very popular as soon as they were registered for grapes in 1997. Unfortunately, by 2002, resistance had already developed to azoxystrobin (Wong and Wilcox 2002). While there are different compounds available within the QoI class, varying levels of control from chemicals with this

mode of action have not been observed due to complete cross-resistance (Baudoin et al. 2019; FRAC 2020). QoI resistance is caused by a single nucleotide mutation in the mitochondrial cytochrome *b* gene which results in the amino acid change of a glycine by alanine at position 143 (Gisi et al. 2002). This mutation, commonly called the G143A allele, does not result in a loss of fitness, meaning the likelihood of *E. necator* to survive in a population and reproduce is not compromised, and can therefore subsist in the population even without frequent spraying (Rallos et al. 2014). Since Wong and Wilcox (2002) first reported QoI resistance in *E. necator* in New York State, QoI resistance has been reported in Michigan (Miles et al. 2012) and Virginia (Baudoin et al. 2008). The G143A allele has even been found in vineyards that have not sprayed with this mode of action in over four years (Rallos et al. 2014). Because of the lack of fitness cost associated with this mutation and its ability to remain in the population indefinitely, it is usually recommended to remove QoI products from a spray program once resistance develops, leaving vineyard managers with less tools to control powdery mildew.

DMI Fungicide Resistance. Demethylation inhibitors (DMIs, FRAC 3) are effective chemicals for control of many fungal pathogens of both animals and plants, including many powdery mildews. These chemicals inhibit the biosynthesis of ergosterol, an essential constituent in fungal cell membranes, by altering the pathway encoded by the cytochrome P450 (*CYP51*) gene (Burden et al. 1989). Resistance mechanisms found to overcome this class of fungicides can include overexpression of the *CYP51* gene, overexpression of ATP binding cassette transporters, or a point mutation in the *CYP51* gene (Dufour et al. 2011). In *E. necator*, Délye et al. (1997) found a point mutation in *CYP51* changing a tyrosine to phenylalanine in codon 136, referred to as the Y136F mutation, which was associated with increased DMI tolerance. In addition to the Y136F mutation found, another mechanism found to be present in *E. necator*, and one that may

be working in conjunction with the mutation, is an increase in copy numbers of the *CYP51* gene (Frenkel et al. 2014; Rallos and Baudoin 2016). The presence of the Y136F mutation and the presence of multiple *CYP51* genes with this mutation is likely the cause of increased DMI tolerance in *E. necator.*

DMI resistance in *E. necator* has been reported in various areas throughout the United States including California (Gubler et al. 1996), New York (Erickson and Wilcox 1997), and Virginia (Colcol et al. 2012). Cross-resistance among different DMIs has not been observed, with reports of different chemicals losing efficacy in different areas. In California and New York, resistance to triadimefon and triadimefonol was observed (Gubler et al. 1996; Erickson and Wilcox 1997), but in Virginia there was decreased efficacy from tebuconazole and myclobutanil rather than triadimefon (Colcol et al. 2012).

Additional Resistance Development. While QoI and DMI resistance is the most frequently reported type of fungicide resistance in *E. necator* throughout the world, there have been reports of other types of resistance that will likely become an increasing problem. Succinate dehydrogenase inhibitors (SDHIs) (FRAC 7) are frequently used in grape disease management and are still performing well in most areas; however, resistance development could increase and spread. Cherrad et al. (2018) first reported resistance development to boscalid, an SDHI. Resistance to quinoxyfen (FRAC 13) has the potential to develop, and this chemical has been reported to have decreased efficacy in some regions (Deliere et al. 2010). In Italy, the emergence of metrafenone (FRAC 50) resistance has been observed by Kunova et al. (2015). Problems resulting from resistance development are likely to continue to increase in severity as resistance development becomes more prevalent throughout the world.

JUSTIFICATION AND OBJECTIVES

As reported above, two major fungicide classes used to control GPM are the QoIs and DMIs. Fungicide resistance to these chemical classes has been reported in *E. necator* populations in multiple areas of the United States as well as in other countries, but resistance has not been previously reported in Georgia. If QoI fungicide resistance is prevalent for *E. necator* populations in Georgia, then growers should consider ceasing application of these products completely, as resistance to QoIs has been confirmed for two other major pathogens of grape in Georgia, *Botrytis cinerea* and *Plasmopara viticola*. This could further complicate control of other diseases being controlled by QoIs such as black rot, caused by *Phyllosticta ampelicida*. As with the QoIs, the DMIs are also used for control of both powdery mildew and black rot. Resistance development in one or both of the fungi associated with these diseases would add yet another level of complexity when selecting fungicides for season-long disease management. To address these concerns and help grape growers in Georgia (and potentially other southeastern states) select the best fungicide application programs, the objectives of this research are to:

1. Determine the frequency of fungicide resistance in Georgia commercial vineyards by conducting a survey for QoI- and DMI-resistant strains of powdery mildew,
2. Determine efficacy of eleven currently available fungicides for grapevine powdery mildew control using field trials,
3. Test varying sulfur additions for potential synergy with DMI fungicides, and
4. Establish whether cross-resistance is present among different DMI fungicides.

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CHAPTER 2

SURVEY OF GEORGIA VINEYARDS FOR QOI AND DMI FUNGICIDE RESISTANCE-ASSOCIATED MUTATIONS IN ERYSIPHE NECATOR[2]

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Abstract

Grapevine powdery mildew (GPM) is a fungal disease caused by *Erysiphe necator*, and it is a recurrent issue for grape growers worldwide. In Georgia, United States (U.S.), GPM is one of the most prevalent and difficult to manage grape diseases, and the potential for *E. necator* to develop fungicide resistance is very high. In the U.S., resistance to quinone outside inhibitor (QoI) fungicides has been reported in Michigan, New York, and Virginia; and resistance to demethylation inhibitor (DMI) fungicides has been reported in California, New York, and Virginia. This survey determined the prevalence of the G143A allele, associated with complete QoI resistance, and the Y136F mutation, associated with increased DMI tolerance, in *E. necator* populations in Georgia vineyards. Of the 12 commercial vineyards surveyed from 2018 to 2020, 9 showed *E. necator* populations with the G143A allele. Of these, 7 vineyard populations showed the resistance associated mutation for 100% of the samples. The Y136F mutation was present in 4 of the 6 vineyards sampled from 2019 to 2020, with the majority of samples consisting of a mixed population for the mutation. Overall, QoI resistance is highly prevalent throughout Georgia's wine grape-producing regions. DMI resistance is likely present as well, but as DMI resistance is less well-defined by or directly associated with the presence of the Y136F mutation, and additional research needs to be conducted in order to fully understand the significance of this mutation in Georgia vineyards.

Introduction

Grapevine powdery mildew (GPM) is a global disease caused by the fungus *Erysiphe necator* – an ectoparasitic, obligate biotroph that can reproduce both asexually through conidia and sexually through chasmothecia. Once established on a grapevine, *E. necator* is a highly efficient parasite, and it can produce conidia in as little as five days (Gadoury et al. 2012). Conidia can occur on any green plant tissue, are wind dispersed, and will germinate upon landing on any suitable plant (Willocquet et al. 1998). Chasmothecia (formerly referred to as cleistothecia), the hardened structures containing ascospores, are produced by sexual reproduction when both mating types are present on a plant (Smith 1970; Gadoury 1988). Chasmothecia are the primary overwintering structures, and ascospores discharged in the spring serve as the primary source of inoculum for disease establishment. In Georgia, chasmothecia with ascospores can be produced multiple times throughout the season, leading to reinfection and promoting a high level of genetic diversity (Gadoury 1988).

Managing GPM requires both cultural and chemical practices. While there are more tolerant *Vitis* spp. and hybrid varieties than the European wine grape, *V. vinifera*, growers typically choose what to grow based on wine quality and not disease tolerance (Bavaresco 2019). In addition to GPM, wine grapes are susceptible to an extensive list of diseases caused by fungi and oomycetes around the United States including Botrytis bunch rot, black rot, ripe rot, downy mildew, anthracnose, *Phomopsis* cane and leaf spot, and many others (Wilcox et al. 2015). Fungicides are the main method of defense against the filamentous pathogens that cause these diseases, and in many areas, fungicides provide the only practical way that growers can produce a crop. Even without the presence of fungicide-resistant pathogens, developing an efficient spray

program can be difficult, as growers must incorporate products that protect against many different organisms.

The development of QoI fungicides (FRAC 11) was particularly important for grape disease management on the East Coast and Midwest, as they have activity against multiple grape diseases caused by fungi, such as black rot, powdery mildew, and ripe rot, while also providing control of downy mildew, an oomycete-caused disease. The first QoI registered for use on grape was azoxystrobin in 1997. As early as five years after market introduction, *E. necator* with resistance to azoxystrobin was reported in a commercial vineyard in New York (Wong and Wilcox 2002). Less than a decade later, resistance was also reported in Virginia (Baudoin et al. 2008) and Michigan (Miles et al. 2012). QoI resistance in *E. necator* is associated with a single nucleotide change from glycine to alanine within cytochrome *b* referred to as the G143A allele (Gisi et al. 2002). This mutation is associated with complete QoI resistance, meaning that when this mutation is present in the fungus, it is completely resistant to the fungicide. This type of resistance is referred to as qualitative resistance – meaning the fungus is either resistant or sensitive based on the presence or absence of this mutation, respectively. This mutation has no known associated fitness cost to the fungus, allowing the mutation to persist in the population for multiple years even in the absence of selection by the fungicide (Rallos et al. 2014).

Demethylation inhibitors (DMIs) (FRAC 3) are also a class of fungicides targeting a specific fungal metabolic pathway. Fungicides in this group target 14α-demethylase, which is encoded by *CYP51,* a cytochrome P450 gene that is necessary for ergosterol production (Schwinn 1984). Many phytopathogenic fungi, including *E. necator*, have developed a point mutation that changes phenylalanine to tyrosine at position 136 in CYP51, reducing the ability of the fungicide to bind 14α-demethylase (Délye et al. 1997). This mutation has been associated

with increased DMI tolerance, but it does not confer complete resistance like the G143A allele (Rallos and Baudoin 2016). Resistance to DMI fungicides is quantitative, meaning that there is a range of fungal sensitivities (McGrath 2009). In addition to the Y136F mutation, *CYP51* copy number plays a large role in DMI fungicide resistance in *E. necator* (Frenkel et al. 2014; Jones et al. 2014). DMI resistance in *E. necator* has been reported in California (Gubler et al. 1996), New York (Erickson and Wilcox 1997), and Virginia (Colcol et al. 2012). Within these reports, cross-resistance among all FRAC 3 fungicides has not been observed, suggesting that some chemicals may be more efficient at binding the target site even in the presence of the Y136F mutation.

The hot, wet, and humid climate in Georgia results in high disease pressure, and growers must spray fungicides frequently to control GPM and numerous other diseases, such as downy mildew and Botrytis bunch rot, caused by *Plasmopara viticola* and *Botrytis cinerea*, respectively. *Plasmopara viticola* and *B. cinerea* have already developed widespread QoI resistance in Georgia (Campbell et al 2020; Fernández-Ortuño et al. 2014), so it is possible that QoI resistance is present in *E. necator* as well. Along with QoIs, DMI fungicides are sprayed frequently on grapes in Georgia. Mutations associated with QoI and DMI resistance (G143A and Y136F) have been found together in *E. necator* in both the United States and France (Dufour et al. 2011; Rallos et al. 2014). In Dufour et al. (2011), 72% of samples in French vineyards that had the G143A allele also had the Y136F allele. In Rallos et al. (2014), the G143A allele was found along with the Y136F allele even four years after the last application with a QoI fungicide, leading them to hypothesize that DMIs could help maintain QoI resistance.

Our study aimed to determine the presence or absence of the G143A and Y136F mutations in *E. necator* populations in Georgia's commercial vineyards. *E. necator* samples were

collected from commercial vineyards in Georgia's wine-grape producing region, genotyped, and the allele frequencies were compared to a research vineyard where known QoI- and DMI-resistant *E. necator* is known to be present.

Materials and Methods

Sample Collection. Either ToughSpots (Diversified BioTech, Dedham, MA) or Cap-Shure Sterile Cotton Swabs (Puritan Medical Products, Guilford, ME) were used to collect *E. necator* samples once colonies of *E. necator* were clearly established on fruit or leaves. Samples were collected from *V. vinifera* or interspecific hybrids (*V. vinifera* x *Vitis* spp.) grown in northern Georgia counties: Gilmer, Habersham, Lumpkin, Rabun, Towns, Union, and White (Tables 2.1 and 2.2). When *E. necator* colonies were observed, ToughSpots were placed on a colony with forceps and lightly pressed to collect fungal tissue. The ToughSpot was then removed from the colony with forceps and placed in a sterile tube with labels including information such as GPS coordinates, grape phenology, variety, and date of sampling. If using Cap-Shure Sterile Cotton Swabs, GPM lesions on either fruit or leaves were swabbed with the tip of the cotton swab and placed back into the bag and labeled in the same manner as the ToughSpot samples. Samples were taken arbitrarily throughout each vineyard with total sample numbers per vineyard ranging from 3 to 75 dependent on the severity of the disease at a location. If disease was found in multiple areas throughout the vineyard, samples to represent that population were taken. While each ToughSpot and each swab are considered a single sample, each sample could account for multiple *E. necator* individuals depending on the severity of the infection at the location. Samples were stored at 4°C until processed.

DNA extraction and qPCR analysis. DNA was extracted using a modified (Miles et al. 2021) Chelex extraction method (Brewer and Milgroom 2010). Each swab was placed in a sterile 2 mL microcentrifuge tube containing 400 µL Chelex solution (5% w/v Chelex 100 sodium form 50-100 mesh [Sigma Aldrich] suspended in molecular grade water). Handles of swabs were removed and the tips of the swabs were fully immersed in the Chelex solution. The samples were vortexed at max speed for 5 min and centrifuged at 17,000 × *g* for 20 sec. Samples were incubated at 95°C for 10 min, vortexed for 5 sec, centrifuged at 17,000 × *g* for 20 sec, heated at 95°C for another 10 min, and allowed to cool to 22°C. Samples were centrifuged for a final 2 min at 17,000 × *g* and the liquid containing DNA was transferred to sterile 1.5 mL microcentrifuge tubes. DNA samples were analyzed with the G143A qPCR assay the same day they were extracted and then stored at -20°C until further analysis could be performed.

The DNA was analyzed using a competitive TaqMan qPCR assay to detect the G143A mutation associated with QoI resistance (Miles et al. 2021). Briefly, 1 µL of DNA from each sample was analyzed in 15 µL qPCR reactions containing 7.5 µL of Perfecta qPCR ToughMix ROX (Quanta Biosciences), 6.0 µL molecular-grade H_2O, 75 nM final concentration 83F and 517R forward and reverse primers, and 100 nM final concentration WT-G143A FAM and MT-G143A VIC TaqMan probes with QSY quenchers specific to the QoI sensitive G-143 and QoI resistant A-143 alleles, respectively (Table 2.3). Each sample was analyzed in duplicate on a StepOne Plus qPCR machine (Applied Biosystems) using the following cycling conditions: initial 2-min activation at 95°C followed by 55 two-step cycles of 95°C (15 sec) and 67°C (1 min). Every reaction plate contained a G143A-WT, G143A-MT, wild type (WT) and mutant type (MT), and a non-template control sample. The StepOne Software v2.3 was used for data acquisition and cycle threshold analysis (Ct) after manually setting the threshold for both

markers to allow for plate-to-plate relative comparisons. Samples were characterized as “QoI Sensitive” if only the G-143 allele amplified, “QoI Resistant” if only the A-143 allele amplified, “Mixed” if both alleles were detected, or “No Amplification” when neither allele was detected.

Samples containing sufficient quantities of *E. necator* DNA (G143A Ct value of 32 or lower, roughly equivalent to 100 conidia or more) were further analyzed with a competitive multiplex qPCR assay to test for both the presence of the Y136F mutation and for differences in copy number of the *Cyp51* gene shown to be associated with increased DMI tolerance. Each sample was analyzed in duplicate on a QuantStudio5 qPCR machine (Applied Biosystems) using the following cycling conditions: initial 5-min activation at 95°C followed by 45 two-step cycles of 95°C (15 sec) and 64°C (45 sec). Each 15 µL qPCR reaction consisted of 1 µL of DNA added to a mix of 7.5 µL TaqPath ProAmp Multiplex Master Mix with Mustang Purple (Applied Biosystems), 4.7 µL molecular-grade H2O, 200 nM final concentration Y136F Forward and Reverse primers and EnEF1 Forward and Reverse (En-g1817 F and R, Jones et al. 2014) primers, and 100 nM final concentration 136-Y-WT FAM, 136-F-MT VIC, Cyp51_noSNP JUN, and EnEF1 ABY TaqMan Probes (Table 2.4). The QuantStudio Design and Analysis Software was used for data acquisition and Ct analysis after manually setting the thresholds for each marker. Samples were characterized as “Wild Type” if only the Y-136 allele amplified, “Mutant Type” if only the F-136 allele amplified, or “Mixed” if both alleles were detected.

Results

QoI resistance survey. During 2018, 2019, and 2020, samples were collected from a total of 12 different commercial vineyards in seven counties (Table 2.1). An additional research vineyard with known QoI and DMI resistance was also sampled and labeled as ‘R’ for

comparison. Sampling was reliant on GPM being present in vineyards and sufficient mildew growth appearing on fruit or leaves. Of the counties surveyed, Lumpkin and White had the most vineyards with GPM levels high enough to sample. In Lumpkin County, 3 out of 4 vineyards sampled had 100% presence of the G143A allele. Only sampled in 2019, the fourth vineyard had a lower percentage (33%) of the mutation found in sampled colonies. In White and Union counties, the G143A allele was present in 100% of samples from each assessed vineyard.

Lower frequencies of the G143A allele were found in Gilmer, Rabun, and Towns counties. In Towns, populations sampled in one vineyard had 100% of the mutant allele, while the other had 0%. In Gilmer, only one vineyard was sampled in one year, and samples from that vineyard also had 0% of the mutant allele. In Rabun County, only 20% of samples taken from one vineyard had the mutation. Lower frequencies of the mutant allele were generally associated with newer, isolated vineyards where QoIs had not been sprayed over multiple years.

DMI resistance survey. Samples were collected from a total of 6 different commercial vineyards across 5 counties in 2019 and 2020 (Table 2.2). For comparison, a research vineyard with known DMI resistance was included and labeled as 'R'. As with the QoI survey, sampling was reliant on GPM being present in vineyards and sufficient mildew growth for collection. Of the vineyards sampled, only the research vineyard and one commercial vineyard had samples with full 'mutant type', meaning all colonies in that sample had the Y136F mutation. A majority of samples taken throughout Georgia were 'mixed', meaning that at least one individual in the colonies collected with the swab had the Y136F mutation, while at least one did not. Unfortunately, the frequency of the mutant allele in a mixed sample cannot be determined with our current method; mixed samples were obtained from 5 of 6 vineyards, but full 'wild type'

samples consisting of colonies completely lacking the Y136F mutation were only obtained from 3 of 6 vineyards.

Discussion

QoI resistance in *E. necator* is an increasing problem in Georgia. In this study, 9 out of 12 commercial vineyards with GPM had *E. necator* colonies that possessed the G143A allele associated with complete QoI resistance. Of these, 7 vineyards had populations of *E. necator* consisting entirely of G143A mutant individuals. Once this allele becomes fixed in a population, it is unknown if the individuals will ever lose the G143A allele and become sensitive to QoIs again, since previous work by Rallos et al. (2014) showed that a vineyard still had high levels of QoI resistance even four years after the last QoI spray event.

In our study, the vineyards without the G143A allele were typically isolated from other vineyards and more recently established (less than three years in production) (Figure 2.1). The two vineyards that had a mixed population (some samples were QoI resistant and others were QoI sensitive) are known to not spray single-site fungicides at the same frequency as other commercial growers in the area in an attempt to use organic contact protectants. In other commercial vineyards where grapes are heavily managed using all available fungicides, high levels of resistance were found.

In our research vineyard (Vineyard R, Table 2.2), DMI resistance has been confirmed through field efficacy trials along with the presence of both mutant and mixed samples (Chapter 3). Therefore, we hypothesize that similar efficacy results would be observed in commercial vineyards with similar frequencies of resistance in their populations. Out of the six vineyards screened, only one vineyard (Vineyard J, Table 2.2) had DMI resistance allele frequencies as

high as in the research vineyard. In the other five vineyards where the Y136F was present, the populations sampled had lower frequencies of this mutation. While this mutation does not confer complete DMI resistance, its presence in all vineyards sampled in Georgia is alarming. If this mode of action is relied upon and sprayed multiple times in a growing season, it could possibly lead to a buildup of a more tolerant *E. necator* population and cause a control failure.

Vineyards with high numbers of individuals with the G143A allele in their *E. necator* populations also showed a higher frequency of the Y136F mutation. In vineyards where the *E. necator* population lacked the G134A mutation, a lower frequency of the Y136F mutation was observed (Table 2.1 and 2.2). There were no vineyards where the G143A allele was found and the Y136F mutation was not. This was also observed in Rallos et al. (2014), where it was suggested that DMI applications could maintain the G143A allele. This could explain how the G143A allele is still present in high numbers in Georgia, even though many commercial vineyards have chosen to leave QoIs out of their spray programs.

The number of samples in this study was dependent on the presence of GPM in a commercial vineyard. Results were shared with growers to help them understand resistance and develop management strategies. The number of examined commercial vineyards with GPM decreased each year with n=8 in 2018, n=5 in 2019, and n=3 in 2020. As growers stop using QoIs, it is important that other modes of action are used in a proper rotation. Even in a proper rotation, there is a high risk of resistance developing in *E. necator* towards other fungicidal classes. *E. necator* populations should continue to be monitored to look for emerging fungicide resistance before widespread failures occur in commercial vineyards.

Conclusions

The G143A allele lacks an apparent fitness cost (Rallos et al. 2014). In Georgia, this mutation was found to be widespread in *E. necator*, even with growers leaving QoIs out of their spray programs. The Y136F mutation was found in much smaller numbers but was still present in all vineyards sampled. Grape growers in Georgia are not advised to use QoI fungicides for GPM unless they are in an isolated area with known QoI sensitivity. They should also be cautious about using DMI fungicides and may want to include a contact fungicide, such as sulfur, in their mix to prevent a possible control failure. Future work determining the frequencies of the Y136F mutation needed to result in DMI field failures would be helpful to determine if the presence of small numbers of this mutation should be concerning to growers.

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Table 2.1. QoI resistance survey of commercial vineyard in Georgia's wine-grape producing regions from 2018-2020.

County	Vineyard[a]	Year collected	QoI-resistant samples/total sampled[b]	Percent resistant
Lumpkin	A	2018	5/5	100%
	B	2018	5/5	100%
	C	2018	3/3	100%
	A	2019	11/11	100%
	F	2019	2/6	33%
	A	2020	15/15	100%
Towns	E	2018	3/3	100%
	L	2020	0/4	0%
Habersham	G	2018	4/4	100%
Rabun	H	2018	1/5	20%
White	D	2018	7/7	100%
	I	2018	3/3	100%
	I	2019	5/5	100%
Union	J	2019	10/10	100%
	R	2019	75/75	100%
	J	2020	10/10	100%
	R	2020	20/20	100%
Gilmer	K	2019	0/5	0%

Letters represent an independent vineyard with some vineyards sampled multiple years.
[a]Vineyard 'R' is a research vineyard with known QoI resistance and is shown for comparison.
[b]QoI resistant samples are determined by the presence of the G143A allele in the *cytochrome b* region. Samples labeled as resistant consisted of swabs containing colonies with the G143A allele present among all of them.

Table 2.2. DMI resistance survey of commercial vineyards in Georgia's wine-grape producing regions from 2019-2020

County	Vineyard[a]	Mutant[b]	Mixed[c]	Wild Type[d]	Year Collected
Lumpkin	A	-	11	-	2019
	F	-	2	3	2019
	A	-	14	-	2020
White	I	-	5	-	2019
Union	J	4	6	-	2019
	R	17	58	-	2019
	J	8	2	-	2020
	R	9	11	-	2020
Gilmer	K	-	1	4	2019
Towns	L	-	-	3	2020

[a]Letters represent an independent vineyard with some vineyards sampled multiple years. Vineyard 'R' is a research vineyard with known DMI resistance and is shown to compare.
[b]Mutant type refers to samples tested with all individuals on the swab having the Y136F mutation.
[c]Mixed refers to samples consisting of a mixture of wildtype and the Y136F mutation individuals.
[d]Wild type refers to samples consisting entirely of individuals that do not posess the Y136F mutation.

Table 2.3. Primers and probes used in the G143A qPCR for detection of the G143A mutation in the *cytb* gene (Miles et al. 2021).

Primers	Sequence (5′-3′)[a]
83F	CGCTACAGACTGGGTCACTG
517R	AGTCTCTTAGGGCCCCCATT
TaqMan Probes	
WT-G143A FAM	[6FAM] AGCCTATGGG**G**TGCAACCGT [QSY]
MT-G143A VIC	[VIC] AGCCTATGGG**C**TGCAACCGT [QSY]

[a]Bold and underlined nucleotides highlight the single nucleotide polymorphisms (SNP) binding locations for the competitive probes.

Table 2.4. Primers and probes (Miles and Jones et al. 2014) used in the qPCR for the real-time detection and quantification of the Y136F mutation and copy number differences in the *Cyp51* gene.

Primers	Sequence (5′-3’)[a]	Target gene
Y136F Forward	AAAGTAACAGTCTATCTGGGACT	*Cyp51*
Y136F Reverse	TACTCGACCATTTACGGACCT	*Cyp51*
EnEF1 Forward (En-g1817 F[1])	TGGAAAGTCTATTGAGGCAACTCC	*EnEF1*
EnEF1 Reverse (En-g1817 R[1])	CAACACACATAGGTTTAGATGGAATCA	*EnEF1*
TaqMan Probes		
136-Y-WT FAM	[6FAM] TTGGACAATCA**T**ATACAA [MGBNFQ]	*Cyp51*
136-F-MT VIC	[VIC] TTGGACAATCA**A**ATACAA [MGBNFQ]	*Cyp51*
Cyp51_noSNP JUN	[JUN] TCAACGCCGAAGAGATTTACACTAATTTAACA [QSY]	*Cyp51*
EnEF1 ABY	[ABY] TTAACAATTGCTGCGTCACCAGACTTAA [QSY]	*EnEF1*

[a]Bold and underlined nucleotides highlight the single nucleotide polymorphisms (SNP) binding locations for the competitive probes.

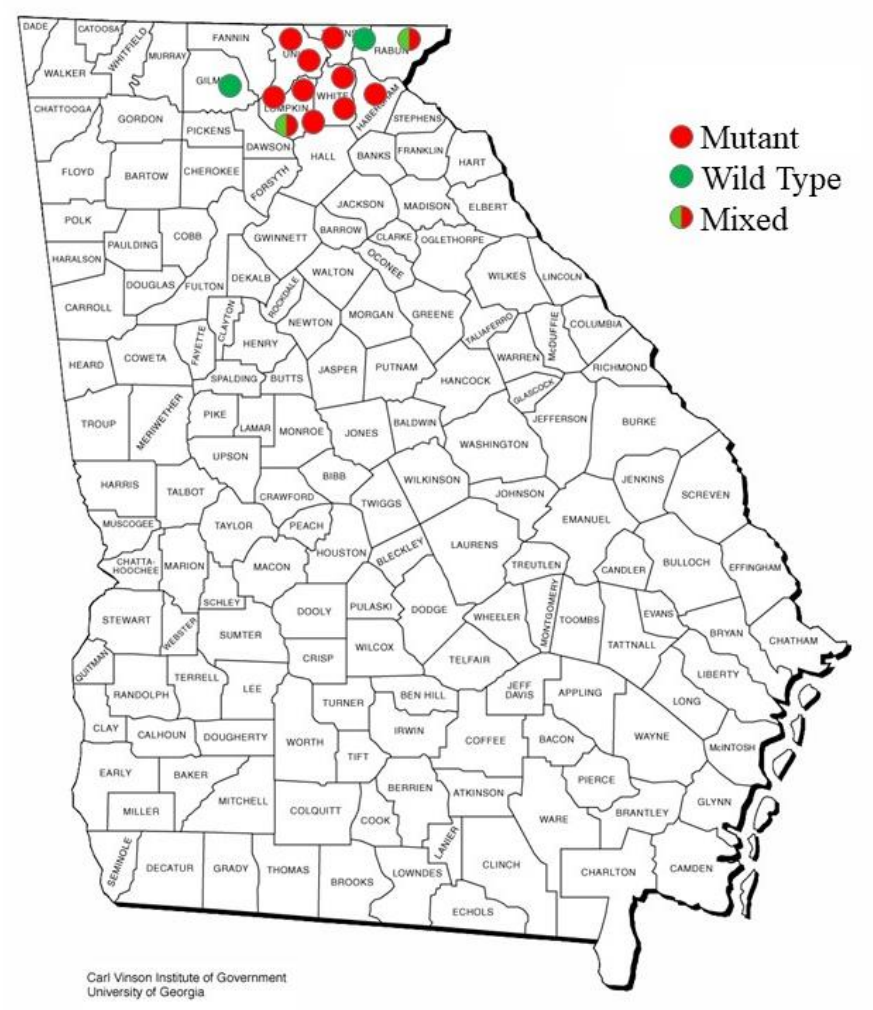

Figure 2.1. Map of QoI resistance survey results from commercial vineyards in Georgia's wine-producing region sampled in 2018 – 2020. Mutant is characterized by a sampled vineyard having the G143A allele in all individuals on a swab, wild type is characterized by a sampled vineyard lacking the G143A allele in all individuals on a swab, and mixed is characterized by some individuals in a vineyard having the G143A allele, while others did not.

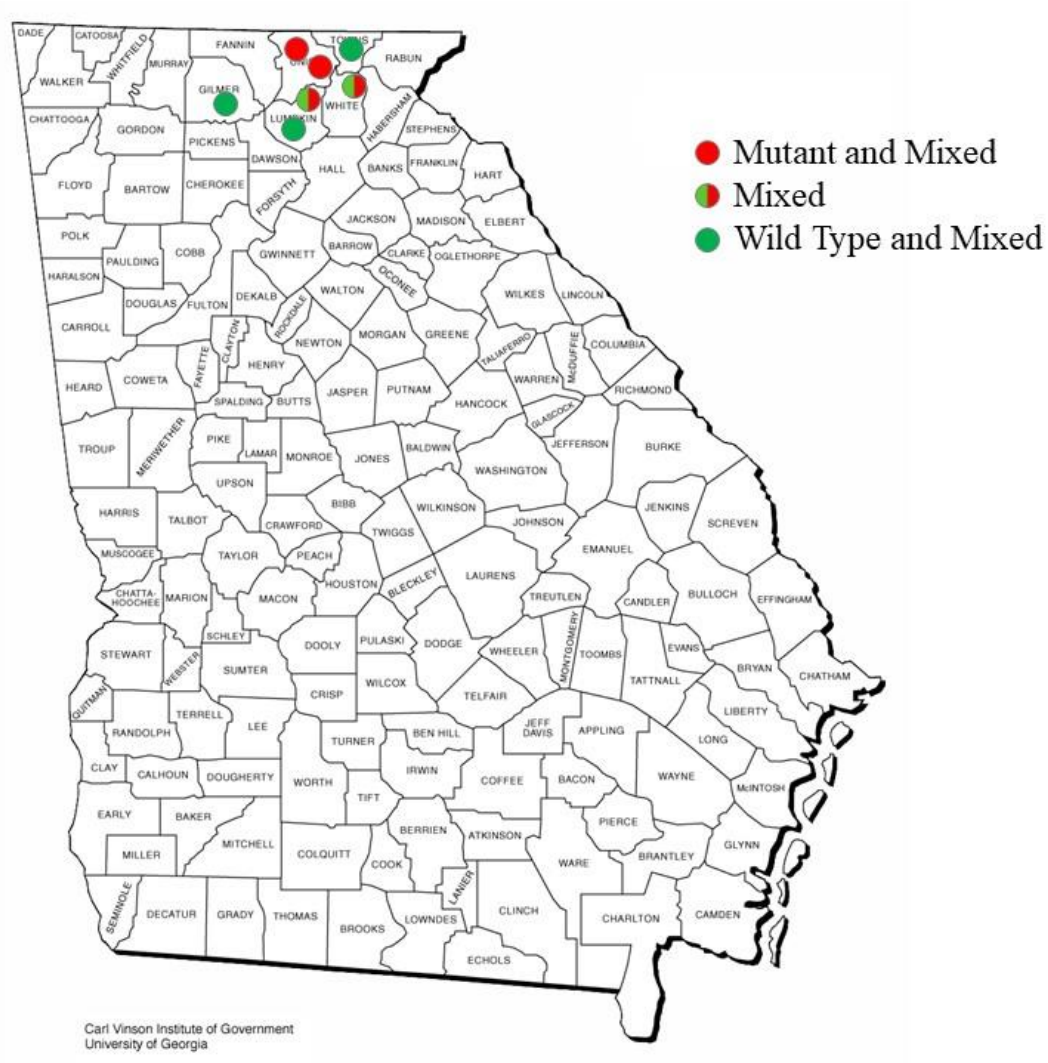

Figure 2.2. Map of DMI resistance survey results from commercial vineyards sampled during 2019 – 2020 in Georgia's wine-producing region. Mutant and Mixed refers to vineyards with a mixture of samples having either all mutant type (Y136F mutation present) or a mixture of individuals (some with Y136F mutation, some without Y136F mutation). Mixed refers to a vineyard with all samples consisting of a mixture of mutant (Y136F mutation present) and wild type (Y136F mutation absent) individuals. Wild Type and Mixed refer to vineyards with samples consisting of either all wild type (Y136F mutation absent) individual or with a mixture of Y136A mutant individuals and wild-type individuals.

CHAPTER 3

FUNGICIDE EFFICACY FOR CONTROL OF GRAPEVINE POWDERY MILDEW IN THE PRESENCE OF A QUINONE OUTSIDE INHIBITOR- AND DEMETHYLATION INHIBITOR-RESISTANT *ERYSIPHE NECATOR* POPULATION IN GEORGIA[3]

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Abstract

Grapevine powdery mildew (GPM), caused by the fungal pathogen *Erysiphe necator*, is a global disease of wine grapes that is difficult to manage. Throughout the world, resistance to many different single-site fungicides has been reported. In Georgia, resistance to quinone outside inhibitors (QoIs) has been reported in *Plasmopara viticola* (grapevine downy mildew) and *Botrytis cinerea* (Botrytis bunch rot). However, a comprehensive assessment of fungicide efficacy has not been conducted on GPM in Georgia. In this work, we conducted a field study that was repeated over two consecutive years (2019 and 2020) at the UGA Mountain Research and Education Center in Blairsville, GA to determine the efficacy of eleven different fungicides for GPM control. Field failures were observed with QoI and DMI fungicides. *E. necator* colonies were collected and genotyped for the G143A and Y136F alleles in *cytochrome b* and *14α-demethylase*, respectively. Both alleles were found in high numbers at the field site in both 2019 and 2020, indicating QoI and DMI resistance in the *E. necator* population. Other chemicals, including succinate dehydrogenase inhibitors (SDHIs), metrafenone, and quinoxyfen, provided excellent control of GPM.

Keywords: grape; *Vitis vinifera;* grapevine powdery mildew; *Erysiphe necator;* fungicide resistance

1. Introduction

Georgia may not be the main producer of wine grapes in the United States, but the industry is growing rapidly. The most recent economic report on the Georgia wine industry stated that it contributed over $81.6 million to the state economy, including wine sales and agritourism (Wolfe et al. 2013). With more consumers in the Southeast looking to spend their weekends at local wineries and vineyards, more vineyards are being established in this region; however, Georgia is a very difficult state in which to grow grapes. As elsewhere, one of the top concerns for grape growers in Georgia is grapevine powdery mildew (GPM). GPM is caused by *Erysiphe necator,* a biotrophic fungal parasite that is found in all grape-growing regions of the world (Gadoury et al. 2012). This fungus can overwinter both asexually as mycelium and sexually as ascospores in chasmothecia on dormant grapevines (Willocquet et al. 1998; Gadoury and Pearson 1998). In the spring, overwintered mycelium covers emerging buds, referred to as flag-shoots, and produces conidiospores. Conidia are wind dispersed, and once a spore lands on a vine, it will germinate, penetrate plant cells by means of a haustorium, begin feeding on plant cells, and produce yet more spores in a cycle that can occur every five days (Willocquet et al. 1998). As the infection becomes more severe and if both mating types are present, the fungus can reproduce sexually and form chasmothecia (thick-walled structure containing ascospores) that will release spores in the spring.

European wine grapes (*Vitis vinfera*) are the most susceptible to GPM, as they did not co-evolve with the fungus. Grapevine varieties developed from species native to North America, where *E. necator* is native, have a natural tolerance (Brewer and Milgroom 2010). All species of grape, however, have ontogenic or age-related resistance. Fruit is most susceptible to GPM at bloom and becomes less susceptible 2 to 3 weeks post bloom (Gee et al. 2008). About four

weeks past fruit set, if the cluster has not already been infected with *E. necator*, the cluster develops resistance and is highly unlikely to become infected during the remainder of the season (Ficke et al. 2002). While leaves can develop ontogenic resistance about 10-16 days after emergence, new leaves emerge constantly, leaving the plant susceptible throughout the entire growing season (Doster and Schnathorst 1985; Calonnec et al. 2018).

E. necator has the ability to produce abundant asexual propagules and recombine multiple times by sexual reproduction throughout the growing season with the potential to rapidly evolve fungicide resistance should a beneficial mutation occur. Single-site fungicides can provide excellent systemic control but have a high risk of leading to resistance development, resulting in lack of or significantly lowered efficacy (Baudoin et al. 2019; FRAC 2020). Quinone outside inhibitors (QoIs) (FRAC 11) and demethylation inhibitors (DMIs) (FRAC 3) are two single-site fungicide classes that are widely used in viticulture. QoI resistance in *E. necator* has been reported in New York (Wong and Wilcox 2002), Virginia (Baudoin et al. 2008), and Michigan (Miles et al. 2012). Additionally, decreased sensitivity to DMI fungicides in *E. necator* has been reported in California (Gubler et al. 1996), New York (Erickson and Wilcox 1997), and Virginia (Colcol et al. 2012). QoI resistance has been reported in other countries around the world including France (Dufour et al. 2011), New Zealand (Beresford et al. 2016), and Hungary (Taksonyi et al. 2013), with co-occurrences of DMI resistance in both France and New Zealand. In addition to resistance to QoI and DMI fungicides, there have been reports of resistance to chemicals including boscalid (FRAC 7) in France (Cherrad et al. 2018), metrafenone (FRAC 50) in Italy (Kunova et al. 2014), and decreased efficacy from quinoxyfen (FRAC 13) in France (Deliere et al. 2010).

Resistance to QoIs and DMI fungicides are caused by point mutations. QoI resistance is often caused by the G143A mutation in the cytochrome *b* region of the mitochondrial genome (Grasso et al. 2006). This mutation is qualitative, meaning that when *E. necator* possesses this mutation, it is able to completely overcome all QoI fungicides. By contrast, DMI resistance is quantitative. Increased tolerance to DMI fungicides in *E. necator* is associated with the Y136F point mutation in the cytochrome P450 (*CYP51*) gene, overexpression of the *CYP51* gene, or overexpression of ATP-binding cassette transporters (Délye et al. 1997; Frenkel et al. 2014). Even with these mutations, some FRAC 3 fungicides are still able to provide control of GPM as they are suspected to have a higher affinity to bind the 14α-demethylase enzyme, the end product of the *CYP51* gene. This resistance is referred to as quantitative because varying levels of resistance or increased tolerance are observed following these mutations (Deising et al. 2008).

Currently, there have not been any reports of fungicide-resistant *E. necator* in Georgia, but it is highly likely to be present due to the frequent use of fungicides to control many fungal and oomycete diseases of grape. Other damaging pathogens, such as downy mildew (*Plasmopara viticola*) and Botrytis bunch rot (*Botrytis cinerea*), have recently developed QoI resistance, leaving grape growers unsure of which modes of action to include in their spray programs (Campbell et al., 2020; Fernández-Ortuño et al., 2014). With the eastern United States being the center of diversity for *E. necator* (Brewer and Milgroom 2010), it is possible that resistance could develop more rapidly in this region than in other areas. In our study, we assessed many of the currently available fungicides registered for grape in Georgia to determine their field efficacy for, or their ability to significantly control, GPM and to determine if resistance has developed. Eleven different products with varying modes of action, including single- and multi-site fungicides, were tested over two years in Blairsville, Georgia, USA on *Vitis vinifera* grapes.

2. Materials and Methods

2.1 Fungicide efficacy trials

Fungicide efficacy trials were conducted at the University of Georgia Mountain Research and Education Center in Blairsville, Georgia, United States, in 2019 and 2020 to assess currently available fungicides registered on grape for their ability to control GPM. Fungicide treatments were chosen to represent a variety of modes of action registered for use on grape in Georgia. Treatments and group names were as follows: 1) Abound (QoI); 2) Aprovia (succinate dehydrogenase inhibitor [SDHI]); 3) Inspire Super (DMI + aniline-pyrimidine [AP]); 4) Luna Experience (DMI + SDHI); 5) Microthiol Disperss (inorganic electrophile); 6) Pristine (SDHI + QoI); 7) Quintec (aza-naphthalene); 8) Rally 40WSP (DMI); 9) Stylet Oil (not specified); 10) Torino (phenyl-acetamide); 11) Vivando (aryl-phenyl-ketone); and 12) an untreated control (no active GPM treatment applied) (Table 3.1). The experimental design was a randomized complete block design consisting of five replications per treatment with one plant used per replication. Unsprayed/untreated border rows between each treatment row acted as a buffer to prevent chemical drift between blocks and to increase GPM disease pressure. Treatments were applied in a water volume of 473 L/ha using a CO_2 sprayer (R & D Sprayers, Opelousas, LA) with a TeeJet adjustable cone tip nozzle (5500- PPX12) (TeeJet Technologies, Wheaton, IL) at a pressure of 172.4 kPa until runoff.

In 2019, treatments were applied five times to *V. vinifera* ‘Chardonnay’ vines on the following dates: at bloom (23 May), post-bloom (11 June), first cover (25 June), bunch closure (9 July), and second cover (23 July). An additional treatment was added in 2020 due to a cool spring, resulting in delayed phenology (longer bloom time), as well as delayed GPM development in the vineyard. In 2020, treatments were applied six times on *V. vinifera* ‘Merlot’

vines: at pre-bloom (27 April), bloom 1 (11 May), bloom 2 (26 May), post-bloom (8 June), bunch closure (22 June), and first cover (6 July).

In addition to the treatments applied as specified above, maintenance sprays to mimic commercial vineyard practices (Table 3.2) were applied to all grapevines to protect against other pathogens that would compromise the trial results. These maintenance sprays were applied every two weeks from bud break until pre-harvest with a John Bean Redline 537T Air Blast sprayer (Durand-Wayland, Inc., LaGrange, GA) at a 473 L/ha total spray volume.

2.2 Disease ratings

Fruit clusters were rated for disease incidence (percent of infected clusters per vine) and severity (percent of powdery mildew per cluster) prior to leaf ratings, as berries are less susceptible to GPM later in the season due to the development of ontogenic resistance. In 2019, two fruit cluster ratings were conducted two weeks apart on 11 July and 26 July. In 2020, three ratings were conducted a week apart on 8 July, 16 July, and 27 July. GPM coverage on five randomly selected clusters from each vine were assessed using the Powdery Mildew Assessment Tool by Adelaide Research and Innovation Pty Ltd. Five clusters from each vine were used to calculate the incidence and severity for each treatment in each block. Because percent cover data are often dominated by low and high values but deficient in intermediate values, disease severity data was transformed using an arcsine square root transformation to improve variable normality and analyzed using SAS 9.4. Means were compared using Tukey's HSD test.

Leaf incidence (percent of infected leaves per vine) and severity (percent of leaf covered with mildew signs and symptoms) data was collected by pulling 25 leaves from each vine and assessing each leaf for GPM. Each leaf was rated on a scale ranging from 0% to 100% in ten-

point increments. Two leaf ratings were conducted in both 2019 (6 and 13 August) and 2020 (5 August and 19 August). Leaf data was arcsine square root transformed and analyzed in SAS 9.4, and means were compared using a Tukey's HSD test.

2.3 DNA extraction and qPCR analysis

E. necator colonies were collected from a research vineyard in 2019 (n=75) and 2020 (n=20). In 2019, samples were collected specifically from vines treated with either QoIs or DMIs on 13 August, but 2020 samples were collected randomly throughout the vineyard, including unsprayed border rows, on 23 September. *E. necator* colonies were collected by rubbing a Cap-Shure Sterile Cotton Swab (Puritan Medical Products, Guilford, ME) on a GPM lesion found on leaves or fruit to gather fungal material on the cotton tip. If multiple lesions were present on a leaf or fruit cluster, they were collected on the same swab. The Cap-Shure Cotton Swabs were then sealed in zip-lock plastic bags, kept in coolers on ice, and refrigerated (4°C) until overnight shipment on ice.

DNA was extracted using a modified (Miles 2021) Chelex extraction method (Brewer and Milgroom, 2010) as stated in Chapter 2. Briefly, each swab was placed in a sterile 2 mL microcentrifuge tube containing 400 µL Chelex solution (5% w/v Chelex 100 sodium form 50-100 mesh [Sigma Aldrich] suspended in molecular grade water). Handles of swabs were removed and the tips of the swabs were fully immersed in the Chelex solution. The samples were then vortexed at max speed for five minutes and centrifuged at 17,000 × *g* for twenty seconds. Samples were then incubated at 95°C for ten minutes, vortexed for five seconds, centrifuged at 17,000 × *g* for twenty seconds, heated at 95°C for another ten minutes, and allowed to cool to room temperature. Finally, samples were centrifuged for two minutes at 17,000 × *g* and the

liquid containing DNA was transferred to a sterile 1.5 mL microcentrifuge tube. DNA samples were analyzed with the G143A qPCR assay the same day they were extracted and then stored at -20°C until further analysis.

The DNA was analyzed using a competitive TaqMan qPCR assay developed by Miles et al. (2021) to detect the G143A mutation associated with QoI resistance. Briefly, 1 µL of DNA from each sample was analyzed in 15 µL qPCR reactions containing 7.5 µL of Perfecta qPCR ToughMix ROX (Quanta Biosciences), 5.975 µL molecular-grade H_2O, 75 nM final concentration 83F and 517R forward and reverse primers, and 100 nM final concentration WT-G143A FAM and MT-G143A VIC TaqMan probes with QSY quenchers (specific to the QoI sensitive G-143 and QoI resistant A-143 allele, respectively) (Table 2.3). Each sample was analyzed in duplicate on a StepOne Plus qPCR machine (Applied Biosystems) using the following cycling conditions: initial 2 min activation at 95°C followed by 55 two-step cycles of 95°C (15 sec) and 67°C (1 min). Every reaction plate contained a G143A-WT, G143A-MT, mixed WT and MT, and a non-template control sample. The StepOne Software v2.3 was used for data acquisition and cycle threshold analysis (Ct) after manually setting the threshold for both markers to allow for plate to plate relative comparisons. Samples were characterized as "Wild Type" if only the G-143 allele amplified, "Mutant" if only the A-143 allele amplified, and "Mixed" if both alleles were detected.

Samples containing sufficient quantities of *E. necator* DNA (G143A Ct value of 32 or lower, roughly equivalent to 100 conidia or more) were further analyzed with a competitive multiplex qPCR assay to test for the Y136F mutation and differences in copy number of the *Cyp51* gene shown to be associated with increased DMI tolerance. Each sample was analyzed in duplicate on a QuantStudio5 qPCR machine (Applied Biosystems) using the following cycling

conditions: initial 5 minute activation at 95°C followed by 45 two-step cycles of 95°C (15 sec) and 64C (45 sec). Each 15 µL qPCR reaction consisted of 1 µL of DNA added to a mix of 7.5 µL TaqPath ProAmp Multiplex Master Mix with Mustang Purple (Applied Biosystems), 4.7 µL molecular-grade H_2O, 200 nM final concentration Y136F Forward and Reverse primers and EnEF1 Forward and Reverse (En-g1817 F and R, Jones et al. 2014) primers, and 100 nM final concentration 136-Y-WT FAM, 136-F-MT VIC, Cyp51_noSNP JUN, and EnEF1 ABY TaqMan Probes (Table 2.4). The QuantStudio Design and Analysis Software was used for data acquisition and cycle threshold analysis (Ct) after manually setting the thresholds for each marker. Samples were characterized as "Wild Type" if only the Y-136 allele amplified, "Mutant Type" if only the F-136 allele amplified, or "Mixed" if both alleles were detected.

3. Results

3.1 Efficacy of fungicides on fruit disease

Disease incidence and severity data was collected from fruit clusters in 2019 and 2020; similar results were observed in both years, but the overall severity of GPM was greater in 2019 (Table 3.3). For grape clusters, incidence did not differ statistically in 2019, as variability was high across ratings; however, the SDHIs and several other classes did provide numerically superior control as compared to the untreated control. In 2020, the incidence of disease was significantly reduced by Torino, Luna Experience, Aprovia, Inspire Super, Pristine, Vivando, and Quintec. In 2019 and 2020, these same fungicides reduced cluster disease severity. However, Abound (azoxystrobin), Rally 40WSP (myclobutanil), and Stylet-Oil (paraffinic oil) provided the worst control overall and did not statistically differ from the untreated control in either year.

3.2 Efficacy of fungicides on leaf disease

Leaf data was collected in 2019 and 2020 (Table 3.4). Powdery mildew leaf infections were observed later in 2020 than in 2019; as a result, the second leaf rating in 2020 is an assessment of the longevity of control, as opposed to both ratings in 2019 and the first rating in 2020, which are more accurate measures of standard fungicidal efficacy. However, similar results were obtained in both years. As observed with fruit ratings, neither Abound (azoxystrobin) nor Rally 40WSP (myclobutanil) provided control at any rating date, and stylet-oil (paraffinic oil) was inconsistent in efficacy. While Microthiol Disperss (sulfur) did not perform well on fruit, it did provide excellent control of GPM on leaves. In 2020, the last leaf rating was taken six weeks after the last spray event, but Luna Experience, Aprovia, Inspire Super, Pristine, Vivando, and Quintec all still provided significant efficacy – even ~1.5 months after application. By this time, Microthiol Disperss no longer provided disease control as compared to the untreated plants, as one might expect of a contact fungicide in a rainy year.

3.3 Resistance mutations

In this research vineyard, 75 of 75 samples in 2019 and 20 of 20 samples in 2020 had the G143A allele in the *cytochrome b* region and were considered mutant or QoI resistant (Table 3.5). No mixed or wild type genotypes were found. When assessing the point mutation in the *cyp51* allele in the research vineyard *E. necator* population, 17 of 75 samples in 2019 and 9 of 20 in 2020 contained only the Y136F mutant allele associated with increased DMI tolerance; the remaining isolates, 58 in 2019 and 11 in 2020, were mixed genotypes with both mutant and wild type alleles. No samples in either 2019 or 2020 had colonies that were all wild type or considered fully sensitive to DMI fungicides.

4. Discussion

In this study, we observed field failures (complete lack of control from fungicides applied) from both Abound (azoxystrobin, a QoI) and Rally 40WSP (myclobutanil, a DMI). These field failures were associated with resistance genotypes of *E. necator* samples taken throughout the vineyard (Table 3.5). Given that all 95 samples from this research vineyard possessed the G143A allele in *cytochrome b* and the lack of statistical difference between azoxystrobin and the untreated control, it was apparent that the field failure observed with Abound (azoxystrobin) was due to the presence of QoI-resistant *E. necator*. Likewise, the Rally (myclobutanil) field failure was associated with a high prevalence of the Y136F mutation (Table 3.5). On the leaves (Table 3.4), only limited disease suppression was observed with Rally (myclobutanil), as compared to azoxystrobin and the untreated control. We believe the myclobutanil field failure was due to the detected presence of the Y136F mutation in *CYP51* which had previously been associated DMI-resistance in *E. necator* isolates (Délye et al. 1997). However, it should be noted that there are other ways *E. necator* can overcome DMI fungicides, including overexpression of the *CYP51* gene, so the presence of this mutation is not purely diagnostic (Frenkel et al. 2015). The combination of the high frequency of the Y136F mutation found in this *E. necator* population with the myclobutanil field failure suggests DMI resistance is present in this location.

While myclobutanil did not provide control, Inspire Super (difenoconazole + cyprodinil) provided excellent efficacy. In this study, excellent efficacy refers to significant disease reduction where almost no mildew was present. Cyprodinil (FRAC 9) is a methionine biosynthesis inhibitor that is mainly used to control *Botrytis cinerea* with minimal ability to suppress *E. necator*. With Inspire Super, difenoconazole (FRAC 3) acts as the main compound providing control of *E. necator* even in the presence of the Y136F mutation. This is believed to

be a result of greater intrinsic activity from difenoconazole as also reported in Baudoin et al. (2019). This result further suggests the absence of complete cross-resistance among FRAC 3 chemicals that has been noted in previous studies (Erickson and Wilcox 1997; Colcol et al. 2012).

Resistance of *E. necator* to additional single-site fungicides, previously seen in other geographic regions, was not observed in this trial, including Quintec (quinoxyfen, FRAC 13), Vivando (metrafenone, FRAC 50), and Aprovia (benzovindiflupyr, FRAC 7). Excellent control was provided by these stand-alone chemicals as well as mixes with single-site fungicides including Luna Experience (fluopyram + tebuconazole, FRAC 7 + FRAC 3) and Pristine (pyraclostrobin + boscalid, FRAC 7 + FRAC 11) in this trial. We hypothesize the control provided by these two chemicals, each with a DMI and QoI respectively in their mix, is likely coming from their SDHI mixing partners. Aprovia (SDHI) provided excellent control on both fruit and leaves as a standalone product, suggesting the absence of resistance to this class in the *E. necator* population at this field site.

Aside from single-site fungicides, two contact or multi-site fungicides were included in these trials: Microthiol Disperss (sulfur) and Stylet-Oil (paraffinic oil). We saw varying levels of efficacy among these two chemicals. Microthiol Disperss provided excellent control on leaves (Table 3.4) but lacked efficacy on fruit (Table 3.3). This was also observed with Stylet-Oil, as it provided less efficacy on the fruit than the leaves but provided significantly less control on the leaves than Microthiol Disperss. The lack of disease control provided by these contact fungicides may be the result of coverage issues, especially on fruit clusters, or frequent rain events washing off the chemicals. Although these fungicides did not perform as well as others, they can still be

used as mixing partners with single site fungicides to delay selection for fungicide resistance as seen in many other pathosystems (Van Den Bosch et al. 2014).

Resistance of *E. necator* to QoIs and DMIs, assuming that it is present in other locations in Georgia and in nearby grape-growing regions of other states, will place further pressure on other fungicide classes that *E. necator* has the ability to develop resistance to. The results for our study indicate field failure of QoI and DMI fungicides for GPM control and illustrate the degree of that failure under field conditions. Comprehensive surveys of production sites in Georgia are needed to determine where, or if, similar resistance is present in vineyards, as the types of field failures observed here could cause grave economic issues for this fledgling industry.

5. Conclusions

At this research vineyard in Blairsville, Georgia, United States, resistance was observed in *E. necator* to both QoI (FRAC 11) and DMI (FRAC 3) fungicides. Cross-resistance among FRAC 3 fungicides was not observed, as difenoconazole still provided significant control of GPM. Other tested single-site fungicides, benzovindiflupyr (FRAC 7), metrafenone (FRAC 50), and quinoxyfen (FRAC 13), still provided high levels of control and did not show evidence of resistance. With the high amount of rainfall observed in Georgia, broad-spectrum, contact fungicides are difficult to use effectively, but can still provide control if utilized often enough, and are a critical component of resistance management programs. Increasing the frequency of application and ensuring proper coverage with broad-spectrum, contact fungicides is critical to their utility. Determining how widespread *E. necator* QoI and DMI resistance is in Georgia and elsewhere will be important, as this information is critical to GPM disease management program development and decisions by grape producers regarding the implementation of further resistance management strategies.

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Table 3.1. List of fungicides compared in field efficacy trials in both 2019 and 2020.

Treatment	Active Ingredient	Distributor	FRAC code	Group name[a]
Abound	Azoxystrobin 22.9 %	Syngenta	11	QoI
Aprovia	Benzovindiflupyr 9.63%	Syngenta	7	SDHI
Inspire Super	Difenoconazole 8.4% + Cyprodinil 24.1%	Syngenta	3 + 9	DMI + AP
Luna Experience	Fluopyram 17.6% + Tebuconazole 17.6%	Bayer CropScience	7 + 3	SDHI + DMI
Microthiol Disperss	Sulfur 80%	United Phosphorus, Inc	M2	Inorganic electrophile
Pristine	Pyraclostrobin 12.8% + Boscalid 25.2%	BASF Corporation	7 + 11	QoI + SDHI
Quintec	Quinoxyfen 22.58%	Gowan Company LLC	13	Aza-naphthalene
Rally 40WSP	Myclobutanil 40%	Corteva AgriSciences	3	DMI
Stylet-Oil	Paraffinic oil 97.1%	JMS Flower Farms, Inc.	NC	Not specified
Torino	Cyflufenamid 10%	Gowan Company LLC	U6	Phenyl-acetamide
Vivando	Metrafenone 25.20%	BASF Corporation	50	Aryl-phenyl-ketone

[a]QoI = quinone outside inhibitor, DMI = demethylase inhibitor, SDHI = succinate dehydrogenase inhibitor, AP = anilino-pyrimidine

Table 3.2. Additional maintenance fungicides applied to the research vineyard during powdery mildew field trials.

Phenology	Fungicide Applied[a]
Bud break	Manzate Pro Stick 3.4 kg/ha
	Microthiol Disperss 3.4 kg/ha
2-inch shoot growth	Manzate Pro Stick 3.4 kg/ha
	Microthiol Disperss 3.4 kg/ha
4-inch shoot growth	Manzate Pro Stick 3.4 kg/ha
Pre-bloom	Manzate Pro Stick 3.4 kg/ha
Bloom	Manzate Pro Stick 3.4 kg/ha
	Elevate 50WDG 1.1 kg/ha
Post-bloom	Manzate Pro Stick 3.4 kg/ha
First cover	Manzate Pro Stick 3.4 kg/ha
Bunch closure	Captan 80WP 2.8 kg/ha
	Revus 0.58 L/ha
	Elevate 50WDG 1.1 kg/ha
Second cover	Captan 80WP 2.8 kg/ha
	Zampro 0.98 kg/ha
Veraison	Captan 80WP 2.8 kg/ha
	Revus 0.58 L/ha
	Elevate 50WDG 1.1 kg/ha
Pre-harvest	Captan 80WP 2.8 kg/ha
	Revus 0.58 L/ha
	Elevate 50WDG 1.1 kg/ha

[a]Distributors, active ingredients, and target pathogens for the respective fungicides are as follows: 1) Manzate Pro Stick (United Phosphorus Inc; Mancozeb; *Phyllostica ampelicida* [black rot], *Botrytis cinerea* [Botrytis bunch rot]), *Plasmopara viticola* [downy mildew] 2) Microthiol Disperss (United Phosphorus; sulfur; *Erysiphe necator* [powdery mildew]), 3) Elevate 50WDG (United Phosphorus; Fenhexamid; *B. cinerea* [Botrytis bunch rot], 4) Captan 80WP (Albaugh LLC; Captan; *Phomopsis viticola* [Phomopsis cane and leaf spot], *P. viticola* [downy mildew]), 5) Revus (Syngenta; Mandipropamid; *P. viticola* [downy mildew]), 6) Zampro (BASF, Ametoctradin, *P. viticola* [downy mildew]

Table 3.3. Fruit disease incidence and severity from efficacy trials in 2019 and 2020.

	2019				2020					
	Powdery mildew cluster incidence [a]		Powdery mildew cluster severity [a]		Powdery mildew cluster incidence [a]			Powdery mildew cluster severity [a]		
Treatment and amount/Ha	11 July	26 July	11 July	26 July	8 July	16 July	27 July	8 July	16 July	27 July
Untreated	80.5 a	79.4 ab	10.1 a	17.4 a	69.6 a	81.0 a	90.5 a	3.9 a	7.8 a	12.5 a
Abound 1.1 L	52.0 ab	69.6 ab	1.6 ab	6.2 ab	48.0 ab	65.5 a	85.0 a	1.8 ab	4.2 ab	7.8 a
Rally 0.35 kg	57.2 ab	90.5 a	2.1 ab	2.3 ab	39.6 abc	57.2 a	74.0 a	2.0 ab	3.1 ab	5.7 a
Stylet-Oil 9.5 L	61.2 ab	73.2 ab	8.1 ab	7.8 ab	26.8 abcd	52.0 a	60.4 ab	1.2 abc	2.7 ab	4.8 ab
Torino 0.25 L	12.8 ab	30.4 ab	0.6 ab	0.5 b	3.4 bcd	0.0 b	0.9 c	0.2 bc	0.0 c	0.0 c
Luna Experience 0.6 L	3.4 b	12.6 ab	0.1 b	0.2 b	0.0 d	0.0 b	0.0 c	0.0 c	0.0 c	0.0 c
Aprovia 0.8 L	19.0 ab	12.0 ab	0.4 ab	0.1 b	0.0 d	0.0 b	0.0 c	0.2 bc	0.0 c	0.0 c
Microthiol Disperss 11.2 kg	52.9 ab	64.7 ab	1.8 ab	4.1 ab	12.3 abcd	33.8 ab	70.3 a	0.5 bc	1.9 bc	4.5 ab
Inspire Super 1.5 L	48.0 ab	43.6 ab	5.7 ab	7.4 ab	0.0 d	0.0 b	3.4 b	0.0 c	0.0 c	0.2 bc
Pristine 0.9 kg	26.8 ab	33.8 ab	1.5 ab	0.8 b	0.0 d	0.9 b	0.9 c	0.0 c	0.0 c	0.0 c
Vivando 1.1 L	7.1 ab	10.0 ab	0.2 ab	0.3 b	0.9 cd	0.0 b	0.0 c	0.0 c	0.0 c	0.0 c
Quintec 0.5 L	7.5 ab	5.2 b	0.1 ab	0.1 b	0.0 d	0.0 b	0.0 c	0.0 c	0.0 c	0.0 c

[a]Powdery mildew incidence (% infected clusters) and severity (% of cluster covered by powdery mildew) were calculated from 5 clusters per treated plant. Means following the same letter are not significantly different from one another based on Tukey's HSD ($P \leq 0.05$). All data was arcsine square root transformed before analysis. Back-transformed means are shown.

Table 3.4 Leaf disease incidence and severity from efficacy trials in 2019 and 2020.

	2019				2020			
	Powdery mildew leaf incidence [a]		Powdery mildew leaf severity [a]		Powdery mildew leaf incidence [a]		Powdery mildew leaf severity [a]	
Treatment and amount/Ha	6 August	13 August	6 August	13 August	5 August	19 August	5 August	19 August
Untreated	82.0 a	88.3 ab	38.4 a	43.4 a	60.8 a	99.3 a	15.3 a	48.2 a
Abound 1.1 L	82.2 a	87.6 abc	34.3 a	41.7 a	60.4 a	87.6 abc	34.3 a	41.7 a
Rally 0.35 kg	68.1 a	92.9 a	17.3 b	27.7 ab	51.9 a	97.7 ab	8.1 ab	51.5 a
Stylet-Oil 9.5 L	17.6 b	52.0 bcd	3.0 c	14.2 bc	47.9 ab	99.1 a	9.3 ab	48.6
Torino 0.25 L	12.0 b	47.0 cd	2.1 c	8.9 cd	14.2 bc	95.2 abc	2.0 bc	37.1 ab
Luna Experience 0.6 L	2.3 b	19.4 de	0.3 c	3.1 de	6.3 c	91.0 abc	0.6 c	24.2 bc
Aprovia 0.8 L	1.4 b	7.0 e	0.2 c	1.1 de	5.3 c	78.2 cd	0.7 c	19.1 bc
Microthiol Disperss 11.2 kg	0.9 b	7.6 e	0.1 c	1.6 de	11.0 c	89.1 abc	1.2 c	31.4 ab
Inspire Super 1.5 L	1.0 b	3.6 e	0.1 c	0.7 e	5.8 c	80.1 bcd	0.7 c	25.4 bc
Pristine 0.9 kg	0.6 b	0.5 e	0.1 c	0.7 e	1.6 c	56.2 d	0.2 c	12.3 c
Vivando 1.1 L	0.3 b	3.1 e	0.0 c	0.4 e	3.4 c	77.8 cd	0.4 c	20.6 bc
Quintec 0.5 L	0.6 b	0.5 e	0.1 c	0.1 e	11.0 c	83.2 bcd	1.0 c	20.0 bc

[a] Powdery mildew incidence (% infected leaves) and severity (% of leaf covered by powdery mildew) were calculated from 25 leaves per treated plant. Means following the same letter are not significantly different from one another when using Tukey's HSD ($P \leq 0.05$). All data was arcsine square root transformed before analysis. Back-transformed means are shown.

Table 3.5. Genotypes of *Erysiphe necator* found at research vineyard in 2019 and 2020.

	G143A[a]		Y136F[b]		
Year sampled	Mutant	Wild Type	Mutant	Mixed	Wild Type
2019	75/75	0/75	17/75	58/75	0/75
2020	20/20	0/20	9/20	11/20	0/20

[a] The G143A mutation confers resistance to quinone outside inhibitors (QoIs). A positive result for this mutation indicates complete resistance. A negative result indicates no detection of this mutation and that the sampled individuals are sensitive.

[b] The Y136F mutation is associated with increased demethylation inhibitor (DMI) tolerance. A sample indicated as "mutant" refers to all colonies in that sample having the Y136F mutation. A "mixed" sample means that some individuals in the sample had the Y136F mutation while others did not. A "wild type" sample means the Y136F mutation was not found in any individuals in the sample.

CHAPTER 4

EFFICACY OF DEMETHLYATION INHIBITOR (DMI) FUNGICIDES IN THE PRESENCE OF A DMI-TOLERANT *ERYSIPHE NECATOR* POPULATION AND INVESTING POTENTIAL SYNERGY BETWEEN DMIs AND SULFUR[4]

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Abstract

Grapevine powdery mildew (GPM) is a disease of wine grapes worldwide caused by *Erysiphe necator*. With fungicide resistance becoming widespread in the population of this pathogen, producers are at risk of losing valuable tools to protect their grapes. Demethylation inhibitors (DMIs) have been an important class of fungicides used in grape production for over 30 years. Resistance to this class has been reported, but complete cross-resistance has not been observed. In this study, we determine which chemicals in the DMI fungicide class are most efficacious in the presence of the Y136F point mutation in *E. necator* associated with DMI resistance. Additionally, we determine if adding sulfur to a DMI fungicide in the presence of pathogen resistance can increase efficacy against GPM. In total, the efficacy of six different DMIs versus GPM were compared and three of these were also tested in combination with sulfur. Our results indicated a high variability between the efficacy provided by the six DMI fungicides against GPM and did not indicate the existence of a synergistic interaction between sulfur and DMIs for GPM control.

Keywords: grape, *Vitis vinifera*, grapevine powdery mildew, *Erysiphe necator*, fungicide resistance, sulfur, demethylation inhibitors

Introduction

Grapevine powdery mildew (GPM), caused by the fungus *Erysiphe necator*, is a global disease affecting *Vitis* spp. over a broad range of natural and agricultural conditions. *E. necator* is an obligate, biotrophic parasite that can infect all green tissue of the grapevine plant. Unlike North American *Vitis* species, European wine grapes (*Vitis vinifera*) are more susceptible to this disease, likely due to their lack of co-evolution with the causal fungus (Brewer and Milgroom 2010) which is native to eastern North America. Even a small amount of GPM on any tissue can decrease yield, affect fruit quality, or produce off flavors in wine (Calonnec et al. 2004). Currently, there are no GPM-resistant varieties of *V. vinifera*, which forces growers to rely on fungicides as their primary method of disease management.

While cultural practices, such as ensuring proper air flow throughout the canopy by removing leaves, can decrease the overall severity of a GPM epidemic, these methods cannot completely prevent the disease (Chellemi and Marois 1992). To properly manage GPM, fungicides are critical; however, developing an efficient spray program can be challenging in conditions that are optimal for disease development such as the warm, humid conditions found in Georgia, United States. In the Southeast U.S., many grape diseases are present in addition to GPM, including downy mildew, Botrytis bunch rot, black rot, and *Phomopsis* cane and leaf spot (Wilcox et al. 2015). For disease management, fungicides targeting a single-site in a fungal-specific pathway are commonly used in Georgia, as their systemic properties are ideal for a climate with frequent rainfall during the growing season. In Georgia, the pathogens causing both downy mildew (*Plasmopara viticola*) and Botrytis bunch rot (*Botrytis cinerea*) have already evolved resistance

to a widely used single-site fungicide class, the quinone outside inhibitors (QoIs) (Campbell et al. 2020; Fernández-Ortuño et al. 2014). While there have been no reports of fungicide resistance in *E. necator* in Georgia, there have been reports of resistance in *E. necator* to single-site fungicides elsewhere in the United States including QoI resistance in New York, Virginia, and Michigan (Wong and Wilcox 2002; Baudoin et al. 2008; Miles et al. 2012), and demethylation inhibitor (DMI) resistance in California, New York, and Virginia (Gubler et al. 1996; Erickson and Wilcox 1997; Colcol et al. 2012).

DMI fungicides target the 14α-demethylase enzyme in the ergosterol biosynthesis pathway, which is encoded by *CYP51*. The efficacy of DMIs is due to their ability to bind to this enzyme and inhibit this critical pathway (Délye et al. 1997). DMI resistance among plant-pathogenic fungi is quantitative, meaning there is a continuous distribution of phenotypes throughout a population, and this can cause varying levels of sensitivity among chemicals with the same mode of action (McGrath 2009). In *E. necator*, two mechanisms of resistance have been observed. First, a point mutation in the *CYP51* gene that results in a tyrosine to phenylalanine substitution at codon 136 (Y136F) is associated with increased DMI tolerance (Délye et al. 1997). It has been suggested that some DMI fungicides appear to have a higher affinity to bind to 14α-demethylase as they can still provide disease control in the presence of the Y136F point mutation (Baudoin et al. 2019). In addition to the Y136F mutation, increased copy number of the *CYP51* gene has also been observed to cause DMI resistance in some cases (Frenkel et al. 2014; Jones et al. 2014; Rallos and Baudoin 2016).

Due to the frequency of plant pathogens developing resistance to single-site fungicides, it has been recommended that sulfur, a broad-spectrum, multi-site fungicide, be added in a tank mix with these other chemicals to increase the level of protection for many crops. For instance, for

control of brown rot of peach and late leaf spot of peanut, it has been reported that there is a synergistic relationship between sulfur and DMI fungicides (Holb and Schnabel 2008; Culbreath et al. 2019). Holb and Schnabel (2008) found that mixing sulfur with propiconazole resulted in more efficacy than either treatment on its own for the control of *Monilinia fructicola* in the presence of DMI resistance. Additionally, Culbreath et al. (2019) observed that sulfur mixed with cyproconazole provided better control of *Nothopassalora personata* than either treatment alone. While the cause of this synergistic relationship between sulfur and DMIs is unknown, these studies suggest that such mixtures have the potential to increase control of fungal diseases. With sulfur already being efficacious against GPM, it is possible that mixing sulfur and DMIs could increase control in the presence of fungicide resistance in this system as well.

Accordingly, the objective of this study was to compare six different DMI fungicides to determine which provided better field control of GPM in the presence of the Y136F mutation. In addition, varying additions of sulfur with multiple DMI fungicides were compared to look for synergistic impacts on GPM control. As grape growers are at risk of losing another fungicide class to widespread resistance, these strategies have the potential to be valuable for disease management.

Materials and Methods

DNA extraction and qPCR analysis to determine the DMI resistance profile of the research vineyard. *E. necator* samples were opportunistically collected from the research vineyard at the University of Georgia Mountain Research and Education Center in Blairsville, Georgia, United States using either ToughSpots (Diversified BioTech, Dedham, MA) or Cap-

Shure Sterile Cotton Swabs (Puritan Medical Products, Guilford, ME) as mentioned in Chapter 2. Briefly, samples were collected once colonies of *E. necator* were clearly established on fruit or leaves. When *E. necator* colonies were observed, ToughSpots were placed on a colony with forceps and lightly pressed to collect fungal tissue. The ToughSpot was then removed from the colony with forceps and placed in a sterile tube with labels including information such as GPS coordinates, grape phenology, variety, and date of sample. If using Cap-Shure Sterile Cotton Swabs, GPM lesions on either fruit or leaves were swabbed with the tip of the cotton swab and placed back into the bag and labeled in the same manner as the ToughSpot samples. Collected samples were stored at 4°C until they could be further processed.

DNA was extracted from each sample using a modified (Miles et al. 2021) Chelex extraction method (Brewer and Milgroom 2010). Each swab was placed in a sterile 2 mL microcentrifuge tube containing 400 µL Chelex solution (5% w/v Chelex 100 sodium form 50-100 mesh [Sigma Aldrich] suspended in molecular grade water). Handles of swabs were removed and the tips of the swabs were fully immersed in the Chelex solution. The samples were vortexed at max speed for 5 min and centrifuged at 17,000 × *g* for 20 sec. Samples were incubated at 95°C for 10 min, vortexed for 5 sec, centrifuged at 17,000 × *g* for 20 sec, heated at 95°C for another 10 min, and allowed to cool to 22°C. Samples were centrifuged for a final 2 min at 17,000 × *g* and the liquid containing the DNA was then transferred to sterile 1.5 mL microcentrifuge tubes.

Samples containing sufficient quantities of *E. necator* DNA (roughly equivalent to 100 conidia or more) were analyzed with a competitive multiplex qPCR assay to test for the Y136F mutation and differences in the number of copies of the *Cyp51* gene. Each sample was analyzed in duplicate on a QuantStudio5 qPCR machine (Applied Biosystems) using the following cycling

conditions: initial 5-min activation at 95°C followed by 45 two-step cycles of 95°C (15 sec) and 64°C (45 sec). Each 15 µL qPCR reaction consisted of 1 µL of DNA added to a mix of 7.5 µL TaqPath ProAmp Multiplex Master Mix with Mustang Purple (Applied Biosystems), 4.7 µL molecular-grade H_2O, 200 nM final concentration Y136F Forward and Reverse primers and EnEF1 Forward and Reverse (En-g1817 F and R, Jones et al. 2014) primers, and 100 nM final concentration 136-Y-WT FAM, 136-F-MT VIC, Cyp51_noSNP JUN, and EnEF1 ABY TaqMan Probes (Table 2.4). The QuantStudio Design and Analysis Software was used for data acquisition and cycle threshold analysis (Ct) after manually setting the thresholds for each marker. Samples were characterized as "Wild Type" if only the Y-136 allele amplified, "Mutant Type" if only the F-136 allele amplified, or "Mixed" if both alleles were detected.

Research vineyard maintenance sprays. For all trials in both 2019 and 2020, additional maintenance fungicides were sprayed every two weeks to control other pathogens from bud break to post harvest. The research vineyard was pruned and maintained to commercial standards. The same maintenance sprays utilized in other trials (Chapter 3) and listed in Table 3.2 were applied with a John Bean Redline 537T Air Blast sprayer (Durand-Wayland, Inc., LaGrange, GA) and calculated to correspond with a 473 L/ha total spray volume.

DMI cross-resistance trial. To determine the level of control offered by various DMI fungicides in the presence of high levels of the Y136F mutation, a field trial was conducted at the UGA Mountain Research and Education Center on *V. vinifera* 'Merlot'. This trial utilized a randomized complete block design with five replications per treatment. The treatments consisted of the DMI fungicides shown in Table 4.1 along with an untreated control and a positive control, Aprovia (benzovindiflupyr, FRAC 7), that has provided excellent efficacy against GPM in other trials at this location (Chapter 3). The lowest labeled rates were used for each treatment. Though

Revus Top contains difenoconazole (FRAC 3) and mandipromamid (FRAC 40), only difenaconazole is active against GPM. FRAC 40 chemicals inhibit cellulose synthesis and are used to target oomycetes; therefore, any GPM control observed from this treatment was assumed to be from difenoconazole alone. Treatments were applied a total of six times with a CO_2 backpack sprayer until runoff on the following dates: pre-bloom (27 April), bloom 1 (11 May), bloom 2 (26 May), post-bloom (8 June), bunch closure (22 June), and first cover (6 July).

Both fruit clusters and leaves were rated for disease incidence and severity in the manner described previously for the sulfur and DMI interaction trials. On 8 July, 16 July, and 27 July five clusters were rated from each vine using the Powdery Mildew Assessment Tool by the Adelaide Research and Innovation Pty Ltd. Twenty-five leaves were taken from each vine on 23 July and 5 August and assessed for percentage mildew cover. All data from both the fruit and the leaves were transformed using an arcsine square root transformation and analyzed in SAS 9.4 using a Tukey's HSD with the GLIMMIX procedure.

Sulfur and DMI interaction studies. Two different sulfur and DMI mixture trials were conducted in 2019 and 2020. In 2019, the trial was designed to compare mixtures of one DMI fungicide, Rally 40WSP (myclobutanil) and different amount of Microthiol Disperss (micronized sulfur) to look for synergistic reactions. In this trial, the highest labeled rate of Rally was compared to the lowest labeled rate of Microthiol Disperss, as well as five different combinations of these two fungicides (Table 4.2). The trials were conducted on *Vitis vinifera* 'Merlot'. A randomized complete block was utilized with five replications per treatment. Treatments were calculated to correspond with a 473 L/ha rate and sprayed with CO_2 backpack sprayers until runoff. Applications were made four times: first cover (1 July), bunch closure (9 July), second cover (23 July), and third cover (6 August). On 6 August and 20 August, leaf

incidence (percent of infected leaves per vine) and severity (percent of leaf covered with mildew) data was collected. A total of 25 leaves were arbitrarily pulled from each vine and used to assess percentage of powdery mildew coverage. The leaves were rated on a scale ranging from 0% - 100% in ten-point increments. The leaf data was analyzed using a Tukey's HSD with a GLIMMIX procedure in SAS 9.4. Means were transformed using an arcsine square root transformation.

In 2020, the trial was designed to compare a standardized rate of sulfur with three DMI fungicides. The treatments were as follows: 1) an untreated control, 2) sulfur, 3) myclobutanil high rate, 4) myclobutanil low rate + sulfur, 5) myclobutanil high rate + sulfur, 6) tebuconazole high rate, 7) tebuconazole low rate + sulfur, 8) tebuconazole high rate + sulfur, 9) mefentrifluconazole high rate, 10) mefentrifluconazole low rate + sulfur, 11) mefentrifluconazole high rate + sulfur (Table 4.3). Treatments were applied on *Vitis vinifera* 'Chardonnay' a total of six times: pre-bloom (27 April), bloom 1 (11 May), bloom 2 (26 May), post-bloom (8 June), bunch closure (22 June), and first cover (6 July). Treatments were calculated to correspond with a 473 L/ha rate and were applied using CO_2 backpack sprayers until runoff.

Fruit clusters were rated for incidence (percent of infected clusters per vine) and severity (percent of powdery mildew per cluster) a total of three times. Fruit cluster data was collected on 29 June, 8 July, and 16 July by rating five clusters from each vine using the Powdery Mildew Assessment Tool developed by Adelaide Research and Innovation Pty Ltd. This tool has a standardized method of GPM rating on fruit clusters that were used to keep fruit cluster ratings consistent. Assessments of leaf disease were conducted on 23 July and 5 August using the same process as reported for 2019. Both fruit and leaf data were analyzed using a Tukey's HSD with a

GLIMMIX procedure in SAS 9.4. Means were transformed using an arcsine square root transformation.

Results

Population structure of *E. necator* in research vineyard. In this research vineyard, a total of 95 samples were analyzed for the Y136F mutation (2019, n = 75; 2020, n = 20) (Table 3.5). As stated in Chapter 3, all samples were either full mutant type, indicating that all individuals from a colony had the Y136F allele, or mixed, where some of the individuals from a colony had the Y136F allele. None of the 95 samples taken from this research vineyard completely lacked the Y136F allele or were considered wild type.

DMI cross-resistance trial. When reviewing the activity of various DMI fungicides, treatment efficacies were similar between both fruit (Table 4.4) and leaf (Table 4.5) disease. Rally 40WSP (myclobutanil) and Elite 45WP (tebuconazole) showed the lowest efficacies among all DMI treatments assessed in this trial, but still provided a small amount of disease suppression as compared to the untreated control. Procure 480SC (triflumizole) and Mettle 125 (tetraconazole) both performed slightly better than Rally and Elite, and Mettle provided significantly more control of disease on the leaves. Cevya (mefentrifluconazole) and Revus Top (difenoconazole) were the most effective DMI treatments in this trial and were most similar to the positive control, Aprovia (SDHI, FRAC 7). Difenoconazole demonstrated the highest overall efficacy, with almost no disease present on the fruit or leaves following this treatment.

Sulfur and DMI interactions. In 2019, fruit data was collected and analyzed, but no statistical differences between treatments were observed (data not presented), likely due to the treatments being applied too late to be effective. However, leaf data did clearly show statistical

differences (Table 4.6). Rally (myclobutanil) on its own was not statistically different from the untreated control and showed hardly any efficacy against GPM. As Microthiol Disperss (sulfur) increased in concentration when mixed with Rally, incidence and severity were both lowered in a stepwise, linear fashion, with no indication of synergy between the various sulfur and DMI rates. The most effective treatment in this trial was the full rate (3.4 kg/ha) of Microthiol Disperss, with none of the combinations providing higher efficacy than the standalone sulfur treatment.

In 2020, with the exception of Cevya (mefentrifluconazole), incidence was high in general for fruit treatments (Table 4.7). No clear differences in control were observed between Rally 40WSP (myclobutanil) and Elite 45WP (tebuconazole). When mixed with sulfur, treatments with either Rally or Elite at both the low and high labeled rates were not statistically different in severity or incidence from the standalone sulfur treatment. Cevya (mefentrifluconazole), however, provided much more control than myclobutanil, tebuconazole, or sulfur. The addition of sulfur to Cevya slightly decreased disease incidence and severity, but not significantly.

Leaf incidence and severity data in 2020 (Table 4.8) was similar to fruit incidence and severity data, but with lower incidence levels. All treatments with Rally and Elite were not statistically different from sulfur on its own. Cevya treatments all provided the highest efficacy, but statistical differences between Cevya high rate, Cevya low rate + Microthiol Disperss, and Cevya high rate + Microthiol Disperss were not observed.

Discussion

At this research vineyard, a high frequency of Y136F alleles were observed in the *E. necator* population (Table 3.2). This suggests that the field trials conducted in this research

vineyard were conducted in the presence increased DMI tolerance, further helping to explain the efficacy result observations from these trials. In these trials, we observed decreased efficacy from the DMI fungicides myclobutanil and tebuconazole, similar to that reported in Virginia from Colcol et al. (2012). Triflumizole and tetraconazole performed better than myclobutanil and tebuconazole, but still showed decreased efficacy at this research site. Some of the differences in efficacy could simply be related to higher intrinsic activity, or being more active, but it is unlikely that the degree of difference observed could be explained by this alone suggesting that the Y136F mutation and DMI tolerance impacted these results. Mefentrifluconazole and difenoconazole are hypothesized to have a higher level of intrinsic activity due to an increased affinity to bind to their target (Baudoin et al. 2019). Overall, all treatments had differing levels of control even though they all had the same mode of action. This could be explained by some chemicals being able to bind to the mutated 14α-demethylase target more efficiently than others. With this information, we can hypothesize that commercial vineyards with the same frequencies of Y136F alleles in their *E. necator* populations will see field failures from both myclobutanil and tebuconazole if relied upon for GPM control.

In this study, we did not observe synergistic effects with sulfur and any DMI fungicide across two experimental designs in the presence of the Y136F mutation, but instead saw that sulfur acted additively. Mixing myclobutanil and tebuconazole with sulfur did not provide more control than sulfur on its own regardless of the amount of the DMI fungicide or amount of sulfur. The disease control observed in both trials appeared to be from sulfur alone, except from the Cevya (mefentrifluconazole) treatments. Of note, Cevya performed exceptionally well even without sulfur – even in the presence of resistance to the other DMI fungicides.

On fruit, a greater overall disease incidence was observed even when treated with sulfur (Table 4.7) versus the level of control observed on leaves (Table 4.8). In Georgia, rainfall is frequent during the growing season, making contact fungicides difficult to utilize effectively. While this could be a result of coverage issues on the fruit clusters, it is also possible that sulfur is washed off and leaving fruit more susceptible. These results suggest that sulfur does not perform well on a biweekly fungicide application schedule with Georgia conditions on the fruit but can still provide control of GPM on leaves. As fungal resistance to sulfur is less likely to occur, further studies of sulfur application to fruit clusters may be useful to growers in climates with frequent rainfall during the growing season.

Although DMI resistance is becoming more widespread in *E. necator* populations (Gubler et al. 1996; Erickson and Wilcox 1997; Colcol et al. 2012), newer chemicals (mefentrifluconazole and difenoconazole) can still provide excellent efficacy in the presence of resistance. This is unlike what occurs with other single-site fungicides, like QoIs, where complete cross-resistance has been observed when resistance occurs. Excellent control of GPM from both of these DMI fungicides was observed at our research vineyard despite a high frequency of the Y136F allele. Knowing that these products can still provide efficacy in the presence of resistance can be extremely valuable information for grape growers, as they can still rely on some DMI fungicides in their fungicide rotation. Furthermore, our data suggests that mixing sulfur with a DMI may be still provide some benefit, even without the synergistic effect, as it increases control of GPM when added to a tank. If growers are unsure of the state of their *E. necator* population, mixing sulfur can ensure some level of efficacy even in the presence of fungicide resistance.

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Table 4.1. Active ingredients of FRAC 3 chemicals used in these trials.

Active FRAC 3 Ingredient	Product Name	Distributor
Difenoconazole 21.9%	Revus Top[a]	Syngenta
Mefentrifluconazole 40%	Cevya	BASF
Myclobutanil 40%	Rally 40WSP	Dow AgroSciences
Tebuconazole 45%	Elite 45WP	Bayer CropScience
Tetraconazole 11.6%	Mettle 125 ME	Gowan Company
Triflumizole 42.14%	Procure 480SC	United Phosphorus Limited

[a]Revus Top also includes mandipropamid (FRAC 40) that does not have activity against *E. necator*.

Table 4.2. Sulfur and DMI combinations compared in 2019 preliminary sulfur trial.

DMI + Sulfur combination	Tank mixture (473 L/ha rate)
Myclobutanil full rate	Rally 0.35 kg
7/8 myclobutanil + 1/8 sulfur	Rally 0.31 kg + Microthiol Disperss 0.43 kg
3/4 myclobutanil + 1/4 sulfur	Rally 0.27 kg + Microthiol Disperss 0.85 kg
1/2 myclobutanil + 1/2 sulfur	Rally 0.18 kg + Microthiol Disperss 1.7 kg
1/4 myclobutanil + 3/4 sulfur	Rally 0.09 kg + Microthiol Disperss 2.55 kg
1/8 myclobutanil + 7/8 sulfur	Rally 0.04 kg + Microthiol Disperss 3.0 kg
Sulfur full rate	Microthiol Disperss 3.4 kg

Table 4.3. Sulfur and DMI combinations compared in 2020 trial.

Sulfur and DMI combinations	Tank mixture (473 L/ha rate)
Sulfur	Microthiol Disperss 3.4 kg
Myclobutanil high rate	Rally 40WSP 0.35 kg
Myclobutanil low rate + sulfur	Rally 40WSP 0.21 kg + Microthiol Disperss 3.4 kg
Myclobutanil high rate + sulfur	Rally 40WSP 0.35 kg + Microthiol Disperss 3.4 kg
Tebuconazole high rate	Elite 45 WP 0.28 kg
Tebuconazole low rate + sulfur	Elite 45 WP 0.17 kg oz + Microthiol Disperss 3.4 kg
Tebuconazole high rate + sulfur	Elite 45 WP 0.28 kg oz + Microthiol Disperss 3.4 kg
Cevya high rate	Cevya 0.37 L
Cevya low rate + sulfur	Cevya 0.22 L + Microthiol Disperss 3.4 kg
Cevya high rate + sulfur	Cevya 0.37 L + Microthiol Disperss 3.4 kg

Table 4.4. 2020 DMI cross-resistance trial results on fruit.

	Powdery mildew incidence on fruit 2020[a]			Powdery mildew severity on fruit 2020[a]		
Treatment and amount/ha	8 Jul	16 July	27 Jul	8 Jul	16 Jul	27 Jul
Untreated	95.2 a	99.1 a	96.9 ab	20.6 a	40.0 a	48.0 a
Elite 45WP 0.28 kg	95.2 a	100.0 a	96.9 ab	7.6 ab	16.1 ab	32.2 ab
Rally 40WSP 0.21 kg	77.0 ab	96.9 a	98.1 a	3.6 bc	10.4 bc	24.6 abc
Procure 480SC 0.30 L	56.4 abc	73.2 ab	90.0 ab	2.6 bc	5.7 bc	11.7 bcd
Mettle 125 0.22 L	52.0 abc	62.0 abc	69.6 abc	1.8 bc	3.7 bc	7.5 bcd
Cevya 0.22 L	23.0 bc	38.8 bc	38.8 bc	1.1 bc	2.1 bc	2.1 d
Revus Top 0.52 L	1.9 c	7.1 c	52.8 abc	0.1 c	0.3 c	3.4 cd
Aprovia 0.78 L	5.2 c	7.5 c	22.3 c	0.2 c	0.5 c	1.0 d

[a]Powdery mildew incidence (percentage of infected clusters) and severity (percentage of cluster covered by powdery mildew) were calculated from 5 clusters per treated plant. Means following the same letter are not significantly different from one another when using Tukey's HSD ($P \leq 0.05$). All data was arcsine square root transformed before analysis. Back-transformed means are shown.

Table 4.5. 2020 DMI cross-resistance trial results on leaves.

Treatment and amount/ha	Powdery mildew incidence on leaves[a]		Powdery mildew severity on leaves[a]	
	23 July	5 Aug	23 July	5 Aug
Untreated	56.4 a	87.3 a	11.8 a	36.4 a
Elite 45WP 0.28 kg	25.6 ab	83.6 a	3.4 ab	25.8 ab
Rally 40WSP 0.21 kg	18.9 abc	86.7 a	2.0 bc	27.3 ab
Procure 480SC 0.30 L	10.3 bcd	86.8 a	1.0 bc	25.9 ab
Mettle 125 0.22 L	1.0 cd	54.6 ab	0.1 bc	10.0 bc
Cevya 0.22 L	0.7 cd	14.8 bc	0.1 bc	1.9 cd
Revus Top 0.52 L	0.7 cd	1.6 c	0.1 bc	0.2 d
Aprovia 0.78 L	0.0 d	5.2 c	0.0 c	0.8 cd

[a]Powdery mildew incidence (% infected leaves) and severity (% of leaf covered by powdery mildew) were calculated from 25 leaves per treated plant. Means following the same letter are not significantly different from one another when using Tukey's HSD ($P \leq 0.05$). All data was arcsine square root transformed before analysis. Back-transformed means are shown.

Table 4.6. 2019 results from preliminary sulfur + DMI interactions trial.

	Powdery mildew leaf incidence 2019[a]		Powdery mildew leaf severity 2019[a]	
Treatment and amount/ha	6 Aug	20 Aug	6 Aug	20 Aug
Untreated	65. 9 abc	79.4 abc	25.3 ab	46.2 a
Rally 0.35 kg	81.0 a	89.3 a	38.1 a	54.3 a
Rally 0.31 kg + Microthiol Disperss 0.43 kg	73.0 ab	84.7 ab	28.6 ab	43.0 a
Rally 0.27 kg + Microthiol Disperss 0.85 kg	48.7 bcd	65.0 bcd	16.5 bc	23.1 b
Rally 0.18 kg + Microthiol Disperss 1.7 kg	37.4 cde	55.2 d	8.2 c	18.3 b
Rally 0.09 kg + Microthiol Disperss 2.55 kg	23.5 de	45.6 de	6.4 c	12.6 bc
Rally 0.04 kg + Microthiol Disperss 3.0 kg	33.5 de	58.0 cd	9.7 c	18.5 b
Microthiol Disperss 3.4 kg	17.6 e	28.4 e	4.5 c	4.9 c

[a]Powdery mildew incidence (percentage of infected leaves) and severity (percentage of leaf covered by powdery mildew) were calculated from 25 leaves per treated plant. Means following the same letter are not significantly different from one another when using Tukey's HSD ($P \leq 0.05$). All data was arcsine square root transformed before analysis. Back-transformed means are shown.

Table 4.7. 2020 Sulfur + DMI interactions trial results on fruit.

	Powdery mildew incidence on fruit 2020 [a]			Powdery mildew severity on fruit 2020 [a]		
Treatment and amount/ha	29 Jun	8 Jul	16 Jul	29 Jun	8 Jul	16 Jul
Untreated	100.0 a	100.0 a	100.0 a	28.5 a	38.3 a	54.5 a
Microthiol Disperss 3.4 kg	99.1 a	100.0 a	100.0 a	7.4 abcd	17.3 ab	24.2 b
Rally 40WSP 0.35 kg	94.8 a	100.0 a	100.0 a	7.8 abcd	16.0 ab	24.2 b
Rally 40WSP 0.21 kg + Microthiol Disperss 3.4 kg	99.1 a	100.0 a	100.0 a	7.1 abcd	10.2 bc	24.3 b
Rally 40WSP 0.35 kg + Microthiol Disperss 3.4 kg	90.5 a	100.0 a	100.0 a	4.9 bcd	9.5 bc	17.7 bc
Elite 45 WP 0.28 kg	95.2 a	96.9 a	98.1 a	12.0 abc	22.0 ab	25.4 b
Elite 45 WP 0.17 kg oz + Microthiol Disperss 3.4 kg	100.0 a	100.0 a	100.0 a	16.8 ab	26.2 ab	40.8 ab
Elite 45 WP 0.28 kg oz + Microthiol Disperss 3.4 kg	100.0 a	100.0 a	100.0 a	13.5 abc	24.6 ab	31.3 ab
Cevya 0.37 L	23.7 b	12.6 b	44.0 b	1.2 cd	0.6 c	4.2 cd
Cevya 0.22 L + Microthiol Disperss 3.4 kg	7.3 b	23.0 b	34.2 b	0.3 d	1.4 c	2.5 d
Cevya 0.37 L + Microthiol Disperss 3.4 kg	15.7 b	30.8 b	30.8 b	0.7 cd	1.1 c	1.9 d

[a] Powdery mildew incidence (percentage of infected clusters) and severity (percentage of cluster covered by powdery mildew) were calculated from 5 clusters per treated plant. Means following the same letter are not significantly different from one another when using Tukey's HSD ($P \leq 0.05$). All data was arcsine square root transformed before analysis. Back-transformed means are shown.

Table 4.8. 2020 Sulfur + DMI interactions trial results on leaves.

	Powdery mildew incidence on leaves 2020[a]		Powdery mildew severity on leaves 2020[a]	
Treatment and amount/ha	23 Jul	5 Aug	23 Jul	5 Aug
Untreated	48.8 a	82.9 a	12.5 a	31.8 a
Microthiol Disperss 3.4 kg	1.6 bc	40.0 bc	0.2 bc	9.0 bc
Rally 40WSP 0.35 kg	13.4 b	58.3 ab	3.6 ab	15.6 bc
Rally 40WSP 0.21 kg + Microthiol Disperss 3.4 kg	0.0 c	39.7 bc	0.0 c	8.2 cd
Rally 40WSP 0.35 kg + Microthiol Disperss 3.4 kg	1.0 bc	50.3 bc	0.1 bc	13.4 bcd
Elite 45 WP 0.28 kg	18.5 ab	67.3 ab	3.2 b	22.5 ab
Elite 45 WP 0.17 kg oz + Microthiol Disperss 3.4 kg	1.5 bc	42.0 bc	0.2 bc	11.4 bcd
Elite 45 WP 0.28 kg oz + Microthiol Disperss 3.4 kg	0.9 bc	24.3 cd	0.1 bc	5.0 de
Cevya 0.37 L	0.0 c	8.2 de	0.0 c	1.0 ef
Cevya 0.22 L + Microthiol Disperss 3.4 kg	0.0 c	3.1 e	0.0 c	0.3 ef
Cevya 0.37 L + Microthiol Disperss 3.4 kg	0.0 c	0.6 e	0.0 c	0.1 f

[a]Powdery mildew incidence (percentage of infected leaves) and severity (percentage of leaf covered by powdery mildew) were calculated from 25 leaves per treated plant. Means following the same letter are not significantly different from one another when using Tukey's HSD ($P \leq 0.05$). All data was arcsine square root transformed before analysis. Back-transformed means are shown.

CHAPTER 5

CONCLUSIONS

Grapevine powdery mildew (GPM), caused by the fungus *Erysiphe necator*, is a difficult to manage disease of wine grapes everywhere they are grown. In Georgia, GPM is one of the top concerns for *Vitis vinifera* (European wine grape) growers as this species and its many varieties are highly susceptible. In this research, two fungicide resistance alleles were frequently observed in *E. necator* populations in *V. vinifera* commercial vineyards in northern Georgia (Chapter 2). The G143A allele, associated with complete QoI resistance, was found in 9 of 12 commercial vineyards sampled from 2018 to 2020. The Y136F mutation, associated with increased DMI tolerance, was found in 4 of 6 vineyards sampled from 2019 to 2020. Both mutations were found at a research vineyard in Blairsville, Georgia (United States), where five field trials from 2019 to 2020 were conducted to determine efficacy from varying fungicide treatments against a resistant *E. necator* population.

In one series of trials conducted over two years at the Blairsville, Georgia, research station, field efficacy of eleven fungicides was assessed (Chapter 3). Complete lack of control from Abound (azoxystrobin, FRAC 11) and Rally 40WSP (myclobutanil, FRAC 3) were observed in both 2019 and 2020 on fruit and leaves. Contact, multi-site fungicides including Stylet-Oil (paraffinic oil) and Microthiol Disperss (sulfur) provided moderate control of GPM. Microthiol Disperss provided good to excellent control on leaves but lacked significant control

on fruit. Other single-site fungicides including Quintec (quinoxyfen, FRAC 13), Vivando (metrafenone, FRAC 50), and Aprovia (benzovindiflupyr, FRAC 7) provided excellent control with little GPM found on fruit or leaves. Pristine (pyraclostrobin + boscalid, FRAC 11 + 7) and Luna Experience (fluopyram + tebuconazole, FRAC 7 + 3) also performed well, likely due to the SDHI (FRAC 7) counterpart included in the mixtures. Inspire Super (difenoconazole + cyprodinil, FRAC 3 + 9) also performed well and significantly better than Rally 40WSP even though both fungicides contain a chemical with the same mode of action. This is likely a result of the higher inherent activity of difenoconazole compared to myclobutanil; it is possible that this intrinsic activity may be more readily observed in the presence of DMI resistance.

In other plant-pathogen management systems, mixing sulfur with a DMI fungicide can provide higher levels of control than either stand-alone treatment. To assess this potential synergy for powdery mildew in wine grapes, a field trial was initially conducted to look at varying amounts of sulfur and myclobutanil. In this trial, the treatment with the highest level of efficacy was sulfur at its full rate, without myclobutanil. Sulfur provided a step-wise additive and linear level of control based on increasing rate, whereas the same could not be said of the DMI fungicide; no evidence of synergy was observed. To further compare, another trial was designed in which 1.4 kg sulfur was mixed with either myclobutanil, tebuconazole, or mefentrifluconazole at their low and high labeled rates. Mefentrifluconazole provided more control of GPM than myclobutanil, tebuconazole, and combinations of them with sulfur. Sulfur provided additional efficacy on leaves when mixed with the DMI fungicides, but evidence of a synergistic relationship was not observed (Chapter 4). Additionally, sulfur provided less control on fruit clusters than it did on leaves, suggesting that growers in Georgia should be careful when relying on sulfur for early-season control of GPM.

Six different DMI (FRAC 3) fungicides including difenoconazole, myclobutanil, tebuconazole, triflumizole, tetraconazole, and mefentrifluconazole were compared to determine differences in efficacy throughout this class. Although these chemicals all have the same mode of action and target 14α-demethylase in the ergosterol biosynthesis pathway, varying efficacy between treatments was observed. Myclobutanil and tebuconazole performed the worst in the presence of the Y136F allele on both fruit and leaves. Triflumizole and tetraconazole performed slightly better, but mefentrifluconazole and difenoconazole provided the highest level of control of GPM in the presence of high levels of the Y136F allele (Chapter 4). These results demonstrated that cross-resistance in this class is incomplete, and the presence of this resistance allele may not mean the complete failure of all chemicals with this mode of action.

The information compiled in this research will be used to directly help grape growers in the state of Georgia. If growers are aware of the population structure of GPM in their vineyard, they can make the most informed decisions when developing their spray programs. It is not recommended to rely upon QoIs for control of GPM in Georgia, and DMIs should be used sparingly. Products containing mefentrifluconazole and difenoconazole can continue to be used with confidence for control of GPM and other fungal diseases, but mixing sulfur is recommended for resistance management and added crop protection. With more frequent use of these newer DMIs, it is possible that *E. necator* could develop increased resistance that results in lower efficacy from these products as well as SDHIs and other single-site fungicides. Using various modes of action and alternating between them is essential, and growers are at risk of losing more high performing fungicides to resistance.

APPENDIX

Introduction

Black rot is a fungal disease of both European wine grapes (*Vitis* vinifera) and muscadines (*Vitis rotundifolia*). The causal agent, *Phyllosticta ampelicida* (syn. *Guignardia bidwellii*) is a fungus in the Ascomycota that can infect leaves, canes, and fruit. *P. ampelicida* is considered a hemi-biotroph, as it has been found to live as an endophyte in grape leaves, but it can also switch to a saprobe by producing appressorium to feed on host cells (Kuo and Hoch 1996). Originally from North America, black rot spread to Europe in 1885 (Reddick 1911).

P. ampelicida can overwinter as either conidia in pycnidia on the leaves or as ascospores in pseudothecia on mummified fruit (Pirrello et al. 2019). Once the spores germinate, they produce appressoria and cause necrotic patches on the leaves. Infected fruit will turn brown and shrivel up to produce mummies (Wilcox 2003). Grapes and muscadines are most susceptible from pre-bloom to bunch closure; depending on conditions, ontogenic resistance is seen approximately 8 weeks post bloom (Hoffman et al. 2002). This disease thrives in very humid, warm climates, and it is therefore more of an issue in the Eastern and Midwestern United States. Rain is essential to the spread of this disease, since the primary inoculum, ascospores inside pseudothecia, are ejected during rain events of 1 to 3 mm (Onesti et al. 2017). In addition to these sexually produced pseudothecia, *P. ampelicida* produces asexual pycnidia that also require rain events of at least 1 mm to release conidia (Onesti et al. 2017).

In *V. vinifera* vineyards where spray programs include a variety of synthetic chemicals, black rot is not a huge issue. In muscadines, however, black rot is one of the most destructive diseases in Georgia (Krewer et al. 2019). Many types of fungicides are registered to control black rot, including carbamates, demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), and dithiocarbamates (Molitor and Beyer 2014). In organic vineyards, this disease is one of the most difficult to manage. Copper is the most used organic fungicide to combat black rot but still does not provide satisfactory levels of control comparable to synthetic fungicides (Molitor and Beyer 2014).

Resistance development in *P. ampelicida* to any class of fungicide has not been reported yet, but there have been observations that DMIs are not providing the same control they once did. In Miessner et al. (2011), it was found that there is an intron in the cytochrome *b* region that might prevent the G143A mutation that is associated with complete QoI resistance. Other mutations, however, could occur in the same region, F129L for example, but are usually associated with reduced sensitivity rather than complete resistance (Grasso et al. 2006). In this study, *P. ampelicida* was isolated from muscadines and European wine grapes through Georgia and in a research vineyard in New York. In future studies, we plan to phenotype and genotype isolates for fungicide resistance.

Materials and Methods

Fungal isolation

When black rot was found in a commercial vineyard or on wild grapes, infected leaves or fruit were collected in plastic or papers bags and transported back to the lab. If samples were shipped from other states, they were shipped overnight and stored at 4°C until used for culturing.

To isolate *P. ampelicida* from leaves, small (1 cm × 1 cm) sections of the brown lesions were cut and surface sterilized with 100 mL 10% bleach (Clorox) for 45 sec, immediately washed with 100 mL 70% ethanol (Koptec), and then rinsed twice with 50 mL sterile deionized (DI) H_2O. Lesions were soaked in the final rinse of DI H_2O for 10 min and then dried on sterilized Whatman filter paper for 1 min. Lesions were plated on 100-mm Petri plates containing oatmeal agar or malt extract agar (Difco) amended media with antibiotics (5 mg/L gentamycin and 50 mg/L chloramphenicol) by partially embedding previously cut lesions into the agar. Plates were incubated at 23° C with 12 hours full spectrum light and 12 hours darkness until characteristic growth (Fig 6.1) was observed typically 14 days after plating. If growth was not observed using the above method, new lesions would be surface sterilized the same as above and placed in empty petri dishes. Sterile DI H_2O used to fully cover the surface (~ 200 μL) was then pipetted on the lesions and left to sit for 10 min. After 10 min, the water sitting on top of the lesions was collected through a pipette and plated on oatmeal agar or malt extract agar media amended with antibiotics. Plates were incubated at 23°C with 12 hours UV light and 12 hours dark until fungal growth (~14 days) was observed.

DNA extraction and ITS sequencing

Once a pure colony was obtained, fungal material was placed in a sterile tube, frozen with liquid nitrogen, and then lysed with a GenoGrinder (SPEX Metuchen, NJ) at 850 RPM for 1 min. DNA was extracted from the individual using the DNeasy Plant Mini Kit (Qiagen Valencia, CA). Once DNA was extracted from the cultures, ITS primers (ITS1F and ITS4) (White et al. 1990) were added and placed in a thermal cycler (Eppendorf Enfield, CT). Cycling conditions were as follows: initial denaturation at 95° C for 5 min, followed by 35 cycles of 95° C for 30 sec, 55° C for 30 sec, 72° C for 30 sec, and a final extension of 72° C for 1 min. Amplification of

the ITS region was confirmed through gel electrophoresis where single bands were observed at ~500 kb regions. Samples were stored in 4°C until they were further analyzed. Amplified DNA was sent to GeneWiz LLC (South Plainfield, NJ) where it was sequenced through Sanger sequencing. Geneious Prime (Auckland, New Zealand) was used to align sequences. The sequences were then placed in the United States National Center for Biotechnology Information's Basiv Local Alignment Search Tool (BLAST) to compare with other sequences in the database for similarities.

Results and Discussion

A total of 13 isolates were collected and are shown in Table 6.1. The isolates sequenced were NNY2, NNY6, NNYP, and WM1.

P. ampelicida was easier to isolate from *V. rotundifolia* samples rather than *V. vinifera* samples. The isolates collected from *V. vinifera* were collected from fruit as other organisms from the leaves outcompeted *P. ampelicida* on agar. This organism grows very slowly with a one-month culture not even covering a petri dish, allowing other organisms to grow throughout the petri dish before spores can germinate.

Morphologies among isolates were varied. NNYP had a fluffier, light grey appearance that differed significantly from both NNY2 and NNY6 which were isolated from the same bunch of grapes in the same location. NNY2 was the only isolate of the four that had more similarities to *Phyllosticta vitis-rotundifolia* than *Phyllosticta ampelicida*. To confirm that this was correct and to further look into the different morphologies of *P. ampelicida*, more isolates need to be obtained and sequenced.

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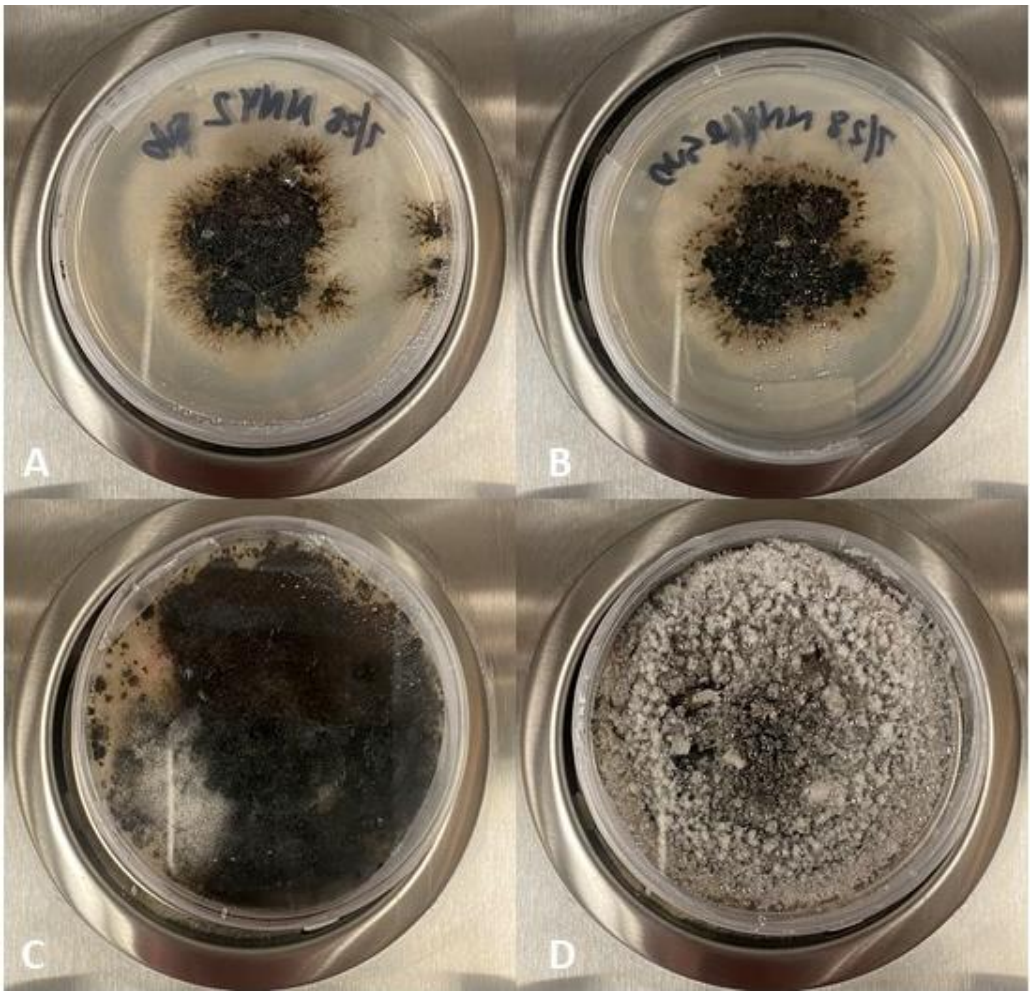

Figure 6.1. *P. ampelicida* cultures that were sequenced. A: NNY2- *Phyllosticta vitis-rotundifoliae* grown on malt extract agar. B: NNY6- *Phyllosticta ampelicida* grown on malt extract agar. C: WM1 *P. ampelicida* grown on oatmeal extract agar. D: NNYP- *P. ampelicida* grown on oatmeal extract agar.

Table 6.1 *P. ampelicida* isolates collected.

Isolate	Host	Location
BRS1	*V. vinifera* 'Chardonnay'	Research Vineyard - Blairsville, GA
BRS2	*V. vinifera* 'Chardonnay'	Research Vineyard - Blairsville, GA
CA	*V. rotundifolia*	Commercial Vineyard - Screven County, GA
NNY2	*V. labrusca* 'Niagara'	Research Vineyard - Geneva, NY
NNY6	*V. labrusca* 'Niagara'	Research Vineyard - Geneva, NY
NNY7	*V. labrusca* 'Niagara'	Research Vineyard - Geneva, NY
NNY8	*V. labrusca* 'Niagara'	Research Vineyard - Geneva, NY
NNY9	*V. labrusca* 'Niagara'	Research Vineyard - Geneva, NY
NNYP	*V. labrusca* 'Niagara'	Research Vineyard - Geneva, NY
SC1	*V. rotundifolia*	Commercial Vineyard - Bogart, GA
WM1	*V. rotundifolia*	Wild - Athens, GA
WM2	*V. rotundifolia*	Wild - Athens, GA
3SM1	*V. rotundifolia*	Wild - Dahlonega, GA near Three Sisters Vineyard