# Fungicide Resistance of *Botrytis Cinerea* from Virginia Wine Grapes, Strawberry, and Ornamental Crops

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ABSTRACT

*Botrytis cinerea* is the principal member of the species complex that causes bunch rot of grapes and gray mold disease on other hosts including fruits and ornamental crops. It has developed resistance to many fungicides, and isolates from eastern US strawberry fields have regularly been identified with resistance to several modes of action. During the 2011-2015 growing seasons, 487 isolates were collected from Virginia wine grapes, strawberries, and ornamental crops and evaluated for sensitivity to eight different fungicides by a germ tube elongation method; for a subset of isolates, a 24-well plate mycelial growth assay was also used, and baseline sensitivity to polyoxin-D was evaluated. Resistance to benzimidazoles and quinone outside inhibitors, and low-level resistance to iprodione were common. Boscalid resistance was common in wine grapes and ornamentals. Resistance to the hydroxyanilide fenhexamid during germ tube elongation was found in only 5% of wine grape isolates, but in 33% of isolates from strawberries and ornamentals. All of the fenhexamid-resistant isolates were identified as *B. cinerea* carrying various mutations in the *erg27* gene. An additional subset of isolates was identified with moderate resistance to fenhexamid during mycelial growth, but not germination and germ tube growth. These were identified as *B. cinerea* HydR2 isolates, which possess an unknown mechanism of resistance towards fenhexamid in mycelial growth. Moderate resistance to cyprodinil was common, but in grape inoculation tests, moderately resistant isolates were controlled by a field rate of cyprodinil. Diminished sensitivity to fludioxonil and fluopyram was rare. Polyoxin-D controlled most isolates in mycelial growth tests at 100 µg/ml.

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## GENERAL AUDIENCE ABSTRACT

The fungus *Botrytis cinerea* is the principal member of the Botrytis species complex, causing bunch rot of grapes and gray mold disease on other hosts including fruits and ornamental crops. These diseases diminish the aesthetic value of ornamental crops and adversely impact the marketability of fruit crops and the wine making potential of grapes. Wine grapes, strawberries, and ornamental plants are important sources of agricultural revenues, which include profits from various forms of tourism, in Virginia. However, due to humid environmental conditions, the use of fungicides is necessary to control *Botrytis* diseases in Virginia. Years of intensive use have led to failures in efficacy of some fungicidal chemical groups, as fungicide-resistant *Botrytis* isolates survive and proliferate in settings where frequent fungicide applications are necessary. During the 2011-2015 growing seasons, 487 *Botrytis* isolates were collected from Virginia wine grapes, strawberries, and ornamental crops and evaluated for sensitivity to eight different fungicides by one or both of two methods. The fungicides we evaluated represent the modes of action available to control *Botrytis*, including quinone outside inhibitors, benzimidazoles, dicarboximides, succinate dehydrogenase inhibitors, hydroxyanilides, anilinopyrimidines, and phenylpyrroles. We also investigated the baseline sensitivity of *B. cinerea* from Virginia to another antifungal chemistry, polyoxin-D, which has recently been registered for use in the United States. In this survey, we found widespread high-level resistance to quinone outside inhibitors, benzimidazoles, and one succinate dehydrogenase inhibitor; low-level dicarboximide resistance was also abundant. High-level resistance to hydroxyanilide and anilinopyrimidine fungicides was uncommon. Moderate resistance to the anilinopyrimidine fungicide we tested against, cyprodinil, was common, but experiments using grapes treated with this fungicide suggest that moderately resistant isolates can be controlled using this active ingredient in a manner consistent with the labelled recommendations. Our results echo reports from surrounding states, where resistance to one or several different fungicides is common in *Botrytis* populations. We also found that *Botrytis* collected during this survey had relatively high baseline tolerance towards polyoxin-D, indicating that this ingredient may not be a very effective compound to combat *Botrytis* in Virginia. However, we also found evidence that hydroxyanilides, phenylpyrroles, and some succinate dehydrogenase inhibitors retain good efficacy against the *Botrytis* populations we surveyed. We did not discover evidence that any species other than *B. cinerea* was causing the bunch rot and gray mold we observed in the field over the duration of this study.

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## Attribution

Some sample collection and fungicide resistance profiling was performed in 2011 and 2012 prior to my involvement in the project. Mizuho Nita contributed statistical analysis to this study.

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## Chapter 1

## Literature Review and Research Objectives

Modern crop production and pathogen control strategies, including the use of site-specific fungicides, have caused the emergence of fungicide-resistant field isolates (Beever et al. 1991; Faretra and Pollastro 1991). In populations with low-frequency, point mutation resistance phenotypes, heavy use of one or a few modes of action can create selection pressure which gives otherwise unfit resistant isolates a survival advantage, hastening their increase and thus increasing the likelihood of broader impacts on the pathogen population (Beever et al. 1991). This represents a serious concern in modern production agriculture, where resistance emergence has often been associated with complete loss of disease control (Faretra and Pollastro 1991; Brent and Holloman 2007). This is of particular concern in the case of *Botrytis cinerea*, the causal agent of gray mold and *Botrytis* bunch rot. A pathogen with a high risk of fungicide resistance development, *Botrytis* may exist in abundant populations in the field, is liable to mutate under laboratory conditions, and is largely controlled by applications of high rates of single-target site fungicides, which are often applied repetitively extensively (Brent and Holloman 2007).

The introduction of systemic fungicides in the 1970's offered new chemistries for the control of gray mold on grapes, but their introduction also hastened the emergence of, and contributed to the degree of fungicide resistance appearing in the field (Brent and Holloman 2007). Among the first modes of action used for the control of gray mold on grapes were the benzimidazoles and dicarboximides. The benzimidazoles, including the methyl benzimidazole carbamate thiophanate methyl, inhibit the assembly of microtubules by competing for binding sites on beta-tubulin in affected fungi (Davidse and Flach 1978). While initially highly effective against gray mold on a number of hosts, benzimidazole resistant field isolates quickly became widespread (Faretra and Pollastro, 1991; Beever et al., 1989). Fields isolates displaying resistance to benzimidazoles commonly carry one of several mutations at amino acid positions 198 and 200 of the B-tubulin gene (Banno et al. 2008; Ziogas et al. 2009). Benzimidazole resistance has been shown to persist in populations of *B. cinerea* long after use has been discontinued (Yourman and Jeffers 1999). Thus, even at sites where benzimidazoles have not been used in recent seasons, applications of thiophanate-methyl could lead to disease management failures.

Dicarboximides were introduced in the 1970’s as an alternative to the rapidly failing benzimidazoles (Brent and Holloman 2007). Several guidelines were provided by the Fungicide Resistance Action Committee Working Group to mitigate the inherent resistance risk presented by the site-specific dicarboximides, and researchers were able to demonstrate that dicarboximide resistance in *Botrytis cinerea* from tunnel-grown strawberries could be delayed by the application of some fungicide mixtures (Brent and Holloman 2007). Despite these efforts, first reports of resistance to dicarboximide fungicides occurred within 5 years of their introduction in the field (Brent and Holloman 2007). Widespread resistance to dicarboxamides, conferred by point mutations in the BcOS1 gene, was detected in vineyards and other crops around the world by the late 1980's (Banno et al. 2008; Beever et al. 1989; Grabke et al. 2013). Our laboratory has documented widespread, low levels of resistance to this modes of action in grapes and other crops in Virginia (Baudoin 2013; Baudoin and Adamo, unpublished data). During mycelial

growth, dicarboximide resistance does not associate with any obvious fitness costs in *Botrytis cinerea* (Raposo et al. 2000). However, resistance to iprodione has been shown to have a significant negative correlation with survival of sclerotia, indicating that long-term survival of resistant isolates may be reduced by dicarboximide resistance (Raposo et al. 2000). Other authors have shown that even in the absence of fungicide pressure, dicarboximide-sensitive populations may develop resistance to vinclozolin, but at the same time demonstrated that populations resistant only to vinclozolin exhibit high phenotype instability and liability to revert to sensitivity (Yourman et al. 2001). These factors make determining the long-term stability and ramifications of previously identified dicarboximide resistance difficult.

Quinone outside inhibitors (QoI) comprise a relatively new class of fungicide available for the control of gray mold, among other pathogens. QoI fungicides target a protein located in the mitochondria, which lack the DNA proofreading and repair capacities present in the nucleus, and are thus more amenable to the accumulation of point mutations (Brent and Holloman 2007). Unsurprisingly, resistance was observed less than two years after QoIs were introduced in the late 1990's, in populations of *Blumeria graminis* (DC.) Speer f. sp. *tritici* Em. Marchal causing wheat powdery mildew in Germany (Brent and Holloman 2007; Angelini et al. 2012). In 2006, resistance to the QoIs azoxystrobin and pyraclostrobin was first reported from a North American vineyard, in grapevine downy mildew, *Plasmopara viticola* (Baudoin and Baldwin 2006). Resistance to QoIs has been discovered in *Botrytis cinerea* from multiple host crops in different parts of the world, including strawberry fields in the eastern United States in 2012, where over 66% of isolates were resistant to pyraclostrobin (Albertini et al. 2012; Fernández-Ortuño et al. 2012).

QoI resistance has been shown by to be associated with the G143A mutation in the *cytb* gene in laboratory mutants and in field isolates of *Botrytis cinerea* displaying resistance to pyraclostrobin (Albertini et al. 2012; Fernández-Ortuño et al. 2012). Unlike some other fungicide resistance phenotypes, QoI resistance appears to be a relatively stable trait (Rallos et al. 2014). In laboratory competition experiments, grape powdery mildew (*Erysiphe necator*) carrying the G143A mutation significantly outcompeted sensitive isolates in the absence of fungicide for several generations (Rallos et al. 2014). In the field, powdery mildew isolates resistant to QoI persisted for four growing seasons, in the absence of any QoI fungicide pressure (Rallos et al. 2014). Thus, historical resistance to QoIs at a given site should be considered when contemplating future fungicide use and resistance mitigation strategies.

Succinate dehydrogenase inhibitors (SDHI) are a group of fungicides that were discovered over 40 years ago, but until recently saw limited use, because few diseases were controlled by first generation SDHI chemistries (FRACa). In 2003, new SDHI chemistries were introduced to the market, and newer compounds have been released since that time, leading to increased use in agriculture (FRACa). The risk of resistance development to SDHIs has grown along with their increased use in the field (FRACa). Boscalid, a newer SDHI, was registered to control gray mold and Sclerotinia diseases in Japan in 2005, as well as Corynespora in cucumbers, and several other pathosystems in subsequent years (Ishii et al. 2011). Despite use reportedly in accordance with labelled recommendations, resistance to boscalid developed rapidly in *Corynespora cassiicola* (Corynespora leaf spot) from cucumbers in Japan, as well as in *Podospharea xanthii* (powdery mildew) affecting cucurbits (Ishii et al. 2011).

Mutations in any of three subunits (B, C, or D) of the succinate dehydrogenase complex confer resistance to different SDHI fungicides in different pathogens (FRACa; Sierotzki and Scalliet 2013). For example, in *Botrytis cinerea* isolates from North and South Carolina strawberry fields, two different mutations at codon 272 conferred resistance to boscalid (Fernández-Ortuño et al. 2012). In other crops, and at other locations, alterations at positions 225, 230, and 278 have also been found to confer resistance (Sierotzki and Scalliet 2013). Complicating matters further is the complexity of cross resistance patterns to SDHI fungicides. The resistance phenotype that is manifest in any particular case depends on the interplay of the SDHI fungicide in question, the target pathogen species, and the point mutation present in that individual (Sierotzki and Scalliet 2013). For instance, of five mutations known to confer boscalid resistance in *Botrytis cinerea*, two are also shown to confer resistance to fluopyram, another SDHI (Veloukas et al. 2013). However, of those two mutations, one also conferred resistance to an additional 6 SDHI chemistries (Veloukas et al. 2013). This makes resistance monitoring and management for SDHI fungicides relatively complicated, but valuable, because some SDHI chemistries remain effective countermeasures against *B. cinerea*.

Anilinopyrimidine (AP) fungicides are a relatively new group with a single-site mode of action that inhibits methionine biosynthesis and production of some enzymes (FRACb). AP fungicides include cyprodinil, mepanipyrim, and pyrimethanil. Isolates resistant to one of these APs are cross resistant to the other APs. but not to other fungicides (FRACb). Three AP resistance phenotypes associated with different resistance mechanisms have been identified in *Botrytis*, Ani$^{R1}$, Ani$^{R2}$, and Ani$^{R3}$ (Chapeland et al. 1999). Ani$^{R1}$ isolates have a high degree of resistance to cyprodinil, believed to be predicated on a single gene, but the mechanism has yet to be characterized (Chapeland et al. 1999; Fernández-Ortuño et al. 2013). Ani$^{R2}$ and Ani$^{R3}$ isolates exhibit moderate to low and low resistance to AP fungicides, and are actually multiple-drug-resistance (MDR) phenotypes (discussed below), now called MDR1 and MDR2 (Chapeland et al. 1999; Kretschmer et al. 2009). MDR1 and MDR2 isolates may readily be distinguished from Ani$^{R1}$ isolates on the basis of cross resistance to other fungicides (Chapeland et al. 1999).

Further complicating fungicide resistance monitoring, several categories of sensitivity to AP's have been described in *Botrytis*, including highly sensitive, sensitive, moderately resistant and resistant (Weber and Hahn 2011). To clarify, these categories delineate the degree of resistance response in a given isolate, while the Ani$^{R}$ distinction refers to the resistance mechanism present in a given isolate. Clearly identifying the resistance category of an isolate that is not obviously sensitive to AP fungicides is difficult in vitro, due not only to the variety of phenotypes that may occur, but also to the shallow and overlapping inhibition curves that result from EC50 profiling of this mode of action against *B. cinerea* (Weber and Hahn 2011). In a 2013 survey, Ani$^{R1}$ resistance was detected in 47% of *Botrytis* isolated from strawberries in North and South Carolina (Fernández-Ortuño et al. 2013). These isolates comprised both resistant and moderately resistant isolates, as defined by Weber and Hahn (2011) (Fernández-Ortuño et al. 2013). Results from inoculation tests on strawberries treated with a field rate of the AP cyprodinil comparing the responses of isolates designated sensitive, moderately resistant, and resistant, Fernández-Ortuño et al. (2013) suggested that the distinction between moderate resistance and resistance was of little practical value. However, the relevance of this distinction has not been explored in grapes.

The hydroxyanilide fenhexamid affects sterol biosynthesis in target pathogens by affecting the

activity of 3-keto reductase, an enzyme involved in C-4 demethylation (Debieu et al. 2001). This mode of action was unique among sterol bioinhibitors, so fenhexamid delivered a novel class of fungicide for the control of *Botrytis* and other related pathogens (Debieu et al. 2001). Early work on inherent risk of resistance development led Ziogas et al. (2003) to conclude that a high intrinsic risk of resistance to fenhexamid existed, but tempered this statement by pointing out that several fitness costs associated with resistance, including reduced pathogenicity and inhibited sporulation and sclerotia production, could adversely impact survival of fenhexamid-resistant *Botrytis* under field conditions. More recently, Billard et al. (2012) demonstrated considerable fitness costs associated with strong resistance to fenhexamid, suggesting that under field conditions, some fenhexamid-resistant isolates may not present a practical disease control threat.

Several different fenhexamid resistance phenotypes have been identified in *Botrytis*, HydR1, HydR2, and HydR3 and HydR3+ (Fillinger et al. 2008; Leroux et al. 1999; Leroux 2007). HydR1 is a low level resistance to fenhexamid during mycelial growth, peculiar to the cryptic species *Botrytis pseudocinerea* (Leroux et al. 1999; Walker et al. 2011). *B. pseudocinerea* was recently identified as a species distinct from *B. cinerea* (Walker et al. 2011). For some time, two groups were identified in *B. cinerea* on the basis of the presence (Group II) or absence (Group I) of two transposable elements, *boty* and *flipper*, which were present in some strains and absent in others (Walker et al. 2011). However, more recent work demonstrated that Group II *Botrytis* could either possess or lack the transposable elements, while the elements were always absent in Group I strains (Walker et al. 2011). Following analysis of several different speciation criterion and the construction of multiple gene genealogies, Walker et al. (2011) were able to demonstrate that Group I strains are *B. pseudocinerea*, while Group II contains only *B. cinerea sensu stricto*. The HydR1 phenotype is intrinsic in *B. pseudocinerea*, which has been found in French vineyards at low rates and, more recently, has been shown to be the primary agent of mold development in German crops including blueberries, apples, strawberries, and peonies (Walker et al. 2011; Plesken et al. 2015). However, even in those German fields, *B. pseudocinerea* is largely displaced by fungicide-resistant *B. cinerea* following fungicide applications (Plesken et al. 2015). Leroux et al. (1999) suggest that the preventative action of fenhexamid against gray mold in the field might explain why HydR1 phenotypes rarely lead to practical resistance--if a fungicide can inhibit the germination of an isolate's spores, that isolate's inherent resistance to the fungicide during a later developmental stage is practically nullified.

HydR2 confers moderate resistance to fenhexamid that has been identified relatively infrequently in *B. cinerea* isolates from Germany, Japan, and more recently, France (Fillinger et al.2008). Like HydR1 isolates, spore germination in HydR2 isolates is inhibited by fenhexamid, while mycelial growth is unaffected (Leroux 2007). Interestingly, mutations known to confer fenhexamid resistance are not present in HydR2 isolates, so the source of their fenhexamid resistance is not clear (Fillinger et al. 2008). Interestingly, MDR2 ($Ani^{R3}$) isolates display moderate resistance to fenhexamid, but the link between MDR genotypes and HydR2 fenhexamid resistance (or any practical resistance to fenhexamid, for that matter) has not been investigated (Kretschmer et al. 2009). In any case, HydR2 isolates share the reduced fitness of *B. pseudocinerea*, and are not seen as a serious disease control issue (Fillinger et al. 2008; Grabke et al. 2013).

HydR3- and HydR3+ are, respectively, moderate and strong fenhexamid-resistance phenotypes

that confer resistance during both germtube growth and mycelial growth (Fillinger et al. 2008; Leroux et al. 2002). As such, isolates with these phenotypes are the greatest cause for concern from disease control and fenhexamid resistance management perspectives (Esterio et al. 2011). Four years after the introduction of fenhexamid in France, HydR3 resistance began emerging in vineyard *Botrytis* populations (Fillinger et al. 2008). The relationship between fungicide use and the abundance of high resistance to fenhexamid in *Botrytis* populations is not clear. In the Champagne region of France, fenhexamid was applied three times a season and resistance was detected in 15-20% of surveyed isolates (Fillinger et al. 2008). However, in France's Loire valley, where fenhexamid was used only once a year, more than half of all *Botrytis* isolates studied displayed HydR3 phenotypes (Fillinger et al. 2008). In the southeastern United States, fenhexamid resistance was found in 17% of isolates from strawberry fields, but the extent to which frequency of fenhexamid use influenced the abundance of resistant isolates was again unclear (Grabke et al. 2013). Grabke et al. (2013) note that while fenhexamid resistance was absent at an organic strawberry field, likely due to the lack of selection pressure, resistance was also not observed at several locations that did incorporate fenhexamid as part of a conventional fungicide spray program. HydR3 resistance to fenhexamid is predicated on point mutations in the *erg27* gene, thought to encode 3-ketoreductase (Albertini and Leroux 2004). Mutations or deletions at numerous amino acid positions in this gene confer HydR3- resistance, while substitutions at positions 63, 412, and 496 are associated with HydR3+ (Fillinger et al. 2008; Grabke et al. 2013).

The polyoxins comprise a family of 12 unique antibiotics with a shared mode of action (Mamiev et al. 2013). Derived from an antibiotic produced by *Streptomyces cacaoi* var. *asoensis*, polyoxins inhibit chitin synthesis, thus inhibiting the assembly of fungal cell walls (Mamiev et al. 2013). These are relatively safe compounds, with limited off target effects and a benign environmental impact profile (Mamiev et al. 2013). Polyoxins have a long history of use in some agricultural regions, and resistance development has been reported by different authors in Japan starting in the 1970's, and more recently in Korea in the late 1990's (Mamiev et al. 2013). In these cases, polyoxins had initially proved highly effective, but intensive use led to resistance development in different pathogens within a few years (Mamiev et al. 2013). However, Mamiev et al. (2013) found that populations of *Botrytis* from Greek basil greenhouses with a history of polyoxin use displayed the same range of EC50s against polyoxin as populations with no history of exposure. Even in untreated populations, baseline sensitivity to polyoxin ranged from 0.4 to 6.4 µl/ml (Mamiev et al. 2013). Mamiev et al. (2013) concluded that while exposure to polyoxin tends to select for more resistant isolates, low-level resistance is common among *Botrytis* populations regardless of selection pressure.

The mechanism conferring low-level resistance to polyoxin in *B. cinerea* is not well understood (Mamiev et al. 2013). In *Cochliobolus heterostrophus*, the causal agent of southern corn leaf blight, five genes associated with polyoxin resistance (*Pol1-5)* have been identified, but no such genes have been studied in *Botrytis* species (Tanaka et al. 2002). MDR mechanisms (discussed below) have been implicated in the abundance of low-level resistance to polyoxin in populations with no history of exposure to the compound (Mamiev et al. 2013). This seems logical, because even if different fungicides are applied, MDR phenotypes with decreased sensitivity towards polyoxin could still become more prevalent (Mamiev et al. 2013).

In any case, field data about the efficacy of polyoxin against *B. cinerea* in grape production are limited. Wilcox and Riegel (2007) indicated Endorse, a formulation of polyoxin-D, failed to provide significant disease control when compared to untreated controls in a vineyard experiencing severe bunch rot pressure. In a different vineyard spray trial, Bay et al. (2011) found that in vines treated with Ph-D, another formulation of polyoxin-D, bunch rot disease incidence and severity was comparable with the control. However, in an assessment of fungicide efficacy in tree fruits, nuts, strawberries, and vine crops, Adaskaveg et al. (2012) considered Ph-D to be "good and effective" if not completely reliable against *B. cinerea*. Assessments of polyoxin efficacy under field conditions in the southeastern United States are not available, to the best of this author's knowledge.

Phenylpyrrole fungicides, including fludioxonil and fenpiclonil, are a relatively new and potent family of fungicides, derived from an antibiotic produced by a species of *Pseudomonas* (Vignutelli et al. 2002). While very different chemically, fludioxonil shares a similar in mode of action with the dicarboximide iprodione and under laboratory conditions, mutants resistant to both compounds can be generated, but cross resistance to both chemistries in field isolates is extremely rare (Leroux 2007; Vignutelli et al. 2002). Research on laboratory mutants resistant to fludioxonil and vinclozolin, a dicarboximide, led Vignutelli et al. (2002) to postulate that the resistance mechanism in isolates of *Botrytis* with fludioxonil resistance induced under laboratory conditions was closely linked to the mechanism acting to confer dicarboximide resistance. However, Vermeulen et al. (2001) had previously shown that ATP Binding Cassette transporter (ABC transporter) *Bcatrb* could be implicated in reduced fludioxonil efficacy against *B. cinerea*, and suggested this and other similar transporters might play a role in MDR. Fernández-Ortuño et al. (2015) demonstrated that deletions in transcription factor *mrr1* were associated with fludioxonil resistance and found that fludioxonil exposure increased expression of the atrb in resistant isolates, confirming the role of MDR mechanisms in fludioxonil resistance.

Field resistance to phenylpyrroles has been slow and rare to emerge. In Switzerland, no fludioxonil-resistant field isolates were observed over a 7-year period of monitoring in vineyards where Switch, a mixture of fludioxonil and cyprodinil, had been applied (Vignutelli et al. 2002). In *B. cinerea* populations from German strawberry, blueberry, raspberry and redcurrant, Weber (2011) found moderate fludioxonil resistance in a small number of isolates. In the United States, Switch was registered to control gray mold for more than 10 years prior to the emergence of fludioxonil resistance in a Virginia strawberry field (Fernández-Ortuño et al. 2013). In 2014, resistance emerged at more strawberry fields in Maryland and North Carolina, as well as at a blackberry field in Georgia (Fernández-Ortuño et al. 2014; Fernández-Ortuño et al. 2014). In 2015, resistance was found at additional sites in Connecticut and South Carolina (Fernández-Ortuño et al. 2015).

Fludioxonil resistance is a phenotypic indicator of MDR (Fernández-Ortuño et al. 2015). MDR has been implicated in resistance development in medically important pathogens of humans, and is associated with upregulated activity of nonspecific, membrane-bound drug efflux transporters (Kretschmer et al. 2009). ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters are the primary proteins implicated in fungal MDR (Kretschmer et al. 2009). Several different MDR phenotypes have been described, including MDR1, MDR1h, MDR2, and MDR 3 (Kretschmer et al. 2009; Leroch et al. 2013). In laboratory evaluations of

fungicide sensitivity, MDR1 isolates display significantly reduced sensitivity to fludioxonil, cyprodinil, and tolnaftate, an antibiotic not used in agriculture, but useful for identifying MDR (Kretschmer et al. 2009). MDR1h provides a stronger resistance to the same chemistries as MDR1, particularly with respect to fludioxonil, to which MDR1h isolates have considerably greater resistance (Leroch et al. 2013). MDR1 and MDR1h phenotypes are associated with increased expression of ABC transporter *atrB*, caused by a number of different augmentations to the *mrr1* transcription factor (Fernández-Ortuño et al. 2015). In MDR1 isolates, expression of the transporter is upregulated up to 124-fold relative to sensitive isolates, while in MDR1h isolates, expression may be up to 260-fold relative to expression in sensitive isolates (Fernández-Ortuño et al. 2015).

Under laboratory conditions, MDR2 strains display resistance to cyprodinil (though considerably less so than MDR1 and 1h isolates), fenhexamid, tolnaftate, and cycloheximide, a dicarboximide protein biosynthesis inhibitor derived from *Streptomyces griseus*, which was once used as an agricultural fungicide (Actidione), but is now used for diagnostic purposes (Kretschmer et al. 2009; Tsuchida et al. 2002). MDR2 phenotypes are associated with increased action of the MFS transporter *M2* (Kretschmer et al. 2009). This increase is associated with two insertions in the *mfsM2* promoter region, similar to uncharacterized long-terminal-repeat retrotransposons found in different fungi (Kretschmer et al. 2009; Mernke et al. 2011). MDR3 isolates display the highest levels of resistance of all MDR phenotypes, and are resistant to fludioxonil, fenhexamid, cyprodinil, tolnaftate, and cycloheximide (Kretschmer et al. 2009). These isolates also display some reduced sensitivity to boscalid and iprodione, relative to other MDR phenotypes (Kretschmer et al. 2009). MDR3 phenotypes show increased expression of both *atrB* and *mfsM2*, and were shown to be recombinants of MDR1 and MDR2 isolates (Kretschmer et al. 2009).

De Waard et al. (2006) postulated that a number of factors, including fitness penalties associated with mutations in ABC transporters in other fungi, could pose obstacles to the spread of some MDR resistant phenotypes in the field, but ultimately argued that stepwise selection for resistance would be inevitable in contemporary crop production. Kretschmer et al. (2009) confirmed this hypothesis, documenting rapid accumulation of MDR in gray mold populations from German and French wine regions in response to fungicide spray pressure. This was further confirmed by field experiments showing rapid selection for an MDR3 isolate introduced to a vineyard utilizing conventional fungicides and spray practices (Kretschmer et al. 2009). MDR1 phenotypes have appeared in different regions in response to different fungicide pressures, as evidenced by the number of different mutations associated with the phenotype (Kretschmer et al. 2009; Leroch et al. 2013). The promoter rearrangements associated with MDR2, on the other hand, are thought to have been unique, single-incident mutations which were selected for and which ultimately spread across France and Germany (Mernke et al. 2011).

Epidemiologically, and from a fungicide resistance management standpoint, the proliferation of MDR phenotypes represents cause for concern, and a considerable challenge. When MDR1 and MDR2 strains exist in sympatry, MDR3 recombinants emerge, which are more fit, and present higher resistance to the widest range of fungicides (Kretschmer et al. 2009). Fernández-Ortuño et al. (2015) recently showed that MDR1 and MDR1h isolates have emerged independently in several different strawberry growing states in the eastern United States.

**Research Objectives**

Fungicide resistance monitoring is an essential measure to ensure the future of crop protection in the southeastern United States. Other labs and authors have tracked and reported on fungicide resistance development in other crops such as strawberry in this region, but information about fungicide resistance in *Botrytis* populations in Virginia strawberries is limited, and data on populations from ornamentals and wine grapes in the state and surrounding regions is almost nonexistent. The goals of this study include a broad survey of fungicide resistance in *Botrytis* populations from different Virginia crops, with a focus on bunch rot in vineyards. This will allow delivery of practically relevant information for growers trying to manage fungicide resistance at their individual sites, while expanding the general body of knowledge about fungicide resistance in *Botrytis* from the eastern United States. Particular attention will be paid to indicators of MDR and the efficacy of relatively new and newly registered chemicals available to control *B. cinerea* including cyprodinil, fludioxonil, fluopyram, and polyoxin-D. In the course of this study, we intend to elucidate the mechanisms of resistance to fenhexamid present in gray mold from Virginia and North Carolina, while also determining whether *B. cinerea* is causing disease in sympatry with *B. pseudocinerea*. The overall goal of this survey is to present a broad overview of fungicide efficacy and resistance risk in Virginia.

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## Chapter 2

## Survey of fungicide resistance of *Botrytis cinerea* from Virginia wine grapes, strawberries, and ornamentals


### Abstract

*Botrytis cinerea* is the causal agent of bunch rot and gray mold on crops including grapes, strawberries, and ornamentals. In some cases, a related fungus, *B. pseudocinerea* may also contribute to gray mold outbreaks. The intensive use of a limited number of site specific fungicides is necessary to control *Botrytis* in the southeastern United States. Widespread resistance to one or more modes of action has been reported in strawberry fields in and around the Carolinas, but data on Botrytis populations from wine grapes are sparse. Isolates of *B. cinerea* were recovered from 68 wine grape, strawberry, and ornamental fields in and adjacent to Virginia and subjected to fungicide sensitivity profiling against seven chemical modes of action using a germ tube elongation assay. For a subset of isolates, a 24-well plate mycelial growth assay was also employed, and baseline sensitivity to polyoxin-D, an active ingredient recently registered in the United States, was evaluated. Resistance to benzimidazoles, QoIs, and SDHIs, as well as low-level resistance to dicarboximides, was common. Resistance to fenhexamid and cyprodinil was rare, although moderate resistance to cyprodinil was relatively common. However, in grape inoculation tests of a field rate of Vangard WG (a.i. cyprodinil), moderately resistant isolates were controlled similarly to sensitive isolates. Fludioxonil and fluopyram retained good efficacy against the *Botrytis* populations characterized in this study, while baseline sensitivity towards polyoxin-D indicates that this ingredient may not be very effective against the same populations.


### Introduction

The use of fungicides for the control of *Botrytis cinerea* Pers. is an essential measure for many growers in order to prevent pre- and post-harvest fruit losses due to gray mold. However, resistance to methyl benzimidazole carbamates (MBC), quinone outside inhibitors (QoI), dicarboximides (DC), and the succinate dehydrogenase inhibitor (SDHI) boscalid was found to be common in Virginia vineyards in a survey conducted in 2011-12 (Baudoin 2013). The same survey also documented limited resistance to the hydroxyanilide (HA) fungicide fenhexamid and ambiguous levels of resistance to the anilinopyrimidine (AP) fungicide cyprodinil (Baudoin 2013). Additionally, resistance to the phenylpyrrole (PP) fungicide fludioxonil was identified in *B. cinerea* from a VA strawberry field in 2013 (Fernández-Ortuño, 2013).

Multiple fungicide resistance is a growing threat to grape and small fruit production world-wide and has been identified in German vineyards (Leroch et al. 2013) and strawberry fields (Leroch et al. 2013), and occurs in commercial strawberry fields in the southern United States (Fernández-Ortuño et al. 2014). Resistance to multiple chemical classes of fungicides can be due to the accumulation of different mutations in target site genes. For example, point mutations in the *Beta-tubulin*, *bos1*, *sdhB*, *cytB*, and *erg27* genes confer resistance to thiophanate-methyl (MBC), iprodione (DC), boscalid, trifloxystrobin, and fenhexamid, respectively (Banno et al. 2008; Banno et al. 2009; Fillinger et al. 2008; Veloukas et al. 2011). Multiple drug resistance

(MDR) mechanisms can also contribute to or cause multiple fungicide resistance phenotypes with decreased sensitivity to fludioxonil, cyprodinil, and fenhexamid by increasing expression of the membrane-bound drug efflux transporters, ATP binding cassette and the major facilitator superfamily (Kretschmer et al. 2009).

In many cases, the fungicide resistance that has been reported is to three or four modes of action, but recently isolates were identified in strawberry fields in Virginia, South Carolina, Connecticut, and Georgia with resistance to seven different classes of chemical fungicides due to a combination of point mutations and an MDR1 mechanism (Fernández-Ortuño et al. 2015). Each isolate carried different point mutation profiles, suggesting that multiple fungicide resistance is emerging independently at each location (Fernández-Ortuño et al. 2015).

Complicating routine fungicide resistance monitoring is the problem of resistance to the AP fungicide cyprodinil. This active ingredient is relatively poorly understood because the mode of action of AP fungicides has not yet been determined (Fernández-Ortuño et al. 2013b). It is difficult to reliably and confidently separate cyprodinil resistance phenotypes using *in vitro* bioassays, due to the abundance of phenotypes and the extent to which these different phenotypes may overlap (Weber and Hahn 2011). Other authors have demonstrated little significance in the distinction between moderate resistance and resistance to cyprodinil in strawberry using *in vivo* inoculation assays with fruit treated with field rates of the AP fungicide pyrimethanil (Fernández-Ortuño et al. 2013b). However, this phenomenon has not been demonstrated in grapes using a field rate of cyprodinil, which is labelled for use on grapes as a single-ingredient formulation, Vangard WG, as well as a mixture with fludioxonil.

Polyoxin is a chemistry developed in Japan comprising several uracil nucleoside antibiotics derived from soil dwelling *Streptococcus*, which act to inhibit chitin synthesis and thus the formation of fungal cell walls (Mamiev et al. 2013; Wilcox 2015). The compound is fungistatic rather than fungicidal in action, and while not yet certified for use in organic agriculture, seems to fit many of the criteria required for classification as such (Smith et al. 2013). One recent assessment of the compound's antifungal activity indicates relatively low efficacy compared to conventional alternatives, but suggests that it can be useful in rotation as part of fungicide resistance mitigation strategies (Wilcox 2015).  While two different polyoxin-D formulations are currently available to grape growers, little data is available about efficacy and resistance development in the field. However, other authors have indicated that low-level resistance to polyoxin can exist in untreated populations of *B. cinerea*, while treatment with the compound can lead to rapid accumulation of resistant isolates in previously unexposed populations (Mamiev et al. 2013).

Bioassays are commonly used to evaluate fungicide sensitivity, and a couple options are available to those studying Botrytis. Germ tube elongation assays are a popular method, in which the growth of germinated conidia on media carrying discriminatory fungicide concentrations is compared with the growth of conidia on unamended control plates of the same medium (Weber and Hahn 2011). The relative growth on fungicide amended media can be used to characterize a given isolates resistance to a given fungicide (Weber and Hahn 2011). The Profile 24 ® disease resistance monitoring tool is an additional resource for fungicide resistance monitoring. In this system, the wells of 24-well plates are filled with fungicide amended media, which are then

inoculated with conidia carried on a sterile wooden toothpick. Instead of measuring germ tube growth, the radial growth within each well of fungicide amended media is compared with the radial growth in one control well (Schnabel et al. 2012).

The objectives of this survey were to determine fungicide resistance profiles for isolates of *Botrytis cinerea* recovered from vineyards, strawberry fields, and ornamental crops in Virginia and at several sites in North Carolina in order to provide practical information about fungicide efficacy in VA and to monitor for the emergence of multiple fungicide resistance in the state. As part of this objective, we intend to compare the results of bioassays using both germ tube elongation and 24-well plate methods. Additional objectives of this study were to better understand the practical implications of cyprodinil resistance phenotypes in grapes by determining the efficacy of a field rate of a formulated, single ingredient AP fungicide on isolates of *B. cinerea* from with variable resistance to cyprodinil. Baseline sensitivity to polyoxin-D was also investigated to provide a better understanding of how this relatively unstudied chemistry might interact with *Botrytis* from VA.

## Materials and Methods

### *B. cinerea* isolates recovered from vineyards, strawberry fields and ornamental crops, and culture conditions.

From 2011 to 2015, single-spored isolates of *B. cinerea* were obtained from vineyards, strawberry fields, and ornamental crops around Virginia and North Carolina (Table S1). Over 500 isolates were collected from wine grape fruits and flower debris, strawberry flower and fruit, and ornamental foliage and flower debris. Flower debris sampled from vineyards comprised desiccated material from single vines separated by at least 5 meters while fruit samples comprised 3-8 berries or a single cluster from single vines separated by at least two panels, although flower and fruit samples may have been sampled from the same vines. For strawberry samples, dead flowers or 3-5 berries showing *B. cinerea* sporulation or dead berries were sampled from single plants separated by at least two rows or several meters within rows. For ornamental samples, samples comprised foliage that was showing *B. cinerea* sporulation, dead foliage material or flowers from individual plants. All samples were kept separate in plastic bags in a cooler for transport. Conidia were collected from sporulating sample material with a sterile inoculation loop, streaked on potato dextrose agar (PDA) and incubated for 12-18 hours at 22ºC. A single conidium was isolated from each sample and transferred to another PDA plate, which was incubated at 22C and the isolate was maintained in culture until fungicide resistance profiling was initiated.

All non-sporulating sample material was incubated in Petri dishes at 18-21ºC for 2-4 days, during which most exhibited signs of gray mold. Petri dishes were kept inside plastic containers and misted initially to maintain high humidity for the first 18-24 hours of incubation. Following incubation, single conidial isolation was performed as described above. All conidia used for fungicide resistance profiling and other experiments were harvested from cultures kept on PDA at 22ºC. For short-term storage, cultures were maintained on PDA slants and as conidial suspensions in sterile water at 10ºC. For long-term storage, conidial suspensions in 20% glycerol were maintained at -50ºC.

**_In_ vitro evaluation of sensitivity of _B. cinerea_ isolates to thiophanate-methyl, iprodione, boscalid, fluopyram, cyprodinil, trifloxystrobin, fludioxonil, and fenhexamid.**
The active ingredients that follow were obtained as formulated products from the manufacturer: the MBC fungicide thiophanate-methyl (Topsin M, United Phosphorus), the DC fungicide iprodione (Rovral 50W; Bayer), the SDHI fungicides boscalid (Endura 70 WW; BASF) and fluopyram (Bayer), the AP fungicide cyprodinil (Vangard WG; Syngenta), the QoI fungicide trifloxystrobin (Flint; Bayer), the PP fungicide fludioxonil (Medallion WDG; Syngenta), and the HA fungicide fenhexamid (Elevate 50 WDG; Arysta). Malt extract agar (1%) (MEA) was used for testing fenhexamid, iprodione, thiophanate-methyl, and fludioxonil. Trifloxystrobin was tested at 100 µg/ml in MEA that was also amended with the alternative oxidase inhibitor salicylhydroxamic acid (SHAM) at 10 µg/ml. Boscalid and fluopyram were evaluated in 0.5% yeast extract agar (YEA). Cyprodinil was initially tested in mineral medium and 0.5% sucrose agar (SA) but eventually these were replaced by 4% Czapek-Dox agar (CDA). For each medium, control plates without added fungicides were also produced. All media were prepared in 60 x 15 mm Petri dishes (Fisher Scientific; Waltham, MA).

Fungicides were added to media cooled to 55ºC at discriminatory concentrations (Table 1) allowing determination of resistance classes including sensitive (s), less sensitive (ls), moderately resistant (mR), and resistant (R) using a spore germination assay developed and described by Weber and Hahn (2011). Briefly, an ca. 20-µl drop of conidial suspension was streaked on a plate for each isolate and fungicide concentration. Growth of 10 representative germ tubes for each treatment was measured after 12-18 hours, and was then expressed as a percentage of growth on unamended control plates for each medium.

These relative growth values were used to assign each isolate to a resistance class for each fungicide as based on work described by Weber and Hahn (2011). Resistant isolates grew without inhibition at 100 µg/ml thiophanate–methyl or 25 µg/ml trifloxystrobin, while sensitive isolates were strongly inhibited at these concentrations. Isolates sensitive to iprodione did not grow more than 30% relative to control at 50 µg/ml of iprodione, while less sensitive isolates grew from 30-50%, moderately resistant isolates grew from 50-80%, and resistant isolates showed uninhibited growth at 50 µg/ml of iprodione. Fenhexamid-resistant isolates were able to germinate and grow without inhibition at 10 µg/ml of fenhexamid. Cyprodinil-resistant isolates grew without inhibition at 1 µg/ml of cyprodinil and grew to at least 25% of control at 25 µg/ml. Moderately resistant isolates grew from 30-100% of control at 1 µg/ml but did not grow at 25 µg/ml of cyprodinil, while sensitive isolates grew less than 30% of control at 1 µg/ml. Boscalid-resistant isolates grew without inhibition at 1µg/ml boscalid and grew to more than 25% of control at 50 µg/ml. Moderately resistant isolates grew without inhibition at 1 µg/ml boscalid but were inhibited at 50 µg/ml, while sensitive isolates grew less than 50% of control at 1 µg/ml boscalid. Isolates sensitive to fludioxonil or fluopyram were inhibited at 0.1 µg/ml of fludioxonil or 3 µg/ml fluopyram, while less sensitive isolates displayed healthy germtube growth at the same concentrations.

While isolates less sensitive to fluopyram and fludioxonil displayed germ tube growth at the discriminatory concentrations listed in Table 2.1, a spectrum of lessened sensitivity against both active ingredients was observed. To better understand the results we obtained from fungicide resistance profiling, effective fungicide concentrations causing a 50% reduction of growth

(EC50) were determined for isolates displaying reduced sensitivity to fluopyram and fludioxonil at discriminatory doses in germ tube elongation assays. A germ tube elongation assay was performed with fluopyram at 0.01, 0.1, 1.0, and 3.0 µg/ml, while fludioxonil was tested at 0.001, 0.01, 0.1, 1.0 and 10 µg/ml. The growth of ten representative germ tubes was recorded for each treatment and expressed as a percentage of the control. These values were used to plot a regression curve with the relative inhibition versus log10 of fungicide was used to determine the EC50 value for a given isolate.

In addition to the germ tube elongation bioassays the Profile 24 ® resistance monitoring tool (Schnabel et al. 2012) was used to test 40 isolates to determine the suitability of its single discriminatory doses for evaluating *Botrytis* fungicide sensitivity in VA. In these assays, only one control is used (CDA) to compare growth on CDA amended with 4 µg/ml cyprodinil; MEA amended with 0.5 µg/ml fludioxonil, 50 µg/ml fenhexamid, 10 µg/ml iprodione, 10 µg/ml trifloxystrobin and 100 µg/ml SHAM, or 100 µg/ml thiophanate-methyl; and YEA amended with 75 µg/ml boscalid or 10 µg/ml fluopyram. The wells (1.93 $cm^2$) of 24-well plates (DOT Scientific; Burton, MI) were filled with ~3 ml of fungicide amended media. Conidia are collected from sporulating *B. cinerea* cultures using sterile cotton swabs, then transferred a well of a 24-well plate using sterile toothpicks. Following a 72-hour incubation period, radial growth in each well is compared visually to the growth on the control media and a score (-, +, ++, or +++) is assigned which corresponds to the relative growth on a given fungicide (no growth, less than 20% growth, 20%-50% growth, or more than 50% growth).

**Evaluation of baseline sensitivity of *B. cinerea* from VA to polyoxin-D**
Polyoxin-D was obtained from Arysta (Cary, NC). Baseline sensitivity of *B. cinerea* isolates from Virginia to polyoxin was assessed by using a mycelial growth assay to determine the EC50s of 29 isolates collected from different hosts (Table S3). Isolates were grown on 60 x 15 mm Petri plates containing CDA for 4 days in the dark at 22ºC. Plugs were removed from these plates using a sterile 4-mm cork borer and transferred to 100 x 15 mm Petri plates containing plain CDA or amended with 0.01, 0.1, 1.0, 10, or 100 µg/ml of polyoxin. Each plate was inoculated with three plugs and incubated in the dark at 22ºC for three days. Two independent tests were performed for each isolate and treatment. The mean colony diameter for each treatment minus the diameter of the agar plug was calculated and expressed as a percentage of the control. The EC50 for each isolate was calculated in Microsoft Excel by regressing the relative inhibition of growth against the log10 of fungicide concentration.

***In vivo* evaluation of cyprodinil sensitivity**
*B. cinerea* isolates with various levels of resistance to cyprodinil were assessed in their ability to produce disease symptoms on table grapes that had been treated with a field rate of cyprodinil. Commercially grown table grapes obtained from a grocery store (Kroger; Blacksburg, VA) were removed from clusters with pedicels intact and disinfested in 20% bleach/0.1% Tween for 2 min and then rinsed three times for 30 s each time with sterile water. Grapes were placed in new, one-gallon polyethylene bags and soaked in 200 ml of 560 mg/liter Vangard WG, 150 mg/liter Vangard WG, or sterile water for untreated controls. After 30 minutes of soaking with occasional agitation, grapes were removed with sterile forceps and allowed to dry on wire racks in a clean transfer hood. After drying completely, grapes were submerged in conidial suspensions ($10^6$ conidia/ml) containing 0.13% water agar (WA) for three to four s and then transferred to a wire

rack inside a clean plastic box. Untreated controls were dipped in 0.13% water agar in the same manner. These boxes were incubated at 20ºC for 4 days, with the initial 48 h inside a plastic incubation tent to maintain high relative humidity. Lesion coverage on treated and untreated grapes was determined after 4 days, and negative controls were assessed for any disease symptoms. For each isolate and treatment, five grapes were used, and each experiment was repeated twice. Statistical analysis was performed using SAS and JMP (SAS Institute, Cary, NC).

## Results

### Sensitivity of *B. cinerea* isolates to thiophanate-methyl, iprodione, boscalid, fluopyram, cyprodinil, trifloxystrobin, fludioxonil, and fenhexamid.

During the 2011-2015 growing seasons, 488 Botrytis isolates were collected and examined for this study. The germ tube elongation method and the Profile 24 ® system provided concurring results when the same isolates were evaluated using both systems (Table S.2). While frequency of resistant isolates varied among different host crops, the same resistance phenotypes (i.e. s, ls, mR and R) were found in isolates recovered from different host crops, except for iprodione R (Table 2.2). Iprodione R was not detected in isolates from strawberries, which constituted a small sample size (*n*=33). Resistance to thiophanate-methyl and trifloxystrobin was found in 70% and 64% of isolates from ornamentals and in 67% and 81% of isolates from wine grapes, respectively. Resistance to both modes of action was also relatively common in isolates from strawberry. Boscalid resistance and moderate resistance was detected in 55% and 19% of isolates from wine grapes, respectively. In the smaller sample recovered from strawberries, resistance and moderate resistance was found in 10% and 34% of isolates; in isolates from ornamentals, which also comprised a smaller sample size (*n*=60), 38% of isolates were resistant to boscalid, while 5% were moderately resistant. Lessened sensitivity to iprodione was detected in 24-32% of isolates from different crops. Iprodione resistance and moderate resistance was detected less frequently, in only 1% and 8% of isolates from wine grapes, and in 7% and 19% of isolates from ornamentals. Resistance to iprodione was absent in the isolates we recovered from strawberries, but moderate resistance was detected in 18% of these isolates.

Cyprodinil resistance was detected in 3% of the *Botrytis* isolates collected from wine grapes and ornamentals in this survey, and in 7% of isolates from strawberry. Moderate resistance to cyprodinil was found in 45%, 47%, and 57% of isolates from wine grapes, ornamentals, and strawberries. Fenhexamid resistance was uncommon. While fenhexamid-resistant phenotypes made up relatively large proportions of isolates collected from strawberries and ornamentals (33% in both cases), sample sizes from these crops were small. Among isolates collected from wine grapes, resistance was found in only 5% of isolates. Diminished sensitivity to fluopyram and fludioxonil was detected in 14% and 5% of isolates from wine grapes, respectively, and in 7% and 9% of isolates from strawberry.

Resistance frequencies have fluctuated from year to year but no major shifts in resistance frequencies occurred over the duration of this study (Table 2.3). Some general trends emerged from these data, but these should not be regarded as definite trends, because sample sizes varied considerably each year. Resistance to thiophanate-methyl and trifloxystrobin is common and seems to be increasing, as is resistance and moderate resistance to boscalid. Resistance and moderate resistance to cyprodinil is common but trending downwards. On the other hand,

resistance to fenhexamid and resistance and moderate resistance to iprodione is relatively uncommon, but seems to be increasingly common, while lessened sensitivity to fluopyram and fludioxonil is rare and does not appear to be increasing.

In 2014 and 2015, a number of isolates were collected at the same nine sites from wine grape clusters (September 2014) or wine grape flower debris (June 2015) to determine the persistence of fungicide resistance phenotypes over the winter months. With respect to cluster samples, resistance to thiophanate-methyl and trifloxystrobin were found in 72% and 80% of isolates, respectively (Table 2.4). However, all isolates from two sites were sensitive to both modes of action. Fenhexamid resistance was found in only 11% of isolates and was found at only three sites, at two of which only one isolate was resistant; at the third site, all isolates collected were resistant to fenhexamid. Frequencies of resistance to boscalid, iprodione, and cyprodinil in cluster-derived isolates closely resembled the larger trends that were manifest in 2014. Lessened sensitivity to fluopyram or fludioxonil was found in 4% of isolates, in each case in single isolates collected from four different sites.

Trends in fungicide resistance in flower samples resembled those seen in cluster samples, with a few notable differences (Table 2.4). Strong resistance to boscalid was less common for flower isolates than moderate resistance, but resistant phenotypes accounted for the majority of isolates collected for both clusters and flowers. Moderate sensitivity to iprodione was relatively uncommon compared with flower isolates and when compared to the cumulative dataset assembled over the course of the survey. Diminished sensitivity to fluopyram and fludioxonil was present in 3% and 5% of isolates from flowers and was present in single isolates from two and three sites, respectively. Resistance to fenhexamid was present in only 3% of isolates from flowers, occurring in only two isolates, collected from two sites. Only 26% of isolates from flowers had moderate resistance to cyprodinil, compared with 57% of isolates from cluster debris.

Isolates less sensitive to fluopyram or fludioxonil were subjected to EC50 determination for the mode of action in question. EC50 values for fluopyram ranged from 0.002 to 1.25 µg/ml (Figure 2.1). Isolates less sensitive to fluopyram germinated and displayed some healthy germ tube growth at 3 µg/ml fluopyram, while isolates less sensitive to fludioxonil showed some healthy growth at 0.1 µg/ml fludioxonil. With respect to fluopyram, most isolates grew without inhibition at 0.01 µg/ml. At 0.1 µg/ml fluopyram, most isolates exhibited 10 to 25% reductions in germ tube growth relative to control, while 1 µg/ml generally reduced growth by as much as 75%, although in some cases, the inhibition was more modest and approached only 50%. At 3 µg/ml, many isolates were completely inhibited or grew to less than 10% of the control, but there were several notable exceptions.

With respect to fludioxonil, concentrations of 0.001 and 0.01 µg/ml caused variable inhibition of germ tube growth, ranging from no reduction to over 50% reduction in growth. 0.1 µg/ml fludioxonil caused variable inhibition ranging from 60% reduction in germ tube growth to complete inhibition. Concentrations of 1 and 3 µg/ml inhibited growth entirely in all but a few isolates, which were able to germinate and grow to 5 to 6% at 1 µg/ml fludioxonil of the control. EC50 values for fludioxonil did not exceed 0.14 µg/ml (Figure 2.2).

**Baseline sensitivity of VA *Botrytis* to polyoxin-D**

EC50 values for polyoxin-D in isolates of *Botrytis* with no known previous exposure to polyoxin-D ranged from 0.14 to 3.9 µg/ml (Figure 2.3). The EC50 value for one isolate was particularly high at 8.34 µg/ml polyoxin-D. In mycelial growth tests, concentrations of 0.01 and 0.1 µg/ml polyoxin-D had little to no impact on growth in most isolates, but caused up to 25% and 43% inhibition of growth in more sensitive isolates, respectively. At 1 µg/ml polyoxin-D, variable responses were observed, ranging from nearly uninhibited growth to strongly inhibited growth; however, all isolates did grow at this concentration. Similarly, at 10 µg/ml, responses ranged from about 50% reduction in growth to complete inhibition. No growth occurred for most isolates at 100 µg/ml, while some isolates grew to about 9% of control. The isolate with an EC50 value of 8.34 µg/ml polyoxin-D grew to 31% of the control at 100 µg/ml.

***In vivo* evaluation of cyprodinil sensitivity**

Isolates classified as sensitive, moderately resistant, and resistant to cyprodinil all grew and sporulated at similar rates in *in vitro* experiments on PDA (Table 5). After four days of incubation, isolates of all types produced similar sized lesions, which coalesced, and covered the surface of untreated control table grapes to a similar extent. On table grapes treated with an approximate field rate of cyprodinil at 560 µg/ml and a lower rate of 150 µg/ml cyprodinil, disease severity (expressed as table grape surface lesion coverage) was significantly greater for resistant isolates when compared with moderately resistant and sensitive isolates. However, disease incidence (the percentage of table grapes that showed disease) was comparable between resistant and moderately resistant isolates at the lower rate of cyprodinil, while at the higher rate, significant differences in disease incidence were seen between sensitive, moderately resistant and resistant isolates. Interestingly, at this lower rate, the ability of sensitive and moderately resistant isolates to grow in the presence of the fungicide increased 3- to 7-fold. Resistant isolates grew better at this lower rate but only by about 30% relative to growth at the higher concentration of active ingredient.

**Discussion**

Both of the bioassay methods we evaluated produced the same results. The Profile 24 ® method provided accurate fungicide resistance characterization with minimal setup and evaluation time; however, there was a three-day incubation period before data could be generated (Table 2.6). The germ tube elongation method, on the other hand, provided more quantitative data, but required much more active involvement, particularly during data collection. We think that the 24-well plate system is an excellent technique for routine fungicide resistance monitoring, while germ tube elongation assays retain great value as sources of quantitative data for purposes such as determining EC50 values and minimum inhibitory concentration. The germ tube elongation assay also provides another metric of sensitivity by allowing approximation of the proportion of germinated conidia, in addition to germ tube growth.

Resistance to thiophanate-methyl and trifloxystrobin was widespread in the *B. cinerea* isolates collected from various host crops in VA and in limited sampling in North Carolina. This is not surprising: benzimidazoles long ago lost their efficacy against Botrytis, and benzimidazole resistant phenotypes can persist even in the absence of any fungicide use (Yourman et al. 2001). More recently, MBC-resistant *Botrytis cinerea* has been found in strawberry and winegrapes

from South Carolina and Virginia, respectively (Baudoin 2013; Fernández-Ortuño and Schnabel 2012). Resistance to the QoI fungicide pyraclostrobin is widespread among *Botrytis* populations in southern state strawberry fields and is frequently associated with the use of the formulated fungicide Pristine, a mixture of pyraclostrobin and boscalid (Fernández-Ortuño et al. 2012). Interestingly, rates of boscalid resistance were relatively low among the isolates we collected from strawberry, while resistance was quite common among isolates from grapes, but the number of samples collected from strawberry was small in this study. Boscalid is labelled for use as a single ingredient on grapes as the product Endura, in addition to in tandem with a QoI fungicide. However, Pristine has historically been applied in VA vineyards to combat powdery mildew (Myers and Baudoin 2006). While it is difficult to determine what role Pristine applications might play in the development of resistance to boscalid in *B. cinerea* from grapes, the use of boscalid as a component of powdery mildew control is expected to lead to increased contact between Botrytis populations and boscalid. This is due to the overlap of the critical period for controlling grapevine powdery mildew in the canopy, which begins at flower and continues for several weeks, coinciding with the emergence of Botrytis at bloom. A study of boscalid resistance development in powdery mildew in Japan suggested that boscalid applications to control *Corynespora* leaf spot of cucumber contributed to the development of boscalid resistant powdery mildew on the same plants (Miyamoto et al. 2010).

Resistance to iprodione was rarely observed among isolates from any host crop. DC fungicides, such as iprodione, were introduced and used to control *Botrytis* in the 1970's. Resistance development occurred rapidly and was widespread following their introduction in the field, but DC resistant populations are also prone to phenotype shifts that may cause resistant populations to become sensitive and vice versa under different ecological and fungicide pressures (Yourman et al. 2001). Surveys of fungicide sensitivity in *Botrytis cinerea* isolates from southern U.S. strawberry fields revealed similarly low rates of iprodione resistance and moderate resistance in seven southern states in 2012 and 2013 (Fernández-Ortuño et al. 2014).

Fenhexamid resistance has been identified in about 17% of *B. cinerea* from 11 strawberry fields in the Carolinas (Grabke et al. 2013). We saw fenhexamid resistance in a third of the isolates we collected from 14 strawberry fields and ornamental and herbaceous locations, but in only 5% of the isolates we collected from over 50 vineyards. Labelled rates for fenhexamid on strawberries allow for application of up to 3 lb ai/A per season and from 2 to 4.5 lb ai/A for use on ornamentals, compared to 1.5 lb ai/A per season for use on grapes. So, it makes sense that increased use of fenhexamid could be a factor in increased resistance in *Botrytis* populations from strawberries and ornamentals. However, it was unclear what relationship existed between fenhexamid applications and resistance frequency in this study. Fenhexamid was applied at some locations where no resistance was detected, while resistance was present at some locations without a history of fenhexamid use.

Resistance occurring as a result of point mutations in the target site gene presents a serious threat for Botrytis control with field rates of fenhexamid in strawberries (Grabke et al. 2013). However, laboratory tests have led other authors to hypothesize that high resistance to fenhexamid in *B. cinerea* isolates is associated with fitness costs that could be detrimental to survival under field conditions (Billard et al. 2012; Saito el al. 2014). Saito et al. (2014) have demonstrated that a *Botrytis* population in a vineyard treated with fenhexamid may not experience an increase in the

proportion of isolates with high EC50s for fenhexamid as a response to fungicide spray pressure. Thus it is difficult to determine the practical threat the fenhexamid resistant isolates we identified will present in the field. Similarly, it is difficult to determine what role resistant populations in other host crops may play in the development of resistance in other crops, for instance, grapes. Geography and physical distance have been shown to be insignificant in genetic differentiation of *Botrytis cinerea* populations in France, but the host plant could present a significant barrier to gene flow between populations evolving in sympatry (Fournier and Giraud 2008). The exact impact of these factors on fungicide resistance development and fenhexamid sensitivity in particular remains unclear.

In a 2013 survey of *B. cinerea* from strawberry fields in North and South Carolina, Fernández-Ortuño et al. (2013b) found that 17% of isolates were resistant to cyprodinil and 30% were moderately resistant. We detected a similar, relatively low abundance of resistance to cyprodinil in our survey, with 3% to 7% of isolates from different host crops cyprodinil resistant, while moderate resistance was present in 45% to 57% of isolates from different hosts. In another survey Fernández-Ortuño et al. (2014) reported that resistance or moderate resistance was present in 27% of isolates recovered from strawberry fields in the states including the Carolina's, Maryland, Virginia, Arkansas, Florida, and Georgia. Over the five-year duration of our study, moderately resistant and resistant isolates accounted for 26% to 68% of isolates collected annually. This was the greatest variation in sensitivity to a mode of action we observed over the course of our study. Fernández-Ortuño et al. (2013b) found that on strawberries treated with a field rate of pyrimethanil (another AP fungicide comparable to cyprodinil), moderately resistant isolates and resistant isolates behaved similarly and were not well controlled compared with sensitive isolates. We found that moderately resistant isolates behaved similarly to sensitive isolates on grapes treated with cyprodinil in terms of disease severity. However, disease incidence was comparable between moderately resistant and resistant isolates at a low rate of cyprodinil, while at an approximate field rate of cyprodinil, significant differences in disease incidence existed between isolates of all types. This suggests that the *in vitro* distinction between isolates sensitive, moderately resistant, and resistant to cyprodinil may be of practical value to *Botrytis* management in vineyards.

Veloukas and Karaoglanidis (2012) showed that baseline EC50 values for isolates sensitive to fluopyram range from 0.03-0.29 µg/ml. About half (49%) of the isolates that could grow at 3 µg/ml fluopyram in germ tube elongation tests had EC50s in excess of 0.29 µg/ml fluopyram (Figure 2.1), indicating resistance development to fluopyram in the *Botrytis* populations we studied. While no correlation was found between boscalid resistance status (s, mR, or R) and EC50 values against fluopyram, two boscalid resistance mutations also confer resistance to fluopyram (Veloukas et al. 2013). Boscalid resistant isolates carrying either of these two mutations in the *Sdh*B subunit may have been initially responsible for the development of fluopyram resistance in VA fields, but further study is necessary to characterize such mutations in these *Botrytis* isolates. Characterizing these mutations would be valuable, because isolates carrying different point mutations manifest considerably different sensitivities to various SDHI fungicides (Veloukas et al. 2013).

Other authors have reported EC50 values for fludioxonil of low to moderately resistant isolates ranging from 0.26 µg/ml to 0.40 µg/ml (Fernández-Ortuño et al. 2013a; Fernández-Ortuño et al.

2015). Moderate to high resistance phenotype isolates had EC50s against fludioxonil ranging from 1.7 to 3.1 µg/ml (Fernández-Ortuño et al. 2015). These low and high resistance groups are MDR phenotypes MDR1 and MDR1H phenotypes, respectively (Fernández-Ortuño et al. 2015). Meanwhile, sensitive isolates had EC50s of 0.01 µg/ml against fludioxonil (Fernández-Ortuño et al. 2013a). Of the isolates we tested, 31 had EC50s below this threshold, indicating sensitivity (Figure 2.2). The remainder of the isolates tested displayed diminished sensitivity to fludioxonil, but not to the degree consistent with MDR phenotypes. This evidence, combined with the lack of isolates displaying elevated cyprodinil and fludioxonil resistance (Table 2.7) makes a convincing case that that MDR phenotypes were not detected in this study, despite the abundance of isolates displaying resistance to multiple fungicides.

*Botrytis cinerea* collected in Israel from previously untreated vineyards, and from greenhouse crops with long histories of polyoxin use had EC50 values for polyoxin ranging from 0.4 to 1.5 µg/ml for sensitive phenotypes, while low level resistant isolates had EC50s ranging from 4.0 to 6.5 µg/ml (Mamiev et al. 2013). While the same phenotypes were present both at locations previously exposed and previously unexposed to polyoxin, the proportion of low level resistant isolates was double that at the locations with a history of polyoxin use when compared with the vineyards with no history of polyoxin use (Mamiev et al. 2013). The same authors showed that polyoxin use could lead to this relative abundance of low-level resistance in *B. cinerea* populations within only two seasons (Mamiev et al. 2013).

To the best of our knowledge, none of the fields we sampled had been treated with polyoxin-D at the time of sample collection. More than half of the isolates we collected fell within the range of sensitivity to polyoxin established in previous studies, but the frequency distribution of EC50 values we observed was skewed towards higher values, with one high outlier, unlike the bimodal distribution seen by other authors. MDR mechanisms have been suggested to play a role in the low level resistance to polyoxin seen in other populations (Mamiev et al. 2013). However, most of the isolates we evaluated also had high levels of resistance to other modes of action not usually associated with MDR phenotypes (or lacked the high resistance to cyprodinil and fludioxonil characteristic of some MDR phenotypes) making it difficult to argue for the presence of MDR mechanisms on the basis of phenotype alone. In any case, it has been suggested that low level resistance to polyoxin is of little practical importance (Mamiev et al. 2013). However, the labelled recommendation of foliar application every 7 to 10 days may be excessive in light of the known risk of resistance phenotype shift, and polyoxin-D may be better used as just one component of a fungicide resistance management and disease control strategy (Wilcox 2015).

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**Figures and Tables Chapter 2**

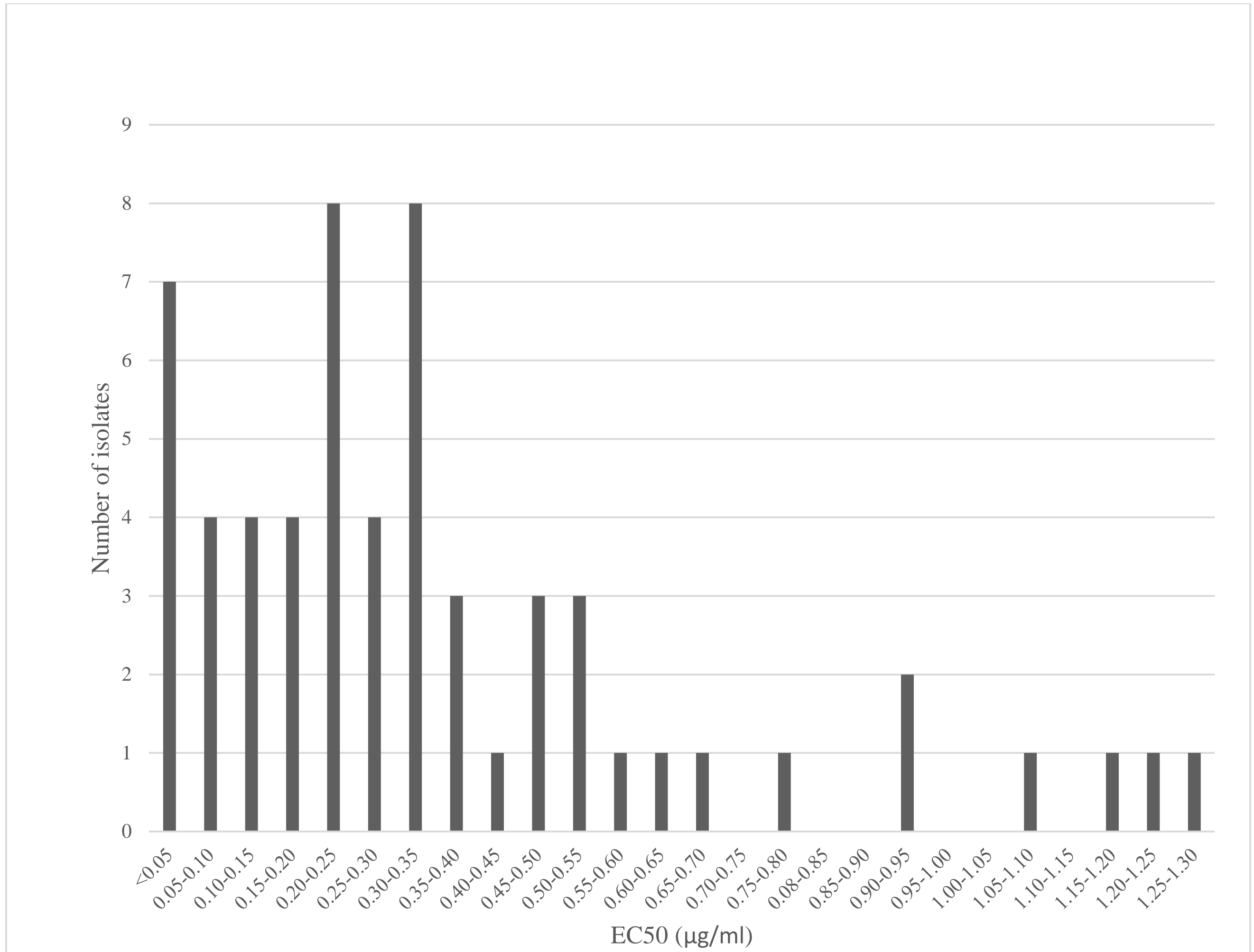


**Figure 2.1.** Frequency distribution of EC50 values for fluopyram against 59 isolates of *B. cinerea* collected from VA crops.

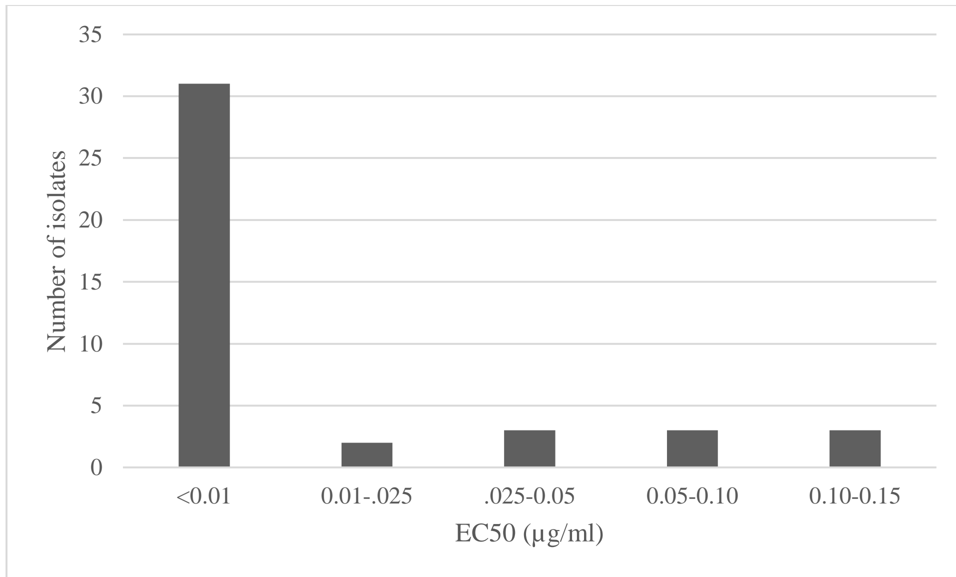


**Figure 2.2.** Frequency distribution of EC50 values for fludioxonil against 42 isolates of *B. cinerea* collected from VA crops.

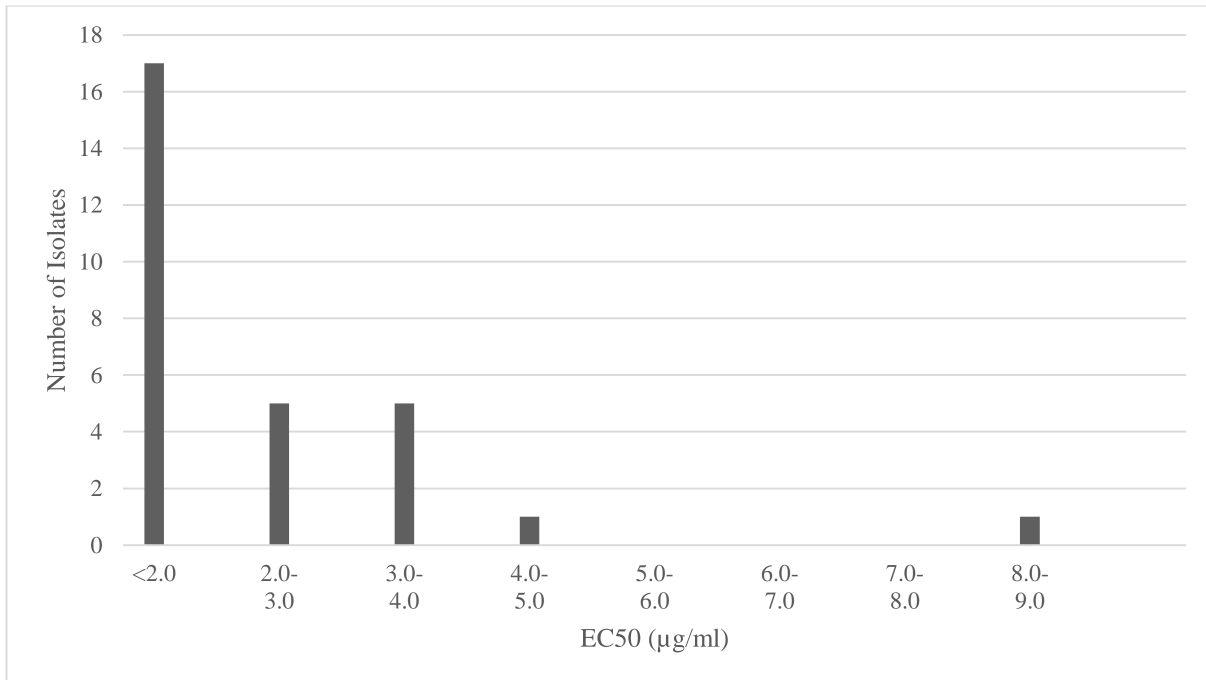


**Figure 2.3.** Frequency distribution of EC50 values for polyoxin-D against 29 isolates of *B. cinerea* collected from VA and NC crops.

**Table 2.1.** Discriminatory fungicide concentrations used in conidial germination tests for fungicide resistance profiling (from Weber and Hahn 2011).

| Fungicide | Discrim. Conc. 1 (ppm) | Discrim. Conc. 2 (ppm) |
|---|---|---|
| Thiophanate methyl | --- | 100 |
| Iprodione | --- | 50 |
| Fluopyram | 3 | --- |
| Boscalid | 1 | 50 |
| Cyprodinil | 1 | 25 |
| Trifloxystrobin | --- | 20 |
| Fludioxonil | 0.1 | --- |
| Fenhexamid | 10 | --- |

**Table 2.2.** Frequencies of different fungicide resistant and sensitive phenotypes in different host crops.

| | Ornamental Isolates (%) | | | | Strawberry Isolates (%) | | | | Wine Grape Isolates (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **N$^{z}$** | **(60)** | | | | **(33)** | | | | **(395)** | | | |
| | **s*** | **Ls** | **mR** | **R** | **s** | **ls** | **mR** | **R** | **s** | **ls** | **mR** | **R** |
| Thiophanate-methyl | 30 | | | 70 | 48 | | | 52 | 33 | | | 67 |
| Trifloxystrobin | 36 | | | 64 | 50 | | | 50 | 19 | | | 81 |
| Fenhexamid | 67 | | | 33 | 67 | | | 33 | 95 | | | 5 |
| Boscalid | 57 | | 5 | 38 | 56 | | 34 | 10 | 26 | | 19 | 55 |
| Fluopyram** | 78 | 22 | | | 93 | 7 | | | 86 | 14 | | |
| Cyprodinil | 50 | | 47 | 3 | 36 | | 57 | 7 | 52 | | 45 | 3 |
| Iprodione | 42 | 32 | 19 | 7 | 55 | 27 | 18 | | 67 | 24 | 8 | 1 |
| Fludioxonil | 74 | 26 | | | 91 | 9 | | | 95 | 5 | | |

*s=sensitive, ls=less sensitive, mR=moderately resistant, R=resistant

**Fluopyram was not included in initial bioassays for fungicide resistance, hence the lower number of data points

$^{z}$ Numbers in parentheses=number of isolates of *B. cinerea* collected and characterized each year.

**Table 2.3.** Percentage of *B. cinerea* isolates collected in Virginia and North Caroline from 2011-2015 resistant (***), resistant and moderately resistant (**), or less sensitive (*) to eight different fungicides collected in VA from 2011-2015.

| | Isolates (%) | | | | |
|---|---|---|---|---|---|
| | **2011** | **2012** | **2013** | **2014** | **2015** |
| **N$^z$** | **(153)** | **(46)** | **(161)** | **(66)** | **(61)** |
| Thiophanate-methyl*** | 67 | 57 | 64 | 73 | 72 |
| Trifloxystrobin*** | 82 | 62 | 70 | 79 | 87 |
| Fenhexamid*** | 1 | 11 | 19 | 20 | 3 |
| Boscalid** | 72 | 52 | 60 | 76 | 84 |
| Fluopyram* | 17 | 4 | 26 | 3 | 3 |
| Cyprodinil** | 43 | 68 | 53 | 65 | 26 |
| Iprodione** | 6 | 14 | 15 | 25 | 4 |
| Fludioxonil* | 6 | 5 | 14 | 3 | 5 |

$^z$ Numbers in parentheses=number of isolates of *B. cinerea* collected and characterized each year.

**Table 2.4.** Fungicide sensitivities of isolates of *B. cinerea* collected from wine grape clusters or wine grape flowers.

| | **Cluster Isolates (%)** | | | | **Flower Isolates (%)** | | | |
|---|---|---|---|---|---|---|---|---|
| **N$^z$** | **(46)** | | | | **(58)** | | | |
| | **s*** | **Ls** | **mR** | **R** | **s** | **ls** | **mR** | **R** |
| Thiophanate-methyl | 28 | | | 72 | 28 | | | 72 |
| Trifloxystrobin | 20 | | | 80 | 12 | | | 88 |
| Fenhexamid | 89 | | | 11 | 97 | | | 3 |
| Boscalid | 24 | | 7 | 69 | 16 | | 81 | 3 |
| Fluopyram | 96 | 4 | | | 97 | 3 | | |
| Cyprodinil | 43 | | 57 | | 74 | | 26 | |
| Iprodione | 64 | 14 | 22 | | 72 | 26 | 2 | |
| Fludioxonil | 96 | 4 | | | 95 | 5 | | |

*s=sensitive, ls=less sensitive, mR=moderately resistant, R=resistant

$^z$ Numbers in parentheses=number of isolates of *B. cinerea* collected and characterized

**Table 2.5.** Fitness and control of isolates s, mR, and R to cyprodinil *in vitro* and on table grapes amended with different rates of cyprodinil.

| | | ***In vitro*** | | ***In vivo**** | | | ***In vivo*** | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | **Lesion coverage (% of surface)** | | | **Incidence (% diseased berries)** | | |
| | | **3 dai** | **10 dai** | **4 dai** | | | **4 dai** | | |
| **Isolate** | **Type** | **Colony diameter (cm)** | ***N*[z]** | **Control** | **Cyprodinil -Low[a]** | **Cyprodinil -High[b]** | **Control** | **Cyprodinil -Low[a]** | **Cyprodinil -High[b]** |
| 43 | s | 6.6 | 7.0 | 81.0 | 5.0 | 0.0 | 100 | 60.0 | 0.0 |
| 156 | s | 5.3 | 7.5 | 80.5 | 2.5 | 0.0 | 100 | 40.0 | 0.0 |
| 444 | s | 5.2 | 9.0 | 68.0 | 2.0 | 1.0 | 100 | 30.0 | 20.0 |
| 188 | mR | 6.4 | 8.3 | 81.0 | 8.5 | 2.5 | 100 | 80.0 | 40.0 |
| 256 | mR | 5.9 | 6.0 | 86.5 | 10.0 | 2.0 | 100 | 90.0 | 30.0 |
| 446 | mR | 5.0 | 10.6 | 78.0 | 5.0 | 2.0 | 100 | 50.0 | 40.0 |
| 71 | R | 5.7 | 9.8 | 82.5 | 37.0 | 19.0 | 100 | 100 | 70.0 |
| 151 | R | 4.6 | 11.2 | 96.0 | 85.0 | 75.0 | 100 | 100 | 100 |
| 344 | R | 6.6 | 6.7 | 79.0 | 33.5 | 17.0 | 100 | 100 | 70.0 |
| Mean | s | 5.7 | 7.8 | 76.5 b | 3.3 e | 0.3 e | 100 a | 43.3 b | 6.7 c |
| Mean | mR | 5.8 | 8.3 | 81.8 ab | 7.8 e | 2.2 e | 100 a | 73.3 a | 36.7 b |
| Mean | R | 5.6 | 9.2 | 85.8 a | 51.2 c | 37.0 d | 100 a | 100 a | 80.0 a |

*= numbers in the same column followed by the same letter are not significantly different at α=0.05

[a]= 150 μg/ml cyprodinil

[b]= 560 μg/ml cyprodinil

[z]= ($N$ x $10^4$ conidia/ml)

**Table 2.6.** Attributes of germ tube elongation-type and 24-well plate-type bioassays for fungicide sensitivity against *B. cinerea*.

| | **Germ tube elongation** | **24-well plate** |
|---|---|---|
| Media Preparation[a] | equivalent | equivalent |
| Assay Setup[b] | 60 min. | 30 min. |
| Incubation | 12-18 h. | 72 h. |
| Evaluation | 90-180 min. | 15-30 min. |
| Data | quantitative; incidence can be inferred by approximating proportion of non-germinated conidia | qualitative |

[a] When preparing 100 ml of medium per treatment

[b] When evaluating six isolates per assay

**Table 2.7.** Selected fungicide resistance phenotype frequencies of *Botrytis* isolates collected in this study.

| | Phenotype | | | | | | |
|---|---|---|---|---|---|---|---|
| **N $^{z}$** | **Thiophanate.-methyl.** | **Trifloxystrobin** | **Boscalid** | **Cyprodinil** | **Fenhexamid** | **Iprodione** | **Fludioxonil** |
| 49 | S | s | s | s | s | s | s |
| 7 | R | s | s | s | s | s | s |
| 9 | S | R | s | s | s | s | s |
| 1 | R | s | s | s | R | R | ls |
| 10 | R | R | s | s | s | s | s |
| 9 | R | R | R | s | s | mR | s |
| 28 | R | R | R | s | s | s | s |
| 4 | R | R | R | s | R | s | s |
| 15 | R | R | R | mR | s | mR | s |
| 46 | R | R | R | mR | s | s | s |
| 19 | R | R | mR | s | s | s | s |
| 16 | R | R | mR | mR | s | s | s |
| 2 | R | R | R | R | s | s | s |
| 6 | R | R | R | mR | R | s | s |
| 1 | R | R | R | R | R | s | s |
| 8 | R | R | R | mR | R | ls | s |

N $^{z}$ =number of isolates of *B. cinerea* collected and characterized

## Chapter 3

## Molecular species identification and characterization of the Erg27 genes of fenhexamid resistant *Botrytis cinerea* causing bunch rot and gray mold on Virginia crops


### Abstract

Botrytis bunch rot and gray mold, caused by *Botrytis cinerea* and a closely related species, *B. pseudocinerea*, are economically significant diseases of wine grapes and strawberries and ornamentals, respectively. The use of site specific fungicides with high risk of resistance development is necessary to control these diseases in the southeastern United States. One such fungicide is the hydroxyanilide fenhexamid, which is commonly used in vineyards and strawberry fields in and around Virginia. Isolates collected from 68 wine grape, strawberry, and ornamental growers in and closely adjacent to Virginia were subjected to germ tube elongation bioassays to determine their sensitivity towards fenhexamid. Isolates sensitive in germ tube elongation were also subject to mycelial growth assays to determine if phenotypes resistant in mycelial growth, but not germination, were present. Isolates from 17 locations were resistant to fenhexamid in germ tube elongation assays, while isolates with moderate resistance in mycelial growth only were recovered from 33 locations. Resistance to fenhexamid during germination was associated with three previously characterized Erg27 mutations, F412S, F412I, and T63I; one isolate carrying the F412S mutation also had an uncharacterized alteration in *erg27*, G108R. Detached berry trials using commercially grown table grapes treated with fenhexamid showed that a field rate of Elevate 50 WDG controlled sensitive isolates but not isolates possessing the *erg27* mutations.


### Introduction

Gray mold is one of the greatest threats to the production of grapes (*Vitis vinifera*) and strawberries (*Fragaria* x *ananassa*) in the United States--industries worth over $5.5 and $2.5 billion annually (Noncitrus fruits and nuts 2014 summary). *Botrytis cinerea* Pers. is the primary cause of the disease on strawberries in the southeastern United States, although *B. caroliniana* X.P. Li & G. Schnabel sp. nov. is a recently recognized species that may also cause gray mold on strawberry (Grabke et al. 2013). In grapes, *Botrytis cinerea* and *B. pseudocinerea* are most often responsible, although *B. pseudocinerea* has yet to be reported outside of Europe (Walker et al. 2013). Analysis of phylogenetically significant genes such as the glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) gene allow species level separation of the Botrytis species complex (Walker et al. 2011).

Frequent fungicide applications are necessary to combat gray mold in vineyards and strawberry fields, as well as in ornamental and herbaceous crop production and floriculture. Unfortunately, *Botrytis* has proven to be a pathogen at high risk of development of resistance to the single-site modes of action of many available fungicides (Ziogas et al. 2003). For example, resistance to benzimidazoles and succinate dehydrogenase inhibitors is common in gray mold from strawberries and grapes from the southeastern United States (Baudoin 2013; Fernández-Ortuño et al. 2012). Fenhexamid is a hydroxyanilide fungicide with site-specific action that has become widely used in *Botrytis* management, and resistance has developed in gray mold populations on

hosts including grapes, strawberries, and greenhouse crops in the United States (Grabke et al. 2013; Moorman et al. 2012; Saito et al. 2011).

Fenhexamid-resistant isolates exhibit one of four phenotypes-HydR1, HydR2, HydR3, and HydR3+ (Fillinger et al. 2008; Leroux et al. 1999; Leroux 2007). The HydR1 phenotype has only been identified in *B. pseudocinerea* and is associated with an innate resistance to fenhexamid during mycelial growth, which is only found in *B. pseudocinerea* (Leroux et al. 1999; Walker et al. 2011). Although present in French vineyards (accounting for up to 15% of the Botrytis species complex in some areas), *B. pseudocinerea* is thought to play little part in bunch rot development on grapes, because of poor fitness on ripening grapes (Walker et al. 2011). Thus, while *B. pseudocinerea* may survive early-season applications of fenhexamid, it rarely persists through grape development and HydR1 phenotypes remain relatively limited in vineyard gray mold populations (Walker et al. 2011). However, *B. pseudocinerea* has been shown to contribute significantly to gray mold development, particularly in the absence of fungicide pressure, in several crops in Germany including strawberry, apple, peony, and lettuce (Plesken et al. 2015). HydR2 phenotypes have only been identified in *B. cinerea*, but they share the reduced fitness of HydR1 isolates, are considered only moderately resistant to fenhexamid, and are inhibited by fenhexamid during spore germination (Leroux 2007). It has been speculated that cytochrome P450 enzymes are involved in mitigation of fenhexamid toxicity in HydR2 phenotypes, as opposed to mutations in the target site gene of fenhexamid (Leroux et al. 2002).

HydR3 and HydR3+ are moderate- and high-resistance phenotypes associated with amino acid substitutions in the fenhexamid target site gene Erg27 in *B. cinerea* (Fillinger et al. 2008; Leroux et al. 2002). Isolates with HydR3 and HydR3+ phenotypes are resistant to fenhexamid both during spore germination and mycelial growth, presenting a more serious disease management issue than other fenhexamid resistance phenotypes (Esterio et al. 2011). However, Billard et al. (2012) have suggested that moderate fitness costs associated with point mutation-mediated fenhexamid resistance could have a detrimental effect on the ability of resistant isolates to succeed in the field, especially during overwintering.

The goals of this study were to determine the fenhexamid-resistant phenotypes present in the isolates of *B. cinerea* recovered in this study and particularly, to determine the point mutations conferring HydR3 fenhexamid resistance. Additional goals included determining the ability of fenhexamid-resistant isolates to grow on grapes treated with a field rate of fenhexamid.

## Materials and Methods

### Isolates of *Botrytis* and culture conditions

During the 2011-2015 growing seasons, 531 isolates of *Botrytis* were collected from more than 60 locations in Virginia and several locations in North Carolina (Table S1). Of these isolates, 416 (78%) were collected from grapes, while the remaining 115 (22%) were collected from strawberries and ornamental crops from commercial growers. Conidia were collected directly from sample tissue, which was incubated in a moist chamber if no obvious sporulation was observed. With a sterile inoculation loop, conidia were streaked onto potato dextrose agar (PDA) and incubated for 12-18 hours at 22ºC. A single germinating conidium was isolated from each sample and transferred to another PDA plate, which was incubated at 22ºC and the isolate was

maintained in culture until fungicide resistance profiling was completed. Conidial suspensions for fungicide resistance evaluations were obtained from cultures ten days following inoculation.

**Sensitivity of isolates to fenhexamid and other fungicides**

Sensitivity to fenhexamid was assessed by a germ tube elongation bioassay based on the method of Weber and Hahn (2011), with minor modifications to the fungicide concentrations and media used. Conidial suspensions were streaked on fungicide-amended media and allowed to incubate at room temperature (~22ºC) in ambient light conditions for 12-18 hours, at which point the length of ten representative germ tubes was recorded for each isolate. Growth on fungicide-amended media was then expressed as a percentage of a given isolate's growth on unamended control media. Fenhexamid (Elevate 50 WDG, Arysta LifeScience) was suspended in 1% malt extract agar at 10 µg/ml (Weber and Hahn 2011). Resistant isolates were able to germinate and grow normally at 10 µg/ml, while sensitive isolates displayed distorted, arrested germ tube growth. In addition to fenhexamid, each isolate was profiled for sensitivity to the MBC fungicide thiophanate-methyl (Topsin M, United Phosphorus), the DC fungicide iprodione (Rovral 50W; Bayer), the SDHI fungicides boscalid (Endura 70 WW; BASF) and fluopyram (Bayer), the AP fungicide cyprodinil (Vangard WG; Syngenta), the QoI fungicide trifloxystrobin (Flint; Bayer), and the PP fungicide fludioxonil (Medallion WDG; Syngenta).

Isolates not resistant to fenhexamid during germination and germ tube elongation were subjected to a mycelial growth test to determine if HydR1 or HydR2 phenotypes might be present. Isolates were plated on PDA and allowed to grow at room temperature. After four days, two 5mm plugs were removed from the PDA plates and transferred to plates of 1% malt extract agar amended with 2 µg/ml fenhexamid. Identical plugs were plated on unamended 1% malt extract agar as controls. Plates were incubated at room temperature in ambient light conditions for 4 days, and mycelial growth of isolates was evaluated every 24 hours. Isolates that displayed mycelial growth at 2 µg/ml fenhexamid were considered putative HydR1 or HydR2 isolates (Leroux et al. 1999).

**Analysis of the *G3PDH* genes from fenhexamid resistant isolates**

In order to confirm the identity of the fenhexamid-resistant isolates identified in germ tube elongation or mycelial growth assays for fungicide sensitivity as *B. cinerea,* the *G3PDH* locus was amplified, sequenced, and analyzed. DNA was extracted from 57 putative HydR3 isolates (7 additional HydR3 isolates were not included in this experiment because they were not collected until after this experiment concluded) and from 19 putative HydR1or HydR2 isolates (the 19 isolates selected constituted a representative sample of the 53 isolates identified as putative HydR1 or HydR2 isolates in mycelial growth assays). Genomic DNA was extracted from mycelium grown on PDA plates at 22ºC as described by Chi et al. (2009). Oligonucleotides used in our study are listed in Table 1. To amplify the glyceraldehyde 3-phosphate dehydrogenase gene (*G3PDH*) from isolates resistant to fenhexamid in germ tube elongation assays or mycelial growth assays, the primer pair G3PDH for/rev was used. Polymerase chain reaction (PCR) was performed in a Mastercycler ep thermocycler (Eppendorf; Hauppauge, NY) in a final volume of 25 µl, containing 1.25 µl of 10 µM forward primer, 10 µl of 10 µM reverse primer, approximately 50 ng of template DNA, 8 µl of nuclease-free water, and 8 µl of ImmoMix Red 2X polymerase (Bioline; Taunton, MA). PCR parameters were as follows: 3 min at 95ºC; followed by 40 cycles of 95ºC for 30 s, 60ºC for 30 s, and 68ºC for 90 s; with final elongation at

68ºC for 5 min. PCR products were checked for size and quality via electrophoresis on 1.5% agarose gels and then cleaned using the ExoSAP-IT PCR purification system (USB Corporation, Cleveland) following the manufacturer's suggested instructions. DNA sequencing, in both forward and reverse directions, was performed by Eurofins MWG Operon (Huntsville, Alabama). Lasergene software suite (DNASTAR; Madison, WI) was used to view chromatographs of sequences, perform end trimming, to construct forward/reverse consensus sequences for each isolate. Basic local alignment search tool (BLAST, NCBI; Madden 2002) was used to align and compare each consensus sequence with the genomic supercontig SuperContig_2_1 of *B. cinerea* type strain T4 (Accession number FQ790286; Amselem et al. 2011).

**Analysis of the *erg27* genes from fenhexamid resistant isolates**

In order to characterize the point mutations conferring fenhexamid resistance to the isolates we identified as putative HydR3 isolates, the *erg27* gene (the target site of fenhexamid) was amplified, sequences, and analyzed. Genomic DNA extracted as described above was used in PCR reactions to amplify a portion of the *erg27* gene. The thermocycler and reaction mixture were as described above, with the exception of the primers, which in this case were primer pair Erg27beg/end (Table 1). Reaction conditions were as follows: 3 min at 95ºC; followed by 40 cycles of 95ºC for 30 s, 60ºC for 30 s, and 68ºC for 90 s; with final elongation at 68ºC for 5 min. PCR cleanup and DNA sequencing were all performed as described above. Lasergene software suite was used to align forward and reverse *erg27* sequences and create consensus sequences for each sequence as described above. To identify amino acid changes in each isolate, consensus sequences were then aligned and compared with a fenhexamid-sensitive reference strain, AY220532.1 (Albertini and Leroux 2004).

**Detached-fruit trial**

Isolates carrying the different point mutations associated with fenhexamid resistance we identified in this study were challenged with table grapes treated with two different rates of fenhexamid in order to determine whether different point mutations confer different levels of resistance *in vivo*. Isolates BF5, Car10, Mi-PelA1, Cri-S4B, RB-LavM.B, and Cri-S4fB, carrying *erg27* wild type, wild type, F412S, F412I, T63I, and F412S and G108R alleles, respectively, were used in detached-grape trials. Commercially grown Thompson Seedless were obtained and washed in 20% bleach/0.1% Tween 20 solution for two minutes, then washed three times with sterile water. Grapes were then transferred to clean 3.78 L polyethylene bags and soaked in 100 ml of fenhexamid solution at 3600 µg/ml, 1200 µg/ml, or in sterile water; the higher rate reflects an approximate field rate of formulated fenhexamid (assuming 100 g/A spray volume), while the lower rate was included to determine if there is a threshold effect associated with any of the resistance adaptations we detected. After 30 minutes of soaking with occasional agitation to ensure good coverage, grapes were removed and allowed to air dry at room temperature on sterile wire racks in a clean transfer hood. Dry grapes were then dipped in conidial suspensions prepared in sterile 0.13% water agar, which helped ensure more uniform coverage ($10^4$ conidia/ml). Grapes were then placed on sterile wire racks in clean plastic boxes, which were kept inside of a misted polyethylene bag to maintain high relative humidity for 24 hours. After 24 hours, the boxes were allowed to incubate on a benchtop at room temperature for an additional 72 hours, at which point the grapes were scored visually for lesion coverage. For each

isolate and treatment, five grapes were used, and each experiment was performed twice. Statistical analysis was performed using SAS and JMP (SAS Institute, Cary, NC).

## Results

### Sensitivity of isolates to fenhexamid

Of 531 isolates, 64 (12%) were resistant to fenhexamid and were able to grow at 10 µg/ml fenhexamid in germ tube elongation assays. Results of sensitivity profiling of other isolates are reported in Chapter 2. Resistant isolates were found at 17 of 68 locations, including two of three locations in North Carolina (Table 2). The greatest concentration of resistant isolates (84%) was collected from an ornamental grower in Montgomery County, VA. The greatest concentration of resistant isolates among vineyard sites (38%) was found at a location in Nelson County, VA. Fenhexamid resistance was present in 5% of isolates collected from grapes and 33% of isolates from strawberries and ornamentals.

Of the remaining isolates, 53 were able to grow on 1% malt extract agar amended with 2 µg/ml fenhexamid (Table 3). These isolates displayed a spectrum of reduced sensitivity to fenhexamid. A little more than half of these isolates displayed radial growth comparable to control (100% radial growth when expressed as a percentage of growth on unamended media). The remaining isolates ($n$=25) had growth on fenhexamid amended media ranging from about 39% to 90% of control. Isolates sensitive to fenhexamid during mycelial growth were completely inhibited by 2 µg/ml fenhexamid.

### Analysis of *G3PDH* gene sequences from putative HydR1, HydR2, and HydR3 isolates

An approximately 1,046-base pair portion of the *G3PDH* gene was successfully sequenced from 52 of the fenhexamid-resistant isolates identified in germ tube elongation assays. Sequencing produced truncated sequences that could not be assembled for several other isolates. All 52 isolates were identified as *Botrytis cinerea* with 99% identity and over 97% coverage with E values of 0.0. Similar size amplicons of the *G3PDH* gene were successfully amplified from 19 isolates displaying fenhexamid resistance in mycelial growth assays, but not in germ tube elongation assays (putative HydR1 of HydR2 isolates). All 19 isolates were identified as *Botrytis cinerea* with strong identity and coverage and E values of 0.0.

### Analysis of *erg27* gene sequences from putative HydR3 isolates

An approximately 900 bp portion of the *erg27* gene was successfully amplified and sequenced from 26 isolates of *Botrytis cinerea* resistant to fenhexamid in germ tube elongation assays (Table S#). The sequences were assembled, translated, and aligned with the protein sequence of a sensitive reference strain, AY220532.1 (Albertini and Leroux 2004). Protein changes including F412S, F412I, and T63I were detected in all 26 isolates (Table 4). One isolate, Cri.S4fB, had a novel protein change, G108R, in addition to an F412I mutation. Modifications at the F412 location were the most prevalent among the isolates successfully sequenced, while modifications at position T63 were found in only four isolates from two locations.

### Detached Fruit Trial

Both fenhexamid sensitive (*erg27* wildtype allele) and fenhexamid resistant (various *erg27* amino acid substitution alleles) isolates grew well on control, table grapes which were not treated

with fenhexamid (Table 5). Sensitive isolates were inhibited by both an approximate field rate of fenhexamid (3600 µg/ml) and a lower rate (1200 µg/ml). Adapted isolates carrying resistance-conferring mutations were not controlled by low or high rates of fenhexamid. There was no apparent synergistic effect between the F412I mutation and the G108R mutation carried in the *erg27* gene of isolate Cri-S4fB.

**Discussion**

Fenhexamid is widely used in the Carolinas to control gray mold on strawberries Grabke et al. 2013). Data collected in this study indicate that fenhexamid use is an important part of management of gray mold and bunch rot management. Resistance to fenhexamid is widespread in strawberry fields in the Carolinas where Elevate has been applied to combat gray mold, but the degree to which fungicide application frequency influenced resistance development was unclear (Grabke et al. 2013). In German strawberry fields, fenhexamid resistance was observed in 40-79% of isolates (Leroch et al. 2013). We detected fenhexamid resistance in 11% of the isolates recovered from vineyards, strawberry fields, and ornamental distributors, which is relatively low compared to frequencies reported elsewhere, but is comparable to rates observed in strawberries in the southeastern United States (Grabke et al. 2013). Fenhexamid resistance was observed at greater frequencies in isolates collected from strawberries and ornamentals in this survey (chapter 2), but relatively small sample sizes make comparison of resistance frequencies across crops unreliable.

The high resistance risk status of *Botrytis* coupled with frequent use of Elevate to control gray mold and bunch rot creates a scenario in which resistance development is unsurprising. It is difficult to determine whether isolates recovered from ornamental locations have been exposed to fenhexamid because plants at these locations often originate in multiple locations with different fungicide use patterns prior to arrival at the final seller. Some of the strawberry fields and vineyards from which we isolated fenhexamid-resistant isolates applied Elevate as part of current or recent fungicide treatments, potentially selecting for resistance (Leroch et al. 2013). However, fenhexamid resistance was not detected at numerous other vineyards and some strawberry fields with similar histories of Elevate application, so it is difficult to draw firm conclusions about precisely how different fungicide use patterns contribute to fenhexamid resistance development in Virginia. Saito el al. (2014) found that in a vineyard treated with fenhexamid, control was influenced by the frequency and rate of fenhexamid applications, but they did not observe increased selection for isolates with higher EC50 values, suggesting that fitness costs associated with resistance limit the proliferation of highly resistant isolates in the field. Billard et al. (2012) have shown that during cold weather, fenhexamid resistant strains may be displaced by other isolates, due to poor fitness under extreme conditions. However, the extent to which fenhexamid resistance and associated fitness costs affect competitiveness in the field is still unclear (Grabke et al. 2013).

*Botrytis pseudocinerea* is present in French gray mold populations at low rates and accounts for some fenhexamid resistance due to innate resistance (Walker et al. 2011). In German fruit and vegetable crops, *B. pseudocinerea* is a more significant contributor to gray mold development, but fungicide applications lead to its rapid displacement by *B. cinerea* (Plesken et al. 2015). The isolates we recovered with resistance to fenhexamid in germ tube elongation assays were

identified as *Botrytis cinerea* carrying different point mutations in the target site gene, *erg27*. These are HydR3 isolates, which represent the greatest disease control concern in the field (Grabke et al. 2013) While the *G3PDH* locus successfully separates *B. cinerea* and *B. pseudocinerea*, this locus does not clearly separate *B. cinerea* and *B. pelargonii* (Walker et al. 2011). Hence, it is possible that several isolates, particularly those isolated from ornamentals, could be *B. pelargonii.*

We also sequenced the *G3PDH* locus from a representative subset of the 53 isolates we recovered that showed resistance to fenhexamid in mycelial growth, but not during germination and germ tube elongation. All 19 isolates were identified as *B. cinerea*, indicating that these are HydR2 isolates, which are *B. cinerea* isolates that display reduced sensitivity to fenhexamid in mycelial growth, but do not carry resistance conferring point mutations in the *erg27* gene (Albertini and Leroux 2004). These strains do show some resistance to fenhexamid in germ tube growth, but only up to 0.4 µg/ml, as opposed to HydR3 strains, which can germinate and grow healthily at 10 µg/ml fenhexamid (Albertini and Leroux 2004; Weber and Hahn 2011). HydR2 strains are not thought to present disease control problem in the field because of this lack of resistance to fenhexamid during germination (Leroux et al. 2007). The absence of *B. pseudocinerea* in the fenhexamid-resistant subpopulations we identified suggests that this species is not a significant member of the gray mold complex affecting grapes, strawberries, and ornamentals in Virginia.

We observed several previously characterized amino acid changes in the HydR3 fenhexamid resistant isolates we recovered, F412S, F412I, and T63I. In one isolate carrying the F412I alteration, the amino acid substitution G108R was also identified. The F412S alteration is the most common mutation associated with fenhexamid resistance in France, Germany, Chile, and the Carolinas (Grabke et al. 2013). F412I mutation has been found in isolates from France, Germany and the Carolinas (Fillinger et al. 2008). These mutations are associated with HydR3+ phenotypes and confer a high degree of resistance that presents a serious control issue in the field (Fillinger et al. 2008). The T63I alteration was initially described in laboratory mutants, but was detected in field isolates from strawberry in the Carolinas (Grabke et al. 2013). While the function of the domain containing amino acid T63 is unknown, isolates carrying amino acid I63 behave like HydR3+ isolates in mycelial growth tests and present control concerns as a result (Grabke et al. 2013). The variety of different resistance genotypes we identified suggests that fenhexamid resistance has emerged independently at the locations we sampled.

The results of our detached grape assays are consistent with work performed with fenhexamid resistant isolates recovered from strawberries in the southeastern United States. Grabke et al. (2013) found that isolates carrying four different point mutations in the *erg27* gene, including F412S, F412I, and T63I, were not controlled by fenhexamid, while wild type isolates were inhibited. We observed significant differences in both disease incidence and severity (as inferred from surface lesion coverage on table grapes) when the growth of wild types isolates was compared with the growth of adapted isolates on fungicide treated table grapes The one isolate we evaluated carrying a novel mutation, G108R, in addition to the previously characterized F412S mutation, behaved similarly to other adapted isolates in *in vitro* experiments. The G108R substitution is novel in the *erg27* gene of *B. cinerea*, but results of the detached grape trial with fenhexamid suggest that this amino acid substitution does not have an effect on fenhexamid

resistance, because the growth on fenhexamid treated table grapes of the isolate carrying both F412I and G108R was not greater than the growth of the isolate carrying only F412I.

In conclusion, fenhexamid resistance is a growing threat in Virginia. While relatively low abundance suggests that selection for fenhexamid resistance is still in early stages for the *Botrytis* populations we surveyed, growers still need to be aware of this as a variable in spray program design and fungicide selection. The absence of *B. pseudocinerea* and the presence of Hydr3+-conferring point mutations suggest that if fenhexamid use in and around Virginia is not carefully monitored and fungicide resistance mitigation is not prioritized, a serious disease control threat could emerge.

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**Tables Chapter 3**

**Table 3.1.** Oligonucleotide primers used in this study.

| Name | Sequence (5'-3') | Target gene | Location in target gene (5'-3') | Reference |
|---|---|---|---|---|
| G3PDHfor | ATTGACATCGTCGCTGTCAACGA | *G3PDH* | 790–817[a] | 1 |
| G3PDHrev | ACCCCACTCGTTGTCGTACCA | *G3PGH* | 1769–1789[a] | 1 |
| Erg27Beg | TGGGATTACCACCATGGGAGACAAGTG | *erg27* | 2-28 | 2 |
| Erg27End | CAATGGTTCCGCATTTCTTTGCCTCCC | *erg27* | 1556-1582 | 2 |

[a]=base pair position based on homologous gene in *Sclerotinia sclerotiorum.*
1=Staats et al. 2005.
2=Fillinger et al. 2008.

**Table. 3.2.** Locations where fenhexamid-resistant isolates were recovered.

| Site | Location* | Host | N Fen-R | N |
|---|---|---|---|---|
| G8 | Fauquier County, VA | Grape | 5 | 41 |
| G11 | Surry County, NC | Grape | 1 | 4 |
| G17 | Surry County, NC | Grape | 2 | 12 |
| G31 | Nelson County, VA | Grape | 5 | 13 |
| G33 | Westmoreland County, VA | Grape | 1 | 6 |
| G37 | Rappahannock County, VA | Grape | 3 | 17 |
| G41 | Albemarle County, VA | Grape | 2 | 22 |
| G43 | Botetourt, VA | Grape | 1 | 14 |
| G48 | Nelson County, VA | Grape | 3 | 8 |
| G50 | Albemarle County, VA | Grape | 1 | 13 |
| O3 | Suffolk, VA | Titan Lilac | 6 | 13 |
| O6 | Waynesboro, VA | Basil, Pelargonium | 3 | 6 |
| O8 | Montgomery County, VA | Lavender, Helleborus, Gaura | 9 | 11 |
| O10 | Montgomery County, VA | Geranium | 4 | 4 |
| St1 | Floyd County, VA | Strawberry | 6 | 9 |
| St2 | Nelson County, VA | Strawberry | 7 | 22 |
| St3 | Virginia Beach, VA | Strawberry | 4 | 16 |
| St4 | Nelson County, VA | Strawberry | 1 | 4 |

*Locations not designated "County" are independent cities in VA, municipalities that are not politically part of a county, even if they are geographically surrounded by a county.

**Table 3.3.** Locations where isolates resistant to fenhexamid during mycelial growth, but not germination, were recovered.

| Site | Location | Host | N Fen-R (myc) | N |
|---|---|---|---|---|
| G2 | Albemarle Co., VA | Grape | 3 | 9 |
| G3 | Albemarle Co., VA | Grape | 2 | 23 |
| G8 | Warren Co., VA | Grape | 4 | 41 |
| G10 | Winchester, VA | Grape | 3 | 16 |
| G13 | Lynchburg, VA | Grape | 1 | 10 |
| G14 | Roanoke Co., VA | Grape | 3 | 10 |
| G15 | Rockbridge Co., VA | Grape | 1 | 11 |
| G17 | Surry Co., NC | Grape | 1 | 12 |
| G18 | Charlottesville, VA | Grape | 1 | 10 |
| G20 | Nelson Co., VA | Grape | 3 | 9 |
| G21 | Nelson Co., VA | Grape | 1 | 7 |
| G24 | Surry Co., NC | Grape | 1 | 8 |
| G29 | Montgomery Co., VA | Grape | 1 | 6 |
| G30 | Nelson Co., VA | Grape | 2 | 6 |
| G31 | Nelson Co., VA | Grape | 1 | 13 |
| G37 | Washington Co., VA | Grape | 1 | 17 |
| G41 | Charlottesville, VA | Grape | 1 | 22 |
| G43 | Botetourt Co., VA | Grape | 2 | 14 |
| G44 | Bedford Co., VA | Grape | 1 | 4 |
| G45 | Botetourt Co., VA | Grape | 1 | 11 |
| G46 | Nelson Co., VA | Grape | 2 | 7 |
| G47 | Nelson Co., VA | Grape | 2 | 4 |
| G48 | Nelson Co., VA | Grape | 2 | 8 |
| G50 | Albemarle Co., VA | Grape | 1 | 13 |
| O2 | Charlottesville, VA | Strawberry, Geranium | 1 | 22 |
| O5 | Westmoreland Co., VA | Rose | 1 | 3 |
| O6 | Augusta Co., VA | Geranium | 1 | 6 |
| O7 | Harrisonburg, VA | Lavender | 1 | 4 |
| O8 | Montgomery Co., VA | Lavender, Hellebore | 2 | 11 |
| O11 | Hanover Co., VA | Rose | 1 | 4 |
| St3 | Virginia Beach, VA | Strawberry | 2 | 16 |
| St4 | Nelson Co., VA | Strawberry | 1 | 4 |
| St5 | Rockingham Co., VA | Strawberry | 1 | 4 |

*Locations not designated "County" are independent cities in VA, municipalities that are not politically part of a county, even if they are geographically surrounded by a county.

**Table 3.4.** Amino acid substitutions in select fenhexamid resistant isolates collected in this study.

| Isolate | Site | Host | *Erg27* genotype |
|---|---|---|---|
| 178 | O10 | Geranium | F412S |
| 180 | O10 | Geranium | F412S |
| 181 | O10 | Geranium | F412S |
| 184 | G8 | Grape | F412S |
| 187 | St1 | Strawberry | F412S |
| 247 | O3 | Titan Lilac | F412S |
| 278 | G31 | Grape | F412S |
| 293 | G8 | Grape | F412S |
| 304 | G8 | Grape | F412S |
| 330 | O6 | Geranium | F412S |
| 332 | O6 | Geranium | F412S |
| 373 | G37 | Grape | F412S |
| 375 | G37 | Grape | F412S |
| 414 | G17 | Grape | F412S |
| 416 | St2 | Strawberry | F412S |
| 419 | St2 | Strawberry | F412S |
| 460 | G48 | Grape | F412S |
| 471 | G50 | Grape | F412S |
| 185 | G8 | Grape | F412I |
| 276 | G31 | Grape | F412I |
| 317 | St2 | Strawberry | F412I |
| 200 | St3 | Strawberry | T63I |
| 202 | St3 | Strawberry | T63I |
| 204 | St3 | Strawberry | T63I |
| 347 | O8 | Lavender | T63I |
| 319 | St2 | Strawberry | F412I, G108R |

**Table 3.5.** Control of wild-type and fenhexamid resistant isolates carrying different amino acid substitutions in the *erg27* gene on detached grapes treated with fenhexamid.

| | | ***In vivo**** | | | | | | ***In vivo*** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Lesion coverage (% of surface)** | | | | | | **Incidence (% diseased berries)** | | | | | |
| | | **4 dai** | | | | | | **4 dai** | | | | | |
| **Isolate** | **Erg27 genotype** | **Control** | | **Fenhexamid Low[a]** | | **Fenhexamid High[b]** | | **Control** | | **Fenhexamid Low[a]** | | **Fenhexamid High[b]** | |
| 43 | Wild type | 79.0 | abc | 9.5 | g | 0.5 | g | 100 | a | 60.0 | b | 10.0 | C |
| 71 | Wild type | 86.5 | abc | 6.5 | g | 1.0 | g | 100 | a | 60.0 | b | 10.0 | C |
| 330 | F412S | 79.0 | abcde | 79.0 | abcde | 68.5 | def | 100 | a | 100 | a | 100 | A |
| 317 | F412I | 92.0 | a | 81.5 | abcd | 64.0 | f | 100 | a | 100 | a | 100 | A |
| 347 | T63I | 81.0 | abcd | 80.5 | abcd | 73.5 | cdef | 100 | a | 100 | a | 100 | A |
| 319 | F412S, G108R | 87.5 | ab | 74.5 | bcdef | 67.0 | ef | 100 | a | 100 | a | 100 | A |

[*]= numbers in the same column followed by the same letter are not significantly different at α=0.05.

[a]= 1200 µg/ml fenhexamid

[b]= 3600 µg/ml fenhexamid

## Appendix

### Supplemental Tables

**Table S.1.** Isolates of *Botrytis* collected for this study.

| Site | Host | Location | Date | Number of isolates | |
|---|---|---|---|---|---|
| | | | | Berries/Fruit | Leaf/Blossom |
| G1 | Grape | Augusta Co., VA | 2011 | 3 | --- |
| G2 | Grape | Albemarle Co., VA | 2011, 2013 | 9 | --- |
| G3 | Grape | Albemarle Co., VA | 2014, 2015 | 12 | 11 |
| G4 | Grape | Fairfax Co., VA | 2011, 2013 | 5 | --- |
| G5 | Grape | Warren Co., VA | 2011 | 9 | --- |
| G6 | Grape | Warren Co., VA | 2011 | 9 | --- |
| G7 | Grape | Loudoun Co., VA | 2011 | 3 | --- |
| G8 | Grape | Warren Co., VA | 2011, 2013 | 37 | 3 |
| G9 | Grape | Shenandoah Co., VA | 2011 | 2 | --- |
| G10 | Grape | Winchester, VA | 2011 | 16 | --- |
| G11 | Grape | Surry Co., NC | 2011 | 4 | --- |
| G12 | Grape | Surry Co., NC | 2011 | 3 | --- |
| G13 | Grape | Lynchburg, VA | 2011 | 10 | --- |
| G14 | Grape | Roanoke Co., VA | 2011 | 10 | --- |
| G15 | Grape | Rockbridge Co., VA | 2011, 2013, 2015 | 11 | --- |
| G16 | Grape | Roanoke Co., VA | 2011 | 6 | --- |
| G17 | Grape | Surry Co., NC | 2011, 2012, 2014 | 12 | --- |
| G18 | Grape | Charlottesville, VA | 2011 | 10 | --- |
| G19 | Grape | Nelson Co., VA | 2011 | 4 | --- |
| G20 | Grape | Nelson Co., VA | 2011 | 9 | --- |
| G21 | Grape | Nelson Co., VA | 2011 | 7 | --- |
| G22 | Grape | Surry Co., NC | 2011 | 11 | --- |
| G23 | Grape | Franklin Co., VA | 2011 | 7 | --- |
| G24 | Grape | Surry Co., NC | 2011 | 8 | --- |
| G25 | Grape | Surry Co., NC | 2011 | 3 | --- |
| G26 | Grape | Shenandoah Co., VA | 2011 | 1 | --- |
| G27 | Grape | Fauquier Co., VA | 2012 | 6 | --- |
| G28 | Grape | Shenandoah Co., VA | 2012 | 2 | --- |
| G29 | Grape | Montgomery Co., VA | 2013 | 6 | --- |
| G30 | Grape | Nelson Co., VA | 2013 | --- | 6 |
| G31 | Grape | Nelson Co., VA | 2013, 2014 | 3 | 10 |
| G32 | Grape | Washington Co., VA | 2013 | 2 | --- |
| G33 | Grape | Westmoreland Co., VA | 2013 | 6 | --- |
| G34 | Grape | Northampton Co., VA | 2013 | 2 | --- |
| G35 | Grape | Surry Co., VA | 2014 | 1 | --- |
| G36 | Grape | Albemarle Co., VA | 2013 | 2 | --- |
| G37 | Grape | Washington Co., VA | 2013 | 17 | --- |

| | | | | | |
|---|---|---|---|---|---|
| G38 | Grape | Galax, VA | 2013 | 2 | --- |
| G39 | Grape | Wise Co., VA | 2013 | 3 | --- |
| G40 | Grape | Wythe Co., VA | 2013 | 4 | --- |
| G41 | Grape | Charlottesville, VA | 2014, 2015 | 13 | 9 |
| G42 | Grape | Charlottesville, VA | 2014 | 1 | --- |
| G43 | Grape | Botetourt Co., VA | 2014, 2015 | 2 | 11 |
| G44 | Grape | Bedford Co., VA | 2014, 2015 | 3 | 1 |
| G45 | Grape | Botetourt Co., VA | 2014, 2015 | 4 | 7 |
| G46 | Grape | Nelson Co., VA | 2014, 2015 | 2 | 5 |
| G47 | Grape | Nelson Co., VA | 2014, 2015 | 2 | 2 |
| G48 | Grape | Nelson Co., VA | 2014, 2015 | 3 | 5 |
| G49 | Grape | Albemarle Co., VA | 2014, 2015 | 4 | 4 |
| G50 | Grape | Albemarle Co., VA | 2014, 2015 | 8 | 5 |
| G51 | Grape | Amherst Co., VA | 2015 | --- | 3 |
| O1 | Rose | Staunton, VA | 2013 | --- | 2 |
| | Stevia | | | --- | 1 |
| O2 | Rose | Charlottesville, VA | 2013 | --- | 2 |
| | Pansy | | | --- | 2 |
| O3 | Lilac | Suffolk, VA | 2013 | --- | 13 |
| O4 | Basil | Hanover Co., VA | 2013 | --- | 3 |
| O5 | Rose | Westmoreland Co., VA | 2013 | --- | 3 |
| O6 | Basil | Augusta Co., VA | 2013 | --- | 2 |
| | Geranium | | | --- | 3 |
| | Lavender | | | --- | 1 |
| O7 | Lavender | Harrisonburg, VA | 2013 | --- | 4 |
| O8 | Astilbe | Montgomery Co., VA | 2013 | --- | 2 |
| | Beeblossom | | | --- | 2 |
| | Hellebore | | | --- | 2 |
| | Lavender | | | --- | 4 |
| | Heuchera | | | --- | 2 |
| O9 | Heucherella | Nelson Co., VA | | --- | 1 |
| O10 | Geranium | Montgomery Co., VA | 2012 | --- | 4 |
| O11 | Rose | Hanover Co., VA | 2013 | --- | 4 |
| O12 | Lettuce | Montgomery Co., VA | 2015 | --- | 1 |
| O13 | Titan Lilac | Nelson Co., VA | 2013 | --- | 2 |
| | Geranium | | | --- | 2 |
| St1 | Strawberry | Floyd Co., VA | 2012 | --- | 9 |
| St2 | Strawberry | Nelson Co., VA | 2013, 2014 | --- | 22 |
| St3 | Strawberry | Virginia Beach, VA | 2012 | 15 | --- |
| St4 | Strawberry | Nelson Co., VA | 2014 | 4 | --- |
| St5 | Strawberry | Rockingham Co., VA | 2014 | 3 | --- |

*Locations not designated “County (Co.)” are independent cities in VA, municipalities that are not politically part of a county, even if they are geographically surrounded by a county.

Table S.2. Results of germ tube elongation and 24-well plate bioassays.

| Active ingredient | Germ tube elongation* | 24-well plate* | Number |
|---|---|---|---|
| Thiophanate-methyl | +++ | +++ | 20 |
| | +++ | ++ | 4 |
| | - | - | 17 |
| Trifloxystrobin | +++ | +++ | 26 |
| | +++ | ++ | 4 |
| | - | - | 11 |
| Fenhexamid | +++ | +++ | 8 |
| | - | - | 33 |
| Boscalid | +++ | +++ | 24 |
| | ++ | ++ | 1 |
| | ++ | + | 1 |
| | - | - | 15 |
| Cyprodinil | +++ | +++ | 1 |
| | ++ | ++ | 16 |
| | ++ | + | 2 |
| | - | - | 22 |
| Iprodione | +++ | +++ | 3 |
| | ++ | ++ | 1 |
| | ++ | + | 1 |
| | + | + | 12 |
| | - | - | 24 |
| Fludioxonil | + | + | 1 |
| | - | - | 40 |

*(+++)=R in germ tube elongation assays, corresponding to 50-100% radial growth (as a % of growth on control) in 24-well plate assays; (++)=mR in germ tube elongation assays, corresponding to 20-50% radial growth (as a % of growth on control) in 24-well plate assays; (+)=ls in germ tube elongation assays, corresponding to up to 20% radial growth (as a % of growth on control) in 24-well plate assays; and (-)=s in germ tube elongation assays, corresponding to 0% radial growth (as a % of growth on control) in 24-well plate assays.

Table S.3. Isolates used in evaluation of baseline sensitivity of *Botrytis cinerea* to polyoxin-D.

| Isolate | Site | Host | Collected |
|---|---|---|---|
| 21 | G5 | Grape | 2011 |
| 23 | G5 | Grape | 2011 |
| 47 | G22 | Grape | 2011 |
| 208 | St3 | Strawberry | 2012 |
| 221 | G27 | Grape | 2012 |
| 235 | G10 | Grape | 2012 |
| 248 | O3 | Lilac | 2013 |
| 283 | G8 | Grape | 2013 |
| 334 | O7 | Lavender | 2013 |
| 335 | O7 | Lavender | 2013 |
| 371 | G37 | Grape | 2013 |
| 382 | G40 | Grape | 2013 |
| 393 | O1 | Rose | 2013 |
| 412 | G17 | Grape | 2014 |
| 416 | St2 | Strawberry | 2014 |
| 421 | St4 | Strawberry | 2014 |
| 445 | G42 | Grape | 2014 |
| 467 | G49 | Grape | 2014 |
| 469 | G49 | Grape | 2014 |
| 470 | G49 | Grape | 2014 |
| 488 | G41 | Grape | 2015 |
| 492 | G41 | Grape | 2015 |
| 500 | G43 | Grape | 2015 |
| 502 | G43 | Grape | 2015 |
| 510 | G3 | Grape | 2015 |
| 516 | G3 | Grape | 2015 |
| 519 | G48 | Grape | 2015 |
| 524 | G50 | Grape | 2015 |
| 530 | G49 | Grape | 2015 |