FUNGICIDE SENSITIVITY PROFILING OF *PLASMOPARA VITICOLA* IN GEORGIA VINEYARDS

by

SARAH ELIZABETH CAMPBELL

(Under the Direction of Phillip M. Brannen and Harald Scherm)

ABSTRACT

Grapevine downy mildew is a serious disease caused by the oomycete *Plasmopara viticola*. This thesis evaluated the prevalence of fungicide resistance in Georgia populations of *P. viticola* using bioassays and molecular methods, and the field efficacy of fungicides commonly used to manage the disease. Resistance to quinone outside inhibitor (QoI) fungicides (G143A mutation) was widespread throughout the state in this pathogen (present in 82.1% of vineyard-year combinations), whereas no carboxylic acid amide or phenyl amide fungicide resistance was detected. Moreover, QoI fungicides provided the least amount of disease control in on-farm field trials and performed no different from untreated controls when tested in a research vineyard. In contrast, other groups of fungicides tested provided adequate or exceptional control of downy mildew, documenting practical disease control failures associated with QoI use.

INDEX WORDS: *Plasmopara viticola*, downy mildew, grape, fungicide resistance, *Vitis vinifera*

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Grape production in Georgia. With ~ 641 hectares and ~25 wineries (Crawford 2018), the wine grape industry in Georgia is relatively small. However, the industry is rapidly growing with the Farm Gate value tripling between 2012 and 2017 (CAED 2017). Illustrating the recent growth of the industry, Watts (2008) estimated its total annual economic impact as $45 million, whereas Wolfe et al. (2013) - only 5 years later- estimated the total annual value at $81.6 million. These numbers represent the total values of grape production, winery output, and visitor revenue.

Wine grape production in Georgia is concentrated in three main regions; North Geogia, Western Georgia, and Southern Georgia. European wine grapes (*Vitis vinifera*) are grown in the mountainous areas of North Georgia, where the elevation is sufficiently high to allow *V. vinifera* to avoid Pierce's disease, a disease prevalent in lower elevations which limits production of *V. vinifera*. These grapes, which include cultivars such as Cabernet Sauvignon, Chardonnay, and Merlot, are typically grown on phylloxera- and nematode-resistant rootstocks and are highly susceptible to diseases. In addition to the commonly recognized *V. vinifera* cultivars, French-American hybrid cultivars are also grown. These include cultivars such as Blanc du Bois, Lemonto, and Lenoir which are grown in areas where Pierce's disease is prevalent, i.e., the lower elevations of North Georgia as well as the Western and Southern Georgia regions. These regions are also suitable for production of the native muscadine, *Vitis rotundifolia*, which do not generally thrive at higher elevations (Hickey 2017).

Grapevine downy mildew. Grapevine downy mildew, caused by the oomycete pathogen *Plasmopara viticola,* infects all types of cultivated grape grown in Georgia except *V. rotundifolia*. It is prevalent in all grape-growing regions in the state and most of the viticultural regions globally including France, Italy, and Spain (Gessler et al. 2011).

Native to North America, this pathogen can be identified by the fluffy, white growth on the abaxial side of the leaf. Yellow "oil spot" symptoms are produced on the opposing adaxial leaf surface. Eventually, leaves turn necrotic and abscise. Abscission can occur during the growing season, causing reduction in yield or improper maturation of grape berries. The inflorescence may also show signs and symptoms of *P. viticola* infection early in the growing season until the berries develop ontogenic resistance to infection (Kennelly et al. 2005). Beyond yield loss and poor-quality harvest, *P. viticola* can weaken the grapevine and expose it to winter injury which can lead to vine death and delay production for multiple years. Georgia and much of the southeastern United States provide an ideal environment for downy mildew development. Frequent precipitation and warm temperatures lead to downy mildew epidemics that can cause total crop loss if left unmanaged.

Downy mildew epidemiology. Like other oomycete plant pathogens, *P. viticola* has a cell wall composed of cellulose, has coenocytic hyphae, is diploid, and reproduces utilizing sexual oospores and asexual sporangia. The life cycle of the pathogen begins after overwintering as an oospore in leaf litter, although asexual overwintering has also been suggested in a few grape-growing regions including the southeastern United States (Hong 2018). The oospores germinate in the spring utilizing free water and produce a single sporangium which gives rise to 30 to 60 zoospores. Zoospores are biflagellate, motile spores which move in free water and penetrate through the stomata of the host. Germ tubes penetrate the stomatal opening and cause a

swelling in the stomatal cavity. After a latent period of 7 to 10 days, sporulating lesions producing sporangia appear in favorable conditions on the abaxial leaf surface (Rossi and Caffi 2007). Each sporangium can contain 1 to 10 zoospores which are released in the presence of free water and cause secondary infections. Free water is required for a significant portion to the pathogen's lifecycle and is necessary for disease development. Inoculum from both oospores and sporangia can contribute to disease epidemics throughout the season (Hong 2018). Late in the season, sexual reproduction occurs through the fusion of antheridium and oogonium to produce the overwintering structure, the oospore (Gessler et al. 2011, Lafon and Clerjeau 1988).

Management of grapevine downy mildew. Downy mildew is among the top three disease concerns in Georgia wine grape production, others being powdery mildew and Botrytis bunch rot (Brannen 2018). In 2016, downy mildew cost Georgia grape producers $1,109,100, of which $124,000 was related to the cost of disease control measures (Brannen 2018). Control methods include cultural practices such as removing leaf litter from the vineyard floor and keeping weeds mowed; however, downy mildew management largely relies on fungicide applications. Growers typically apply fungicides on a host phenology-based schedule starting at budbreak and continuing until defoliation and dormancy; depending on rainfall patterns, fungicides may be applied on either 7 to 10 (wet) or 10 to 14-day (dry) intervals. In wet years, this can lead to 17 or more fungicide applications per year.

Currently, there are many commercial fungicides available for downy mildew management, but they vary considerably in efficacy and preharvest interval, rendering some more useful than others. Early in the growing season, winegrape producers in Georgia typically use multi-site fungicides such as the dithiocarbamate, mancozeb. These types of fungicides are relatively effective and have a low risk of resistance development due to their multi-site mode of

action. However, they also have a long preharvest interval, which prevents their usage later in the growing season. During the middle and end of the growing season, growers, therefore, may have to use single-site mode of action fungicides, such as quinone outside inhibitors (QoI) and carboxylic acid amides (CAA), for disease management (Gisi and Sierotzki 2015; Nita et al. 2019). These fungicides can be very effective against downy mildew but carry a greater risk of fungicide resistance development (FRAC 2018b).

Fungicide resistance. More than 250 plant pathogens have developed reduced sensitivity or resistance to one or more agricultural pesticides (FRAC 2018a). Pesticide resistance can be defined as the acquired heritable shift of a pest population toward reduced sensitivity to a pest management chemical. This phenomenon occurs in weeds, arthropods, and plant pathogens. Among the various pest organisms, plant pathogens are the most prone to resistance development due to their short generation times with large numbers of propagules produced, long propagule dispersal distances, sexual and asexual reproduction, and/or repeated exposure to pesticides applied during the growing season for disease protection (Brent and Hollomon 2007). The Fungicide Resistance Action Committee (FRAC) is an industry organization which classifies fungicides into groups based on their modes of action, conducts research on fungicide-resistant pathogens, and makes recommendations on fungicide resistance management. Many factors go into the risk assessment of a fungicide, such as the fungicide class and biochemical site of action. Fungicides with a single-site mode of action generally have a higher risk of fungicide resistance development than those with a multi-site mode of action. Beyond the risk assessment of the fungicide active ingredient, there is also a risk assessment based on pathogen biology. Pathogens are considered to have high resistance risk if they can undergo many disease cycles per season,

disperse over long distances, and have a sexual phase in their life cycle. *Plasmopara viticola* is an example of a pathogen with a high resistance development risk (FRAC 2018a, 2018b).

Two types of resistance are generally recognized that can develop in response to fungicides in the pathogen population: qualitative and quantitative. Qualitative resistance is also referred to as disruptive or discrete resistance. In qualitative resistance, the resistant individuals in the population are insensitive to the fungicide at concentrations many times those used to effectively manage a sensitive pathogen. Quantitative resistance, on the other hand, is also referred to as multi-step or continuous resistance. In this case, the pathogen population exhibits a range of sensitivities to a fungicide concentration. In field conditions, applying a higher dose of fungicide may control the pathogen; however, continually increasing the dose would be necessary for long-term control (Brent and Hollomon 2007).

Among FRAC groups, pathogens may also exhibit cross-resistance. Cross-resistance occurs when a pathogen has developed reduced sensitivity to one active ingredient in a given fungicide class and a reduction of sensitivity to other active ingredients in that fungicide class which have similar mechanisms of action is also observed. Cross-resistance has been demonstrated in many classes of fungicides including the quinone outside inhibitors and the phenyl amides. In addition, some pathogens have developed multiple resistance in which resistance arose independently to multiple unrelated fungicides (Brent and Hollomon 2007; FRAC 2018b).

QoI fungicide resistance. Quinone outside inhibitor (QoI) fungicides first appeared on the market in 1996 and are classified as FRAC group 11. This class of fungicides, also referred to as the strobilurins, was originally discovered by isolating antifungal compounds from wood-decaying mushroom fungi. Commercial manipulation of the strobilurins created compounds that

were less likely to break down rapidly and were easier to apply safely. Along with being classified as reduced-risk pesticides, the QoIs affect a very broad range of plant pathogens, from oomycetes to rusts (Gullino et al. 2000). Increased safety, broad-spectrum activity, and their preventative and post-symptom activity made this class of fungicides popular among growers (Wong and Wilcox 2001).

QoIs are single-site fungicides that block the transfer of electrons at the quinone outside site of the bc1 complex of the mitochondrial electron transport chain, also called complex III (Gullino et al. 2000). This single-site mode of action effectively prevents ATP formation, depriving the organism of energy. The target site, cytochrome bc1, is encoded by the *cyt b* gene which resides in the mitochondrial genome. The mitochondrial genome differs from the nuclear genome in many ways. The mitochondrial genome is uniparentally inherited, has lower genetic diversity, can withstand rearrangement, and is believed to be more prone to mutation. Conversely, the nuclear genome, which is biparentally inherited, is less prone to mutations due to more advanced repair mechanisms (Gisi et al. 2002). The site-specific nature of this fungicide class is one of the main reasons for fungicide resistance development in many plant pathogens.

Four years after their initial introduction to the market, the first QoI-resistant pathogens were identified in 2000. These included *Blumeria graminis* f. sp. *tritici* on wheat in France, Germany, and the U.K.; *Pseudoperonospora cubensis* on cucumber in Spain and Japan; and *P. viticola* on grape in Italy (Gisi et al. 2002). Grapevine downy mildew QoI resistance has been reported in European countries including France and Italy, and in the United States in Kentucky, Maryland, New York, North Carolina, Pennsylvania, and Virginia (Colcol and Baudoin 2016; Corio-Costet et al. 2011; Gauthier and Amsden 2013; Gee et al. 2011). Currently, there are over

30 plant pathogens with resistance to QoI fungicides. This class exhibits cross-resistance among different QoI active ingredients such as azoxystrobin and pyraclostrobin.

Resistance has generally been assessed by bioassay, polymerase chain reaction (PCR) testing for specific mutations that confer resistance (Brent and Hollomon 2007), or both. Two mutations for QoI resistance in *P. viticola* that alter the QoI target site have been described previously. The more extensively described mutation, G143A, is a point mutation which changes glycine to alanine at position 143 in the *cyt b* gene of the mitochondrial genome (Baudoin et al. 2008; Chen et al. 2007; Corio-Costet et al. 2011; Gee et al. 2013; Gisi et al. 2002). This single nucleotide polymorphism confers "total" or qualitative resistance to QoI fungicides. The other described mutation, F129L, confers "partial" or quantitative resistance to QoI fungicides and is the result of a single nucleotide polymorphism occurring at position 129 of the *cyt b* gene which changes the resulting amino acid from phenylalanine to leucine (Chen et al. 2007). Neither of these mutations have a significant negative effect on the enzyme activity and the resulting fitness of the organism (Gisi et al. 2002).

CAA fungicide resistance. Carboxylic acid amide (CAA) fungicides reside in FRAC group 40 and have been used in commercial agriculture since 1988 with the introduction of dimethomorph. Iprovalicarb was introduced in 1998 and was followed by flumorph in 2000, benthiavalicarb in 2003, mandipropamid in 2005, and valifenalate in 2008 (Nanni et al. 2016; Toffolatti et al. 2018). Compounds in this group have a single-site mode of action which inhibits the cellulose biosynthesis necessary for cell wall formation after zoospore encystment in host tissue (Blum et al. 2010).

Resistance of *P. viticola* to the CAA dimethomorph was documented in French vineyards in 1994 and 2000 (Gisi et al. 2007). Resistance to mandipropamid, a more current widely used

fungicide, was reported in Europe as early as 2004 (Aoki et al. 2013). To date, CAA resistance has been described in France, India, Italy, and Japan (Toffolatti et al. 2018). Most recently, resistance was reported in the United States in Virginia and North Carolina (Feng and Baudoin 2018).

Plasmopara viticola cellulose biosynthesis is regulated by a group of nuclear genes, *CesA*. Within *CesA3,* a single nucleotide polymorphism at position 1105 confers resistance by changing the resulting amino acid from glycine to serine (G1105S) or glycine to valine (G1105V) (Nanni et al. 2016). This type of resistance is recessive, necessitating that both alleles of the diploid *P. viticola* genome carry the resistance mutation to show a highly resistant phenotype (Gisi et al. 2007).

PA fungicide resistance. The first occurrence of phenylamide (PA) resistance in the field was documented in Europe in 1980 for control of *P. infestans* on potatoes (Staub 1991). Resistance of *P. viticola* to the common PA fungicide metalaxyl was first observed in vineyards in 1981 in France and South Africa. PAs control diseases by inhibiting RNA synthesis, specifically rRNA polymerization. The mechanism of resistance to phenylamides and the number of genes involved is currently unknown (Gisi et al. 2000). No PA resistance has been reported previously in the United States for *P. viticola,* and a recent survey conducted in the southeastern United States failed to detect resistance to mefenoxam, a more biologically active enantiomer of metalaxyl (Colcol and Baudoin 2016).

While fungicide resistance development in *P. viticola* has been documented for many regions globally, no studies have evaluated the sensitivity of populations of this pathogen in vineyards in Georgia. Additionally, no comprehensive vineyard-level efficacy studies have been conducted with routinely used, oomycete-active fungicides for downy mildew management;

efficacy comparisons are important, as producers need to incorporate numerous chemical classes for resistance management, while also maintaining excellent disease control and meeting preharvest interval requirements. This is especially critical for locations where fungicide-resistance has been documented.

The lack of information pertaining to fungicide resistance of *P. viticola* in Georgia necessitates studies with the following objectives:

1) Evaluate the prevalence of fungicide resistance in *P. viticola* on wine grapes throughout Georgia vineyards through sampling, traditional bioassays, and PCR-based methods.
2) Compare the efficacy of fungicides from different FRAC groups against downy mildew progression in field trials in the presence of QoI-resistant populations of *P. viticola*.

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CHAPTER 2

SURVEY OF FUNGICIDE SENSITIVITY IN *PLASMOPARA VITICOLA* POPULATIONS IN GEORGIA VINEYARDS[1]

[1] Campbell, S., Scherm, H., and Brannen, P. M. 2019. To be submitted to *Plant Health Progress*.

ABSTRACT

Grapevine downy mildew, caused by *Plasmopara viticola*, is among the most damaging diseases of grapes globally, including the viticultural regions of Georgia. While management of this disease typically involves fungicide applications, resistance development in *P. viticola* can render chemical management ineffective. The objective of this study was to survey fungicide sensitivities of *P. viticola* populations in vineyards across three regions in Georgia. Samples were collected from 18 vineyards between 2015 and 2018 and tested for phenotypic sensitivity to quinone outside inhibitor (QoI), carboxyl amide acid (CAA), and phenylamide (PA) fungicides using bioassays and the presence of the known resistance-causing mutations G143A (QoI) and G1105S (CAA) using polymerase chain reaction (PCR). This study documented widespread occurrence of QoI resistance in Georgia with 82.9 and 82.9% of isolates and 91.7 and 82.1% of vineyard-year combinations testing positive for QoI resistance based on bioassay and PCR testing, respectively. No CAA or PA resistance was identified. Due to the widespread presence of QoI resistance, QoI fungicides should not be used for downy mildew management in Georgia vineyards, and resistance management practices should be employed to maintain the fungicides still effective on downy mildew management in the state.

INTRODUCTION

Control of grapevine downy mildew is one of the top three disease management concerns for Georgia grape producers, the other two being black rot and powdery mildew management (Brannen 2018). Downy mildew, caused by *Plasmopara viticola*, causes defoliation of the

grapevine, which lowers overwintering potential, delays fruit maturation, and causes direct yield and quality losses (Matasci et al. 2007). In 2016, downy mildew caused an 8% reduction in crop value statewide and cost Georgia grape producers a total of $1.1 million in damage and control costs (Brannen 2018).

Plasmopara viticola is an oomycete plant pathogen that is native to North America. Each year, downy mildew epidemics begin with oospore germination in leaf litter, although asexual overwintering as also been suggested based on evidence of clonal genotypes occurring over multiple years (Hong 2018). Each oospore produces one sporangium, which in turn produces several zoospores. These motile zoospores move in free water and penetrate the stomatal openings to colonize the mesophyll cells of leaves, rachides, and young grape berries (Sapkota et al. 2018). Subsequently, sporangia are produced which emerge through stomata and produce more zoospores as secondary inoculum (Rossi and Caffi 2007). During the epidemic, both oospores in leaf litter and asexual sporangia contribute to disease progression (Kennelly et al. 2007; Hong 2018).

Due to the large number of sporangia produced asexually during an epidemic, the short generation time, and the sexual recombination prior to oospore formation, *P. viticola* is a pathogen with a high risk of fungicide resistance development as defined by the Fungicide Resistance Action Committee (FRAC) (FRAC 2013). As a high resistance risk pathogen, *P. viticola* has shown reduced sensitivity to 5 of the 16 available oomycete-active fungicide classes (FRAC 2018a, Gisi and Sierotzki 2015). In some cases, including the quinone outside inhibitor (QoI) fungicides, resistance development occurred quickly at 4 years after market introduction in 1996 (Bartlett et al. 2002, Gisi and Sierotzki 2015). QoI fungicides are in FRAC group 11 and inhibit mitochondrial respiration by binding at the Qo site of the cytochrome b on the

mitochondrial membrane. Resistance to QoI fungicides in many pathogens, including *P. viticola*, can be caused by a single point mutation in the cytochrome *b* gene at amino acid site 143 which substitutes glycine for alanine, G143A. This mutation confers total QoI resistance in the pathogen (Gisi and Sierotzki 2008).

Another resistance-causing mutation identified in *P. viticola*, G1105S in the *PvCesA3* nuclear gene, confers a recessive carboxylic acid amide (CAA) fungicide resistance. Resistance occurs only if both alleles of this diploid pathogen contain this single nucleotide polymorphism, which substitutes glycine for a serine at position 1105 (Blum et al. 2010). The CAA fungicide class (FRAC group 40) specifically targets oomycetes in the order *Peronosporales* by inhibiting cellulose synthesis, a necessary process in the manufacturing of oomycete cell walls (Blum et al. 2012, Gisi and Sierotzki 2015). While resistance development risk is moderate for *P. viticola*, resistance to CAA fungicides in oomycetes has been documented as early as 1994, shortly after their introduction (FRAC 2013, Gisi and Sierotzki 2015), and it has been confirmed also in *P. viticola* in Europe and the United States (Baudoin et al. 2008, Gisi et al. 2007).

Resistance by *P. viticola* to phosphonate, cyanoacetamide, and phenylamide (PA) fungicides has also been described previously; however, the mechanisms of resistance to these fungicides remain unclear. The phosphonates function primarily by induction of host plant defenses, whereas the mode of action for cyanoacetamides is classified as unknown (FRAC 2018a). PA fungicides belong to FRAC group 4 and inhibit ribosomal RNA biosynthesis, which, in oomycetes, mainly impacts hyphal growth and sporangium formation. Loss of sensitivity to this fungicide class was documented as early as 1981 in European vineyards (Gisi and Sierotzki 2015).

Loss of sensitivity of *P. viticola* to one or more fungicide classes has been documented in viticultural regions globally; however, no fungicide sensitivity testing has been conducted on *P. viticola* populations in the state of Georgia. The objective of this study, therefore, was to survey fungicide sensitivities in three Georgia regions where wine grapes susceptible to downy mildew are produced using both bioassays and – where available – molecular assays.

MATERIALS AND METHODS

Isolate collection. Samples of downy mildew-infected leaves from 18 commercial vineyards throughout Georgia and one research vineyard in North Georgia were obtained between 2015 and 2018 (Fig. 2.1). Most samples were collected from North Georgia (13 vineyards), where downy mildew-susceptible *V. vinifera* cultivars dominate. Three vineyards were sampled from West Georgia and two vineyards were sampled from South Georgia. Leaves with actively sporulating downy mildew lesions were taken from 20 locations within one block in each vineyard, whereby a block was denoted by the grower and contained one cultivar. Cultivars from which leaves were collected included *V. vinifera* cultivars Barbera, Cabernet Sauvignon, Gewürztraminer, Merlot, Syrah, and Touriga Nacional and hybrid cultivars Blanc du Bois and Lenoir. All leaves were placed in individual zipper storage plastic bags and transported to the lab on ice.

In the laboratory, *P. viticola* isolates from single lesions with downy mildew sporulation were subcultured on leaf discs from greenhouse-grown Chardonnay plants. Discs were cut with a 13-mm cork borer; surface-disinfested by a sterile distilled water wash with light fingertip abrasion; sprayed with 70% ethanol and washed again with sterile distilled water; and placed on rifampicin, ampicillin, and pimaricin (RAP) water agar (Wong and Wilcox 2000). Single lesions

on field-collected leaves were washed with 10 µL of sterile water by repeatedly ejecting and regaining water over the leaf surface. The sporangium suspensions were deposited on the leaf discs, and cultures were incubated at 22°C for 24 h in the dark, after which excess water was shaken off and the leaf discs were returned to the incubator at 22°C with a 12-h light and dark cycle. After 7 days, one or two leaf discs were placed in long-term storage and one leaf disc was used to extract *P. viticola* DNA as described below. Isolates were stored long-term by placing leaf discs in 1.5-mL microcentrifuge tubes. Tubes were left open in a laminar flow hood for 24 h, after which the tubes were closed and placed in a -20°C freezer.

Fungicide resistance bioassays. Leaf disc bioassays were conducted with all three fungicide groups of interest, whereby azoxystrobin, mefenoxam, and mandipropamid served as representatives of the QoI, PA, and CAA classes, respectively. Isolates from long-term storage were revived in groups of five to ten. The process consisted of washing the stored leaf disc with distilled water and pipetting the water onto greenhouse-grown Chardonnay leaf discs on RAP water agar as described above. Seven days later, fungicide solutions were prepared. Azoxystrobin (Abound 22.9%, Syngenta Crop Protection, Greensboro, NC) and mefenoxam (Ridomil Gold SL 45.3%, Syngenta Crop Protection) were evaluated at concentrations of 1, 3, 10, 30, 100, and 300 µg/mL. Mandipropamid (Revus 23.3%, Syngenta Crop Protection) was tested at 0.1, 1, 3, 10, 30, and 100 µg/mL. Chardonnay leaf discs were soaked in each concentration of fungicide on a shaker for 1 h; leaf discs soaked in water were used as a control. After 1 h, four leaf disc replicates from each concentration were placed on one RAP water agar dish. Dishes were stored in the dark for 24 h, when 10 µL of a 1.0 x 10^6 sporangia/mL suspension was placed on each disc. Dishes were stored at 22°C in the dark for 24 h, shaken to remove water, and returned to 22°C for a 12-h light/dark cycle. Seven days after inoculation, the

incidence of discs with signs of downy mildew and disease severity (measured as percent disc area with sporulation) were assessed visually on each disc. To indicate resistance, a discriminatory dose of 1.0 µg/mL was used for the QoI active ingredient azoxystrobin (as used previously by Baudoin et al. 2008). A discriminatory dose of 10 µg/mL was used to determine resistance to CAA and PA fungicides.

Molecular fungicide resistance assays. PCR-based fungicide resistance assays were conducted for the QoI and CAA fungicide classes for which certain sequences of resistance-causing mutations are known. Total DNA was extracted from leaf discs using a method provided by Hong (2018). Briefly, 7-day-old sporulating lesions of *P. viticola* were washed with 50 µL of 1× TBE buffer in 1.5-mL microcentrifuge tubes. Tubes were microwaved for 20 s three times, after which they were centrifuged at 15,600 x *g* for 5 min. After centrifuging, the supernatant containing DNA was pipetted off and placed in a new microcentrifuge tube. Samples were stored at -20°C until assays were performed.

Previously published research contains several PCR primer sets for identification of key mutations in the *cyt b* and *PvCesA3* genes relating to fungicide resistance (Aoki et al. 2011; Aoki et al. 2013; Furuya et al. 2010; Furuya et al. 2009; Sirven et al. 2002; Zhang et al. 2017). To identify the G143A mutation of the *cyt b* gene for QoI fungicide resistance, primers published by Baudoin et al. (2008) were utilized. The wild-type and mutant forward primers were 5′ CCT TGG TGA CAA ATG AGT TTT TGG AG 3′ and 5′ CCT TGG TGA CAA ATG AGT TTT TGG AC 3′, respectively. The two forward primers have a common reverse primer, 5′ CAA CTT TTC CAA TTA ATG GGA TAG 3′. A separate PCR reaction was completed with the wild-type and the mutant primers for each DNA sample. Reactions with water were used as a negative control and a resistant sample obtained from Dr. Anton Baudoin (Virginia Polytechnic

Institute and State University) was used as a positive control. End-point PCR was conducted with Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Chicago, IL) using the following thermocycler settings: 95°C for 15 min, 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s (Baudoin et al. 2008). PCR products were electrophoresed on a 3% agarose gel for 90 min and visualized with ethidium bromide.

The G1105S mutation in the *PvCesA3* gene of the nuclear genome is responsible for loss of fungicide sensitivity to CAA fungicides. A tetra-primer PCR approach was published by Zhang et al. (2017) which determines the presence of the mutation in either allele of the diploid nuclear genome. This is a recessive mutation; hence the pathogen will be resistant to CAA fungicides only if both alleles contain the G1105S mutation. For the tetra-primer assay, there are two outer primers, CesaOF (5′ GCA CAG ACA TGG TTT TCC TT 3′) and CesaOR (5′ GTC CAA AAG TGC AAA GTC CAA CG 3′) and two inner primers, Cesa3IW (5′ TAC CTT TAC GGC AAA TGT GTG CG 3′) and Cesa3IM (5′ GAC AAT GTA GAC AAC CAG CAA CGA TCT 3′). The two outer primers create a 612 bp standard fragment for each reaction. Cesa3IW generates a 238 bp fragment with Cesa3OR, indicating a sensitive allele. Cesa3IM generates a 378 bp fragment with Cesa3OR, indicating a resistant allele. The approximate 100 bp difference in size of the sensitive and resistant amplicons allows heterozygous *P. viticola* to be distinguished from homozygous individuals in a single reaction. A reaction with water was used as a negative control and a CAA-resistant sample was obtained from Dr. A. Baudoin as a positive control. Illustra PuReTaq Ready-To-Go PCR Beads were again utilized for end-point PCR, and thermocycler conditions were set as follows: 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 63°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min (Zhang et

al. 2017). PCR products were electrophoresed on 1.5% agarose gel for 45 min and visualized with ethidium bromide.

RESULTS

Isolate collection. A total of 20 *P. viticola* infected leaves were collected from each vineyard. Vineyards ranged in age, management intensity, and disease levels throughout the state. From each leaf collected, a single lesion was subcultured; however, subculturing was not successful for all isolates, resulting in a variable sample number per vineyard (Tables 2.1 and 2.2).

Fungicide resistance bioassays. Although some sporulation of select *P. viticola* isolates was visible on leaf discs treated with 0.1 µg/mL of mandipropamid (CAA fungicide class), no sporulation was visible at the discriminatory dose of 10 µg/mL for the 47 isolates tested. Similarly, no mefenoxam (PA class) resistance was detected at the discriminatory dose by bioassay of 47 isolates representing 12 vineyards in Georgia (Table 2.1). QoI resistance bioassays were conducted with fungicide concentrations increasing until phytotoxicity was observed at the edges of the leaf discs, an occurrence also documented by Baudoin et al. (2008). Using a discriminatory dose of 1.0 µg/mL, azoxystrobin (QoI) resistance was detected in bioassay in 39 isolates representing 82.9% of isolates and 91.7% of vineyard-year combinations tested (Table 2.1). The only vineyard in which QoI sensitivity was detected by bioassay was located in Colquitt County in South Georgia (Table 2.1).

In conducting bioassays, the discriminant dose of 1.0 µg/mL azoxystrobin served as a clear line between sensitive and resistant isolates as there was no intermediate level of resistance

shown. QoI-resistant *P. viticola* isolates were able to grow on all fungicide concentrations tested, making an accurate EC_{50} value determination impossible.

Molecular fungicide resistance assays. The tetra-primer assay to determine CAA fungicide resistance was applied successfully to 99 samples representing 18 vineyards and 12 counties in Georgia, none of which showed resistance (Table 2.2). By contrast, QoI fungicide resistance (G143A mutation) was detected by allele-specific PCR in 82.1% of vineyard-year combinations tested (Table 2.2). The only vineyards in which isolates lacked the G143A mutation (indicating QoI sensitivity) were located in South Georgia (Colquitt and Mitchell counties); interestingly, in the Colquitt County vineyard, where samples were collected from 2015 through 2018, the *P. viticola* population shifted from QoI sensitive to resistant in 2018 (Table 2.2).

Presence or absence of an amplified DNA sequence in QoI PCR revealed three patterns. The first pattern was the presence of a wild-type (sensitive) allele and no mutant allele (n = 42). The second pattern was the reverse, a mutant allele without a wild allele (n = 156). The third pattern was the amplification of both the wild-type allele and the mutant allele in the same sample (n = 64). This indicates the presence of both sensitive and resistant mitochondrial genome in the sample. In bioassay analysis, such isolates displayed a resistant phenotype and were able to sporulate on azoxystrobin concentrations >1.0 µg/mL.

DISCUSSION

Use of fungicides is an integral part of wine grape production in the southeastern United States, with fungicides applied every 7-10 or 10-14 days during the growing season, depending on rainfall. In this study, fungicides with different modes of action were used in bioassays to

evaluate the pathogen population for fungicide resistance. The presence of known resistance-causing mutations was detected utilizing allele-specific and tetra-primer PCR assays for QoI and CAA resistance, respectively. Samples were collected from 18 commercial vineyards throughout Georgia. Sample size per vineyard ranged from 1 to 18 resulting in 158 samples collected during this survey, but an additional 105 samples were available for molecular assay based on previously isolated and stored *P. viticola* DNA (Hong 2018). Thus, samples totaled 262 statewide over 3 years, in line with that used in similar work by Baudoin et al. (2008) where samples per vineyard ranged from 1 to 8 or by Colcol et al. (2016) where total isolates per state was 153. A sample size of 10 to 50 leaves per vineyard was considered adequate for resistance monitoring by Corio-Costet (2015).

This study documented widespread occurrence of QoI resistance in Georgia with 82.1% of vineyard-year combinations testing positive for the G143A mutation. Vineyards with QoI resistance were distributed throughout North and West Georgia, which are the main wine producing regions of the state. QoI sensitivity was restricted to two vineyards in southern Georgia (Mitchell and Colquitt counties). These vineyards are distinct from vineyards in North and West Georgia in that they grow primarily French-American hybrid cultivars; experience a warmer, more humid climate; and experience disease onset considerably later in the growing season. Based on the PCR assay, a shift from sensitivity to resistance was detected in the vineyard in Colquitt County from the 2017 to the 2018 growing season.

For both QoI and CAA fungicides, bioassay results displayed the phenotype predicted by the PCR assay. Samples were taken from a single lesion isolate, which may or may not be derived from a single zoospore infection event. For QoI fungicides, allele-specific PCR often produced amplicons for both the mutant G143A allele and for the wild-type allele. This suggests

two possibilities: 1) those single-lesion samples were isolate mixtures, since previous work evaluating single sporangiophore isolates more consistently yielded either the resistant or sensitive genotype (Gisi and Sierotzki 2015) or 2) those samples contained *cyt b* genes that are heterogeneous within an isolate. As expected, such mixed samples displayed a resistant phenotype in our bioassays.

Some fungicide resistance alleles may decrease the fitness of the pathogen in the absence of the fungicide. This is due to the resistant allele possibly disrupting important biological processes (Delmas et al. 2017). In *P. viticola*, resistance to QoI fungicides does not affect monocyclic fitness parameters such as sporangium production, latent period, or infection frequency (Corio-Costet et al. 2011). The mutation causing CAA resistance reportedly also lacks a fitness cost (Delmas et al. 2017). The lack of fitness cost suggests that even when the fungicide is not applied, the resistant mutations will remain in the pathogen population. The detection of QoI fungicide resistance in *P. viticola* in Georgia in this study indicates that QoI fungicide resistance will continue to impact Georgia's viticulture industry into the future.

Fungicide resistance management, defined as optimizing fungicide usage to delay development or spread of resistant pathogen strains, has two main strategies: active ingredient mixtures and alternation. In a recent study, mixtures were predicted to be the better resistance management practice when using high-risk fungicides such as the QoIs (Elderfield et al. 2018). QoIs and CAAs also show cross-resistance, indicating that resistance development to one fungicide in the class renders the other active ingredients of that class equally ineffective (FRAC 2018b). Employing resistance management strategies such as alternation and fungicide mixtures will be critical to maintain effectiveness of CAA and PA fungicides in this region.

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Table 2.1. Results of leaf disc bioassays to assess resistance of *Plasmopara viticola* against quinone outside inhibitor (QoI; azoxystrobin), carboxylic acid amide (CAA; mandipropamid) and phenylamide (PA; mefenoxam) fungicides in Georgia vineyards.

				QoI		CAA		PA	
County	Region[a]	Vineyard	Year collected	Resistant isolates/total[b]	Percent Resistance	Resistant isolates/total[c]	Percent resistance	Resistant isolates/total[c]	Percent resistance
Cobb	W	B	2017	5/5	100	0/5	0	0/5	0
Colquitt	S	C	2017	0/8	0	0/8	0	0/8	0
Fannin	N	D	2017	4/4	100	0/4	0	0/4	0
Gilmer	N	E	2017	2/2	100	0/2	0	0/2	0
		F	2017	5/5	100	0/5	0	0/5	0
Haralson	W	G	2018	4/4	100	0/4	0	0/4	0
Lumpkin	N	H	2018	2/2	100	0/2	0	0/2	0
		I	2018	1/1	100	0/1	0	0/1	0
		J	2018	3/3	100	0/3	0	0/3	0
Rabun	N	M	2017	7/7	100	0/7	0	0/7	0
White	N	Q	2017	3/3	100	0/3	0	0/3	0
		R	2017	3/3	100	0/3	0	0/3	0
TOTAL				39/47	82.9	0/47	0	0/47	0

[a] Region in Georgia where county is located; N: North, W: West, S: South

[b] Isolates determined resistant when growth at fungicide concentration of 1.0 µg/mL

[c] Isolates determined resistant when growth at fungicide concentration of 10 µg/mL

Table 2.2. Results of polymerase chain reaction assays to assess resistance of *Plasmopara viticola* against quinone outside inhibitor (QoI; G143A mutation) and carboxylic acid amide (CAA; G1105S mutation) fungicides in Georgia vineyards.

				QoI		CAA	
County	Region[a]	Vineyard	Year collected	Resistant isolates/ total[b]	Percent resistance	Resistant isolates/ total[c]	Percent resistance
Carroll	W	A	2018	16/16	100	0/5	0
Cobb	W	B	2017	8/8	100	0/1	0
Colquitt	S	C	2015	0/6	0	--	--
		C	2016	0/15	0	--	--
		C	2017	0/19	0	0/28	0
		C	2018	3/3	100	0/1	0
Fannin	N	D	2017	16/16	100	0/5	0
Gilmer	N	E	2017	6/6	100	0/2	0
		F	2015	10/10	100	--	--
		F	2017	12/12	100	0/2	0
Haralson	W	G	2018	15/15	100	0/3	0
Lumpkin	N	H	2018	5/5	100	0/3	0
		I	2015	4/4	100	0/1	0
		I	2018	9/9	100	0/3	0
		J	2018	14/14	100	0/4	0
Madison	N	K	2018	18/18	100	0/4	0
Mitchell	S	L	2016	0/1	0	--	--
		L	2017	0/4	0	--	--
Rabun	N	M	2015	8/8	100	--	--
		M	2017	6/6	100	0/4	0
Towns	N	N	2018	7/7	100	0/4	0
		O	2018	15/15	100	0/4	0
Union	N	P	2015	6/6	100	--	--
		P	2016	9/9	100	0/6	0
		P	2017	15/15	100	0/10	0
White	N	Q	2017	3/3	100	0/2	0
		R	2015	5/5	100	0/5	0
		R	2017	8/8	100	0/5	0
TOTAL				218/263	82.9	0/102	0

[a] Region in Georgia where county is located; N: North, W: West, S: South
[b] Isolates containing G143A mutation conferring complete resistance out of total isolates tested
[c] Isolates containing a homozygous G1105S mutation conferring resistance out of total isolates tested

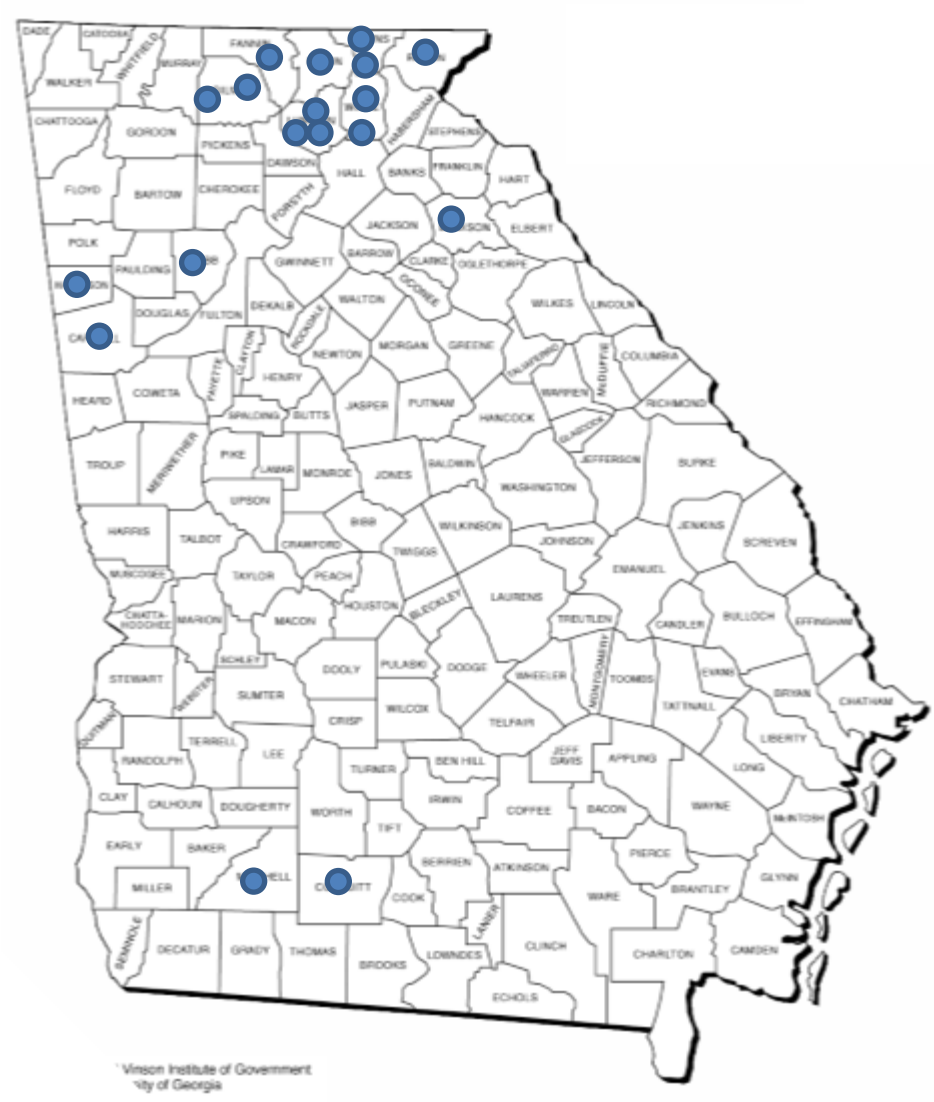

Figure 2.1. Vineyards (*n* = 18) sampled for *Plasmopara viticola* isolates in three grape-producing areas of Georgia (North, West, and South Georgia) between 2015 and 2018.

CHAPTER 3

EFFICACY OF FUNGICIDE TREATMENTS FOR *PLASMOPARA VITICOLA* CONTROL AND OCCURRENCE OF STROBILURIN FIELD RESISTANCE IN GEORGIA[1]

[1] Campbell, S., Scherm, H., and Brannen, P. M. 2019. To be submitted to *Crop Protection*.

ABSTRACT

Grapevine downy mildew, caused by *Plasmopara viticola*, is a major disease of cultivated wine grapes in Georgia (USA), where the climate is favorable for rapid epidemic development. Although loss of fungicide sensitivity has been documented for *P. viticola* populations in other viticultural regions, it had not been confirmed in Georgia. Downy mildew samples from three vineyards, two managed commercially and one located at a research station, were subjected to polymerase chain reaction (PCR) assays to identify the presence of known mutations conferring quinone outside inhibitor (QoI) and carboxylic acid amide (CAA) fungicide resistance; PCR assays detected the presence of the QoI resistance-conferring mutation G143A in 100% of samples (n = 39) and the absence of the CAA resistance-conferring mutation G1105S at all three locations. To determine fungicide efficacy, field trials were conducted in these same vineyards with 11 oomycete-active chemicals or combinations. Significantly reduced efficacy of QoI-containing fungicides (azoxystrobin and pyraclostrobin), with disease levels statistically equivalent to those of untreated vines, was observed. Based on the detection of the QoI resistance-conferring mutation G143A and associated field disease control failures observed where QoIs were applied, QoIs are likely no longer effective for downy mildew management in most Georgia vineyards.

INTRODUCTION

Plasmopara viticola (Berk. & M.A. Curtis) Berl. & DeToni, the causal agent of grapevine downy mildew, is prevalent in Georgia vineyards and can cause complete crop loss if left unmanaged. Direct yield loss through infection of young grape inflorescences and indirect loss from the infection and subsequent abscission of leaves are both observed with downy mildew. Premature defoliation leads to poor fruit maturation and can cause vine winter kill. European wine grapes, *Vitis vinifera*, are highly susceptible to this pathogen. Hybrid grapes (European and American grape crosses) vary in susceptibility, whereas the southeastern United States native muscadine, *Vitis rotundifolia*, is resistant to this pathogen (Lafon and Clerjeau 1988, This et al. 2006).

High precipitation and favorable temperatures in Georgia and much of the southeastern United States provide an ideal environment for downy mildew development. Management relies heavily on fungicide applications made on either 7-10 (wet) or 10-14-day (dry) intervals from mid-April to mid-September. In the United States, numerous fungicide products are labeled for grapevine downy mildew control, but these belong to a limited number of modes of action. Growers in Georgia apply up to 17 fungicide applications per season depending on the amount and frequency of rainfall (Nita et al. 2019). This vigorous spray schedule increases fungicide resistance selection pressure and makes resistance development more likely. Older contact-protectant fungicides, such as mancozeb or captan, and newer active ingredients such as azoxystrobin and madipropamid, are both utilized. Mancozeb and captan are relatively effective against *P. viticola*, and they have a multisite mode of action which increases their durability and

decreases the risk of fungicide resistance development (FRAC 2018a, 2018b). In contrast, single-site mode of action fungicides are often less durable and rapidly become ineffective due to fungicide resistance development in the pathogen population. In the case of the quinone outside inhibitor (QoI) fungicides (e.g., azoxystrobin and pyraclostrobin), which have a single-site mode of action at the cytochrome bc1 complex in the mitochondria, *P. viticola* resistance developed within 4 years after market introduction in France and Italy (Corio-Costet 2011, Gisi et al. 2002, Heaney et al. 2000). Resistance has also been reported to the carboxylic acid amide (CAA) fungicides in 2005 in Europe (FRAC 2018a, Gisi et al. 2007), and both classes are utilized extensively in Georgia for management of grapevine downy mildew.

The objectives of this study were to determine the efficacy of several common downy mildew fungicides through direct comparisons in vineyards, and to relate potential control failures to the presence of resistant *P. viticola* populations as determined by polymerase chain reaction (PCR) assays to detect a specific mutation causing QoI resistance.

MATERIALS AND METHODS

Evaluation of fungicide resistance at field trial sites. Downy mildew samples (leaves with sporulating lesions) were obtained from two commercial vineyards in Lumpkin and White counties and a research vineyard at the Georgia Mountain Research and Education Center (GMREC) in Union County in June of 2017. Twenty *P. viticola*-infected leaves were collected from each site, placed in individual plastic bags with zipper closure, stored on ice in coolers, transported to the lab, and stored for 1 day at 22°C. Single lesions on field-collected leaves were washed with 10 µL of sterile water by repeatedly ejecting and regaining sterile water over the leaf surface. Sporangium suspensions were deposited on fresh *V. vinifera* cv. Chardonnay leaf

discs from greenhouse-grown plants, and cultures were incubated at 22°C for 24 h, after which excess water was shaken off the leaf discs and they were returned to the incubator and incubated at 22°C with a 12-h light-dark cycle. After 7 days, one leaf disc was used to extract DNA of *P. viticola*. Total DNA was extracted from leaf discs using a method provided by Hong (2018). Briefly, 7-day-old sporulating lesions were washed with 50 µL of 1×TBE buffer in 1.5-mL microcentrifuge tubes. Tubes were microwaved for 20 s three times, after which they were centrifuged at 15,600 x *g* for 5 min. After centrifuging, the supernatant with the DNA was pipetted off and placed in a new microcentrifuge tube. Samples were stored at -20°C until PCR assays were performed. Leaf disc culture and DNA extraction was successful for 39 samples across the three sites.

To detect the G143A mutation of the *cyt b* gene for QoI fungicide resistance in the isolated DNA, primers published by Baudoin et al. (2008) were utilized. The wild-type and mutant forward primers were 5′ CCT TGG TGA CAA ATG AGT TTT TGG AG 3′ and 5′ CCT TGG TGA CAA ATG AGT TTT TGG AC 3′, respectively. The two forward primers use a common reverse primer, 5′ CAA CTT TTC CAA TTA ATG GGA TAG 3′. A separate PCR reaction was completed with the wild-type and the mutant primers for each sample. Reactions with water were used as negative controls and a resistant DNA sample obtained from Dr. Anton Baudoin (Virginia Polytechnic Institute and State University) was used as a positive control. PCR was conducted with Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Chicago, IL) using the following thermocycler settings: 95°C for 15 min, 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s (Baudoin et al. 2008). PCR products were electrophoresed on a 3% agarose gel for 90 min and visualized with ethidium bromide.

To determine the presence or absence of the G1105S mutation in the *PvCesA3* gene causing CAA fungicide insensitivity, a tetra-primer PCR approach published by Zhang et al. (2017) was used comprising two outer primers, CesaOF (5′ GCA CAG ACA TGG TTT TCC TT 3′) and CesaOR (5′ GTC CAA AAG TGC AAA GTC CAA CG 3′) and two inner primers, Cesa3IW (5′ TAC CTT TAC GGC AAA TGT GTG CG 3′) and Cesa3IM (5′ GAC AAT GTA GAC AAC CAG CAA CGA TCT 3′). The two outer primers create a 612 bp standard fragment for each reaction. Cesa3IW generates a 238 bp fragment with Cesa3OR, indicating a sensitive allele. Cesa3IM generates a 378 bp fragment with Cesa3OR, indicating a resistant allele. A reaction with water was used as a negative control and a CAA-resistant DNA sample was obtained from Dr. Anton Baudoin as a positive control. Illustra PuReTaq Ready-To-Go PCR Beads were used, and thermocycler conditions were set as follows: 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 63°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min (Zhang et al. 2017). PCR products were electrophoresed on 1.5% agarose gel for 45 min and visualized with ethidium bromide.

Fungicide efficacy trials in the presence of QoI-resistant downy mildew populations. To test the efficacy of various fungicides against grapevine downy mildew, field trials were conducted in the three aforementioned vineyards in 2018. A total of 11 treatments (Table 3.1) were applied to single vines in a randomized complete block design with five (commercial vineyards) or six (GMREC) replications. Additional untreated control plots were included at the GMREC site but not the two commercial sites. At all three locations, the azoxystrobin (Abound) treatment was included twice, replicated independently. The blocks were arranged linearly along rows, and cultivars included Merlot (GMREC), Pinot Grigio (Lumpkin County), and Touriga Nacional (White County).

Treatments were applied four times during the growing season at the bloom, post bloom, bunch closure, and second cover timings; a fifth application was made at GMREC during the first cover time period. Applications were made with a CO_2 backpack sprayer with a TeeJet adjustable cone tip (5500-PPX12) at a pressure of 172.4 kPa to runoff. Fungicide doses corresponded to label recommendations applied at the equivalent of 467.7 L/ha. Additional chemicals were applied to control other pests with an airblast sprayer at the GMREC or by backpack sprayer in the commercial vineyards (Table 3.2).

Disease assessment. Disease incidence of downy mildew was assessed weekly after disease onset by recording the presence or absence of sporulating lesions on 30 leaves per vine (15 on each side of the vine). Severity ratings were taken by visual estimation of percent sporulation coverage on 50 individual leaves per vine weekly after the fourth fungicide application. Disease incidence and severity were used to calculate areas under the disease progress curve (AUDPC). AUDPC values were either untransformed or log- or square-root transformed prior to analysis, as needed. The analysis of variance utilized PROC GLIMMIX in SAS v. 9.4 (SAS Institute, Cary, NC), and means were compared using Tukey's test ($\alpha = 0.05$).

RESULTS

Prevalence of fungicide resistance. Downy mildew samples collected from all three vineyards showed the presence of the G143A mutation conferring QoI resistance in 100% of tested isolates (Table 3.3). The G1105S mutation conferring CAA resistance was not detected in any isolate from these vineyards.

Fungicide efficacy. *Plasmopara viticola* signs were first recorded in mid-June in all vineyards; however, the epidemic progressed differently at each location (Fig. 3.1). Downy

mildew progressed quickly at the GMREC due to the presence of untreated vines within the vineyard and within the trial. Disease progressed more slowly at the commercial vineyards, presumably due to the active disease management program conducted by the grower collaborator on the vines surrounding the trial.

Although overall disease levels were different among vineyards, the overall ranking of fungicide efficacy was largely similar (Table 3.4). In all vineyards, treatments containing the QoI active ingredients, azoxystrobin and pyraclostrobin, showed the least efficacy against downy mildew. Treatments of captan, cyazofamid and potassium phosphite alone provided good efficacy, and potassium phosphite + captan, potassium phosphite + cyazofamid, mandipropamid, mandipropamid plus difenoconazole, and dimethomorph plus ametoctradin provided the best overall efficacy. In the GMREC vineyard where an untreated control was added to the treatment groups, the QoI fungicides did not perform significantly better than the untreated control when analyzed as a complete trial (Table 3.4).

DISCUSSION

When a fungicide is applied to an area where some of the pathogen isolates are resistant, the fungicide reduces the fitness of the fungicide-sensitive individuals in the population. This causes the frequency of resistant individuals to increase faster than that of sensitive individuals (van den Bosch et al. 2015). Vines treated with QoI fungicides or left untreated showed disease earlier than those treated with other modes of action. Furthermore, QoI fungicides provided the lowest level of downy mildew control at all three locations. This is consistent with a population in which QoI resistance has been selected for over time, and where 100% of the tested isolates

were resistant based on PCR assay. The results provide evidence of a true field failure of these fungicides across all trial sites.

The active ingredients of the fungicides used this trial varied in their risk of resistance development as assigned by the Fungicide Resistance Action Committee (FRAC) (FRAC 2018a). Fungicides with a known high resistance development risk were azoxystrobin and pyraclostrobin (Group 11, QoI). Additionally, there were fungicides with a risk that was assumed to be medium to high for resistance development: ametoctracadin (group 45, QoI stigmatellin binding type [QoSI]) and cyazofamid (group 21, quinone inside inhibitor [QiI). The CAA fungicides mandipropamid and dimethomorph (group 40) are classified as having a low to medium resistance risk, although resistance development in *P. viticola* has been documented in many locations (Aoki et al. 2013, Feng and Baudoin 2018, Gisi et al. 2007, Toffolatti et al. 2018). Lastly, fungicides with a low resistance risk included in this trial were captan (group M04) and the phosphonates (group P07) (FRAC 2018a). The findings of this study support these resistance ratings, with *P. viticola* having developed resistance to the most at-risk fungicides with the subsequent result of reduced efficacy against this rapidly reproducing pathogen.

The goal of fungicide resistance baseline determination is to capture the full population variation of sensitivity levels. To adequately accomplish this, Russell (2004) recommends a sample size between 20 and 50 per population. These suggested sample sizes can be limited by available resources and time. For example, a sample size of fewer than 10 isolates per vineyard was used to attribute a loss of field sensitivity to the widespread presence of QoI resistance mutation G143A (Baudoin et al. 2008). Another study evaluating fungicide resistance in *P. viticola* had a sample size of approximately 6 per vineyard with a total of 28 vineyards evaluated

(Colcol and Baudoin 2016). These sample sizes are more in line with a recommendation of 5 to 10 leaves per location made by Sirven et al. (2002).

The presence of the G143A mutation conferring QoI resistance in all three vineyards provides evidence that the loss of sensitivity to QoI fungicides in the efficacy trials is largely due to a genetic mutation in the pathogen and not caused by other external variables. QoI resistance was also found beyond the three field tested vineyards; indeed, commercial vineyards throughout Northern and Western Georgia contain QoI-resistant *P. viticola* populations (Chapter 2). The presence of this QoI resistance mutation has also been documented previously in vineyards in Maryland, New York, North Carolina, Pennsylvania, and Virginia (Baudoin et al. 2008, Colcol and Baudoin 2016, and Gee et al. 2011).

The absence of the G1105S mutation also correlates with the field trial results. Treatments which contained a CAA fungicide, mandipropamid in this study, performed as well as fungicides with a lower resistance development risk and provided adequate disease control. Resistance of *P. viticola* to the CAA fungicides has been reported previously in Virginia due to the G1105S mutation (Feng and Baudoin 2018). With regard to potassium phosphite, it is possible that resistance to this active ingredient was observed in the commercial field site in White County, since the disease levels observed were similar to those of the QoI fungicides, and resistance to potassium phosphite is known to occur (Adaskaveg et al. 2017); this possibility needs to be addressed in future studies.

The G143A mutation conferring QoI resistance does not convey a fitness cost to the pathogen, indicating that once acquired, the mutation may remain in the population until a selection event, such as a repeated application of fungicide, selects for resistance (Delmas et al. 2017). Continuous monitoring and future work are needed to maintain an accurate record of

fungicide sensitivities in Georgia *P. viticola* populations and to record any new sensitivity losses to currently used fungicides. This includes future fungicide trials for in-field testing, PCR assays for known mutations, and bioassays for sensitivity testing to fungicides for which no causal mutation has been identified.

Along with monitoring, fungicide resistance management practices need to be deployed to avoid the selection of new resistant *P. viticola* populations. Fungicide-resistance management principles will not only benefit downy mildew management, but also management of other common vineyard diseases such as Botrytis bunch rot, caused by *Botrytis cinerea,* and powdery mildew, caused by *Erysiphe necator*. All the diseases mentioned have a high risk of developing fungicide resistance and must be monitored and managed with care (FRAC 2013).

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Table 3.1. Treatments applied to three vineyards to test fungicide efficacy against downy mildew caused by *Plasmopara viticola*.

Treatment	Active ingredient	Distributor	FRAC code	Mode of action[a]	Dose
Abound	Azoxystrobin 22.9%	Syngenta Crop Protection	11	QoI	1.1 L/ha
Pristine	Boscalid 25.2 % + Pyraclostrobin 12.8%	BASF Corporation	7 + 11	SDHI + QoI	875.7 g/ha
Ranman	Cyazofamid 34.5%	FMC Corporation	21	QiI	0.2 L/ha
Revus	Mandipropamid 23.3%	Syngenta Crop Protection	40	CAA	0.6 L/ha
Revus Top	Mandipropamid 21.9% + Difenoconazole 21.9%	Syngenta Crop Protection	40 + 3	CAA	0.5 L/ha
Captan	Captan 37.99%	Drexel Chemical Company	M04	Phthalimides	4.7 L/ha
Zampro	Ametoctradin 26.9% + Dimethomorph 20.2%	BASF Corporation	45 + 40	QoSI + CAA	1.0 L/ha
Prophyt	Potassium Phosphite 54.5%	Helena Agri-Enterprises	33	Phosphonates	2.3 L/ha
Ranman + Prophyt	Cyazofamid 34.5% + Potassium Phosphite 54.5%	FMC Corporation + Helena Agri-Enterprises	21 + 33	QiI + Phosphonates	0.2 L/ha + 2.3 L/ha
Captan + Prophyt	Captan 37.99% + Potassium Phosphite 54.5%	Drexel Chemical Company + Helena Agri-Enterprises	M04 + 33	Phthalimides + Phosphonates	4.7 L/ha + 2.3 L/ha

[a]QoI = quinone outside inhibitor, SDHI = succinate dehydrogenase inhibitor, QiI = quinone inside inhibitor, CAA = carboxylic acid amide, QoSI = quinone outside inhibitor stigmatellin binding type.

Table 3.2. Additional fungicides applied to the research vineyard at the Georgia Mountain Research and Education Center in Blairsville, GA, during efficacy trials against grapevine downy mildew caused by *Plasmopara viticola*.

Phenology	Fungicides applied
Bloom	Microthiol Disperss 5.6 kg/ha Elevate 50WDG 1.12 kg/ha Rally 40W 0.37 L/ha
Postbloom	Microthiol Disperss 5.6 kg/ha Topsin M 70WDG 1.68 kg/ha Rally 40W 0.37 L/ha
First Cover	Microthiol Disperss 5.6 kg/ha Topsin M 70WDG 1.68 kg/ha Rally 40W 0.37 L/ha
Bunch Closure	Microthiol Disperss 5.6 kg/ha Elevate 50WDG 1.12 kg/ha Rally 40W 0.37 L/ha
Second Cover	Microthiol Disperss 5.6 kg/ha Topsin M 70WDG 1.68 kg/ha Rally 40W 0.37 L/ha

Table 3.3. Polymerase chain reaction (PCR) results for fungicide resistance identification based on the G143A (quinone outside inhibitor, QoI) and G1105S (carboxylic acid amide, CAA) mutations in *Plasmopara viticola* populations in three Georgia vineyards.

			QoI		CAA	
County	Vineyard	Year collected	PCR resistant isolates/total	% resistant	PCR resistant isolates/total	% resistant
Union	Research	2017	15/15	100	0/10	0
White	Commercial	2017	11/11	100	0/5	0
Lumpkin	Commercial	2018	13/13	100	0/3	0

Table 3.4. Downy mildew area under the disease progress curve (AUDPC) values from one research vineyard (Union County) and two commercial vineyards (White and Lumpkin counties) following application of fungicides four or five times between bloom and second cover.

	Union County		White County		Lumpkin County	
Treatment and dose	Incidence AUDPC [z] (% days)	Severity AUDPC [y] (% days)	Incidence AUDPC [w] (% days)	Severity AUDPC [v] (% days)	Incidence AUDPC [w] (% days)	Severity AUDPC [u] (% days)
Untreated control	3404.4 a	352.8 a				
Abound 1.1 L/ha	3211.3 a	315.7 a	168.7 a	8.7 abc	1089.3 a	86.0 a
Abound 1.1 L/ha (second treatment)	3040.3 a	333.2 a	132.3 ab	9.2 ab	822.3 ab	62.3 a
Pristine 875.7 g/ha	2961.4 a	265.7 a	166.3 a	9.1 a	857.3 ab	75.0 ab
Ranman 0.2 L/ha	1023.9 b	55.3 b	6.0 b	1.0 bc	259.7 abc	12.3 bc
Captan 4.7 L/ha	839.4 bc	49.7 b	6.0 ab	0.2 c	105.33 bc	9.2 bc
Prophyt 2.3 L/ha	434.7 cd	18.1 c	91.0 ab	4.2 abc	138.7 abc	10.8 bcd
Revus 0.6 L/ha	414.7 cd	16.6 c	4.7 b	0.0 c	40.3 c	1.5 d
Zampro 1.0 L/ha	295.8 cd	16.7 c	4.0 b	0.3 c	71.3 c	4.7 cd
Revus Top 0.5 L/ha	208.9 d	11.5 c	14.0 ab	0.6 c	133.7 abc	11.5 bc
Prophyt 2.3 L/ha + Captan 4.7 L/ha	155.8 d	7.0 c	18.7 ab	0.5 c	47.7 c	1.8 cd
Prophyt 2.3 L/ha + Ranman 0.2 L/ha	112.8 d	4.3 c	6.0 b	0.3 c	59.7 c	2.6 cd

[z]Calculated based on 30 infected leaves sampled per plot for the 72-day period from 24 May to 5 August 2018. Means followed by the same letter are not significantly different when using Tukey's test.
[y]Calculated based on 50 leaves sampled per plot for the 19-day period from 17 July to 5 August 2018. Means followed by the same letter are not significantly different when using Tukey's test (square-root transformed values).
[w]Calculated based on 30 infected leaves sampled per plot for the 71-day period from 24 May to 3 August 2018. Means followed by the same letter are not significantly different when using Tukey's test (log-transformed values).
[v]Calculated based on 50 leaves sampled per plot for the 21-day period from 13 July to 3 August. Means followed by the same letter are not significantly different when using Tukey's test (log-transformed values).

[u]Calculated based on 50 leaves sampled per plot for the 18-day period from 16 July to 3 August 2018. Means followed by the same letter are not significantly different when using Tukey's test (log-transformed values).

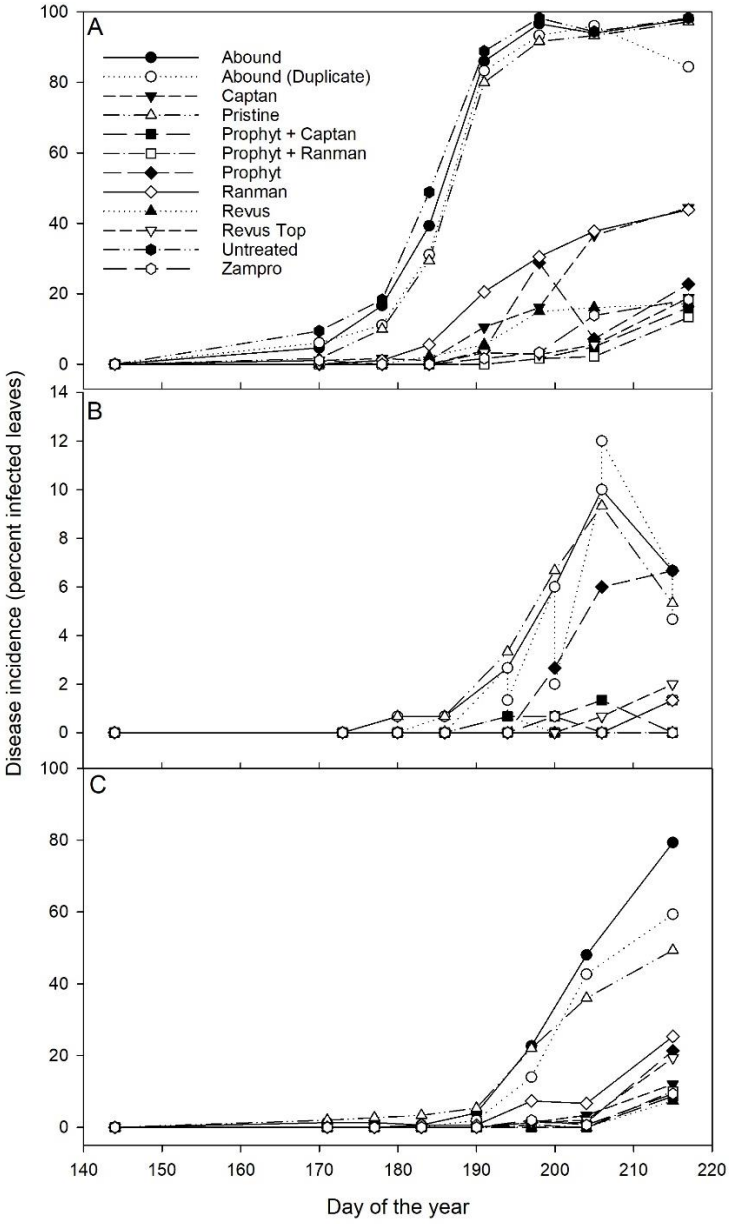

Figure 3.1. Effect of timed fungicide applications (four or five sprays from bloom through second cover) on grapevine downy mildew disease incidence in a research vineyard in Union County (A) and two commercial vineyards in White (B) and Lumpkin (C) counties. Downy mildew incidence was assessed weekly after disease onset in mid-June by evaluating 30 leaves per vine for disease symptoms and signs.

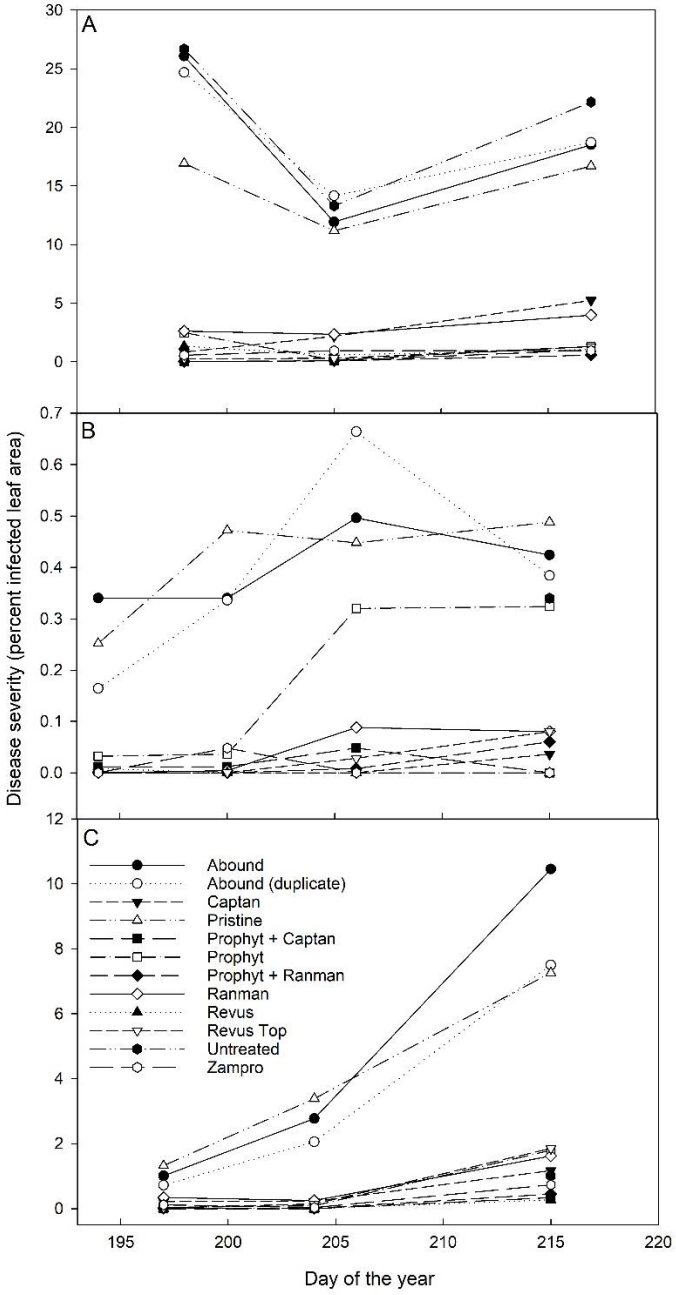

Figure 3.2. Effect of timed fungicide applications (four or five sprays from bloom through second cover) on grapevine downy mildew disease severity in a research vineyard in Union County (A) and two commercial vineyards in White (B) and Lumpkin (C) counties. Downy mildew severity was assessed weekly after final fungicide application in mid-July by evaluating 50 leaves per vine for percentage of disease coverage.

CHAPTER 4

CONCLUSIONS

For this research, I evaluated the efficacy of multiple fungicide classes utilized for management of grapevine downy mildew in the presence quinone outside inhibitor (QoI) fungicide-resistant strains of *Plasmopara viticola*. In addition, I determined the resistance status of Georgia *P. viticola* populations to three fungicidal classes, i.e., the QoIs, carboxylic acid amides (CAAs), and phenylamides (PAs). The former study (Chapter 3) determined the efficacy of commonly used fungicides for management of downy mildew in commercial and research vineyards in Georgia where QoI resistance had been confirmed. Single-vine replicates arranged in a randomized complete block design within the vineyards were treated with 11 fungicides or fungicide combinations four or five times during the growing season. Incidence and severity of downy mildew was significantly higher in treatments of Abound (azoxystrobin 22.9%) and Pristine (boscalid 25.2% and pyraclostrobin 12.8%) which contain a QoI active ingredient. In the research vineyard, QoI treatments were not statistically different from the untreated control vines, which were not sprayed with any downy mildew active ingredients. The next level of efficacy was characterized by treatments of Ranman (cyazofamid 34.5%) and Captan (captan 37.99%). The remaining treatments of Prophyt (potassium phosphite 54.5%), Prophyt + Captan, Prophyt + Ranman, Revus (mandipropamid 23.3%), Revus Top (mandipropamid 21.9% and difenoconazole 21.9%), and Zampro (ametoctradin 26.9% and dimethomorph 20.2%) provided the best level of efficacy. The data obtained was similar among the three trial sites, suggesting

that the loss of efficacy observed with QoI fungicides is prevalent within the North Georgia viticulture industry.

The second study (Chapter 2) documented the fungicide sensitivity levels of *P. viticola* isolates collected from commercial vineyards throughout Georgia. Samples were collected over 4 years from 2015 to 2018 from 18 commercial vineyards from Georgia's three main wine-producinn areas: North Georgia, West Georgia, and South Georgia. Samples were subjected to polymerase chain reaction (PCR) assays to determine the presence or absence of the known resistance causing mutations G143A for QoIs and G1105S for CAAs. A subset of samples from 2017 and 2018 was selected for bioassays to test for fungicide sensitivity to a QoI (azoxystrobin), a CAA (mandipropamid), and a PA (mefenoxam) fungicide. Resistance was determined by the ability to sporulate on a discriminatory fungicide dose. Bioassay results were directly associated with the presence of resistance-causing mutations detected by PCR assay. PA resistance was not detected by bioassay, and CAA resistance was not detected by bioassay or PCR assay in any of the samples tested. QoI resistance was detected by bioassay (91.7%) and PCR assay (82.1%) in the large majority of the vineyard-year combinations tested, being widespread in North and West Georgia vineyards but not prevalent in vineyards in South Georgia. The two South Georgia vineyards were negative for the presence of G143A and sensitive in QoI bioassays, although the G143A mutation was detected in one of the vineyards in the last year of the survey, 2018. While this study did not illuminate the cause of maintenance of QoI sensitivity in South Georgia, possible factors are cultivar selection, local climate, and differences in disease pressure.

In summary, proper fungicide selection in vineyard disease management continues to be critical to successful management of grapevine downy mildew, and understanding the fungicide

sensitivity of local pathogen populations is helpful for effective vineyard management. In this study, the QoI fungicides were determined to be ineffective for most of the *P. viticola* populations sampled based on the presence of a resistance-causing mutation and the corresponding resistant phenotype in field efficacy trials. Based on these studies, this class of fungicides is not recommended for continued use for downy mildew control in North and West Georgia vineyards. The other classes tested, CAA and PA, were considered effective and remain viable management options for vineyard disease management. However, sound resistance management techniques must be utilized by producers if these classes are to be maintained for the future.