THE PREVALENCE AND MOLECULAR MECHANISMS OF SINGLE-SITE FUNGICIDE RESISTANCE IN THE APPLE SCAB PATHOGEN, *VENTURIA INAEQUALIS*

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ABSTRACT

Apple scab, caused by the fungal pathogen *Venturia inaequalis* is among the most prevalent and economically important diseases in commercial apple orchards in regions with temperate climates worldwide. The absence of durable apple scab resistance in the majority of commercially desired cultivars often mandates numerous fungicide applications each season to successfully manage the disease. Highly effective single-site fungicides have enhanced the ability to manage a number of phytopathogenic fungi, including *V. inaequalis*. Unfortunately the highly specific nature and repetitive use of these fungicides has led to their diminished efficacy and widespread resistance in *V. inaequalis*. The identification of the prevalence of single-site resistance in populations of *V. inaequalis* and an understanding of molecular mechanisms of resistance to these fungicides can aid in the rapid detection of fungicide resistance, the deployment of fungicide anti-resistance management strategies, and the development of efficient and effective chemical management programs for apple scab control.

In this dissertation, a combination of applied and basic research was implemented to gain further understanding of the molecular mechanisms associated with practical resistance to DMI and QoI fungicides in isolates and/or populations of *V. inaequalis*. Microscopy-aided mycelial growth assays were used to determine sensitivity phenotypes to the DMI fungicides myclobutanil and difenoconazole and to the QoI fungicide trifloxystrobin. The majority of populations of *V. inaequalis* collected throughout the Northeastern, Mid-Atlantic, and Mid-Western United States

were found to have reduced sensitivity or practical resistance to myclobutanil, but not difenoconazole. Illumina sequencing and reverse-transcriptase quantitative PCR were used to identify the contribution of target gene overexpression and the presence of upstream repeated elements in myclobutanil and difenoconazole resistance. Another molecular technique, allele-specific quantitative PCR was used to evaluate the role of target gene heteroplasmy in the quantitative resistance response and practical resistance to trifloxystrobin in isolates and populations of *V. inaequalis*. Additionally, baseline sensitivity of *V. inaequalis* to three SDHI fungicides was determined, and the *sdhB* gene, a target for SDHI fungicides, was identified and characterized. In summary, the results of this dissertation contribute to the identification and understanding of mechanisms associated with single-site fungicide resistance in *V. inaequalis.*

BIOGRAPHICAL SKETCH

Sara Michelle Villani was born on April 17, 1983 to Constance Villani and the late Dr. Michael G. Villani. She has a younger sister, Kate Villani and a wonderful Soft Coated Wheaten Terrier, Allie, with whom she's been able to share her entire graduate experience.

Sara's interest in applied agricultural research began in 1999 in the entomology laboratory of Dr. Greg Loeb at the Cornell University New York State Agricultural Experiment Station in Geneva, NY. As a summer research technician for seven years in Dr. Loeb's program, Sara had the opportunity to become involved in several projects focusing on insect pests of grapes and small fruit, including the implementation of tydeid mites, *Orthotydeus lambi,* as biological control agents of grape powdery mildew. After earning a B. Sci. in Chemistry from the State University of New York at Geneseo in 2005, Sara joined the plant pathology program of Dr. Herb Aldwinckle at the New York State Agricultural Experiment Station as a research technician and assisted on research of the fire blight pathogen, *Erwinia amylovora*.

Sara joined the Cornell University Tree Fruit and Berry Pathology program led by Dr. Kerik Cox in 2007, where she has continued to conduct research to this day. After conducting research on fungicide resistance in brown rot and apple scab and presenting results at regional and national research and extension meetings with Dr. Cox, Sara decided pursue her doctorate in plant pathology at Cornell University as part of the Employee Degree Program in 2012. Her research goals included investigating the prevalence of single-site fungicide resistance in the apple scab pathogen *Venturia inaequalis,* and gaining better understanding to the molecular mechanisms governing this resistance. In December 2015, Sara will become a member of the Plant Pathology faculty at North Carolina State University.

DEDICATION

In memory of my father:

Your guidance, encouragement, generosity, and love are always with me.

To laugh often and love much;
To win the respect of intelligent people and the affection of children;
To earn the approval of honest critics and endure the betrayal of false friends;
To appreciate beauty;
To find the best in others;
To give of one's self;
To leave the world a bit better, whether by a healthy child, a garden patch, or a redeemed social condition;
To have played and laughed with enthusiasm and sung with exultation;
To know even one life has breathed easier because you have lived…
This is to have succeeded.

-Ralph Waldo Emerson

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CHAPTER 1

INTRODUCTION

Background: The New York Apple Industry, Overview of *Venturia inaequalis* Biology and Disease Development, and Management of Apple Scab

Apple (*Malus x domestica*) is the most economically important fruit crop in the northeastern and mid-Atlantic regions of the United States. Currently, New York is second to Washington State in national apple production with a total yield of 36,300 kg of total harvest per hectare, valued at over 240 million dollars in 2014 (USDA National Agricultural Statistics Service, 2015). Of the total utilized crop, slightly more than 50% goes directly to the fresh market while the remaining crop is destined for processing (New York Apple Association, 2015). Across the state, apples are grown commercially on 16,100 hectares (USDA National Agricultural Statistics Service, 2015) in primarily five distinct growing regions: the Niagara frontier, Champlain Valley, Hudson Valley, Central NY, and the Finger Lakes. Over the past decade, the adaptation of innovative horticultural practices including high density training systems and dwarfing rootstocks (Gregory et al., 2013; Robinson, 2008; Robinson et al., 2013) in addition to progressive disease and insect management programs, has kept New York competitive with the top other states in regards to apple production in profit.

Apple scab, caused by *Venturia inaequalis* (Cooke) G. Winter, is among the most economically important and intensively managed fungal disease of apples in the temperate growing regions worldwide including the northeastern and mid-Atlantic United States (Holb et

al, 2003; MacHardy, 1996; MacHardy et al, 2001). The pathogen's ability to infect and cause extensive damage to both foliar and fruit tissue can drastically reduce crop value or lead to complete losses during the current season if not adequately managed (Holb et al, 2003, MacHardy, 1996, Roberts 1935). Additionally, during severe epidemics, apple bud scales may become infected (Becker et al., 1992) and/or conidia may overwinter in buds (Becker et al., 1992), thereby increasing risks of infection the following spring. Furthermore, severe infection can reduce return bloom, thereby reducing fruit quality and value in subsequent years (Curtis, 1924; Holb, 2007).

Epidemics of apple scab are initiated during the spring when conditions are conducive to infection, or when weather remains cool (< 30 ºC) and substantial wetting events (> 6 h of leaf wetness) occur (MacHardy and Gadoury, 1989). Due to high temperatures in the summer and the onset of ontogenic resistance in both leaves and fruit, infection by *V. inaequalis* later in the season is not common. However, epidemics resulting from secondary infection by conidia can occur during extended cool, humid weather (Holb et al., 2003).

Leaves infected with apples scab overwinter along the orchard floor and serve as the primary source of inoculum in the spring (Holb, 2007, MacHardy, 1996). Pseudothecia develop during the late winter/early spring from loculostromata (stromatic spheres) (MacHardy et al., 2001) that form shortly after leaf abscission during the preceding season. Interestingly, the pseudothecia have the ability to orient themselves in a negative-geotropic position such that mature ascopores are ejected upwards towards the atmosphere, thereby improving probabilities that they will be carried by air currents into young tissue within the tree canopy (Gadoury and MacHardy, 1985). The release of ascopores and subsequent infection commences during a wetting period (rain event) shortly after susceptible tissue is exposed (Gadoury et al., 2004;

MacHardy, 1996) during bud-break (green-tip). Water travels into the pseudothecia through the porous walls of the ascocarp or through the ostiole and reaches the asci through osmosis (Aylor, 1998). Mature ascospores swell and protrude through the ostiole until they are ejected (Gadoury et al., 1992). This ejection period, which results in primary infection of leaf and fruit tissue, can last from six to twelve weeks, and is influenced greatly by temperatures, relative humidity, light exposure, and leaf degradation rate (due primarily to increasing temperatures) (Aylor, 1996; Brooke, 1969; Gadoury et al, 2004,). While primary apple scab infections often result from infection by ascospores, in orchards with high levels of inoculum, conidia overwintering on and within infected apple buds also serve as a source of primary apple scab inoculum in the spring (Becker et al., 1992).

Symptoms of apple scab infection are initially observed as olive-brown colored lesions appearing on cluster/spur leaves, terminal leaves, and on immature fruit. As the season progresses, secondary infections by conidia of *V. inaequalis* may be initiated in the absence of effective management programs (MacHardy, 1996). The consequences of severe infections can have adverse impacts on the apple crop for both the current and following season. In cases of severe leaf infection, premature defoliation will cause a decrease in photosynthesis and may lead to reduced development of flower buds for the following year. Besides the negative implications of reduced photosynthesis on fruit quality (Lakso and Goffinet, 2013), fruit infections by *V. inaequalis* can impact size, taste, and premature fruit drop (MacHardy, 1996). Furthermore, the cracking of even the smallest fruit lesions can initiate infection of fruit rot pathogens including *Botryospherea* spp. and *Colletotrichum* spp. (Roberts, 1935).

Reduction in apple scab infections can be accomplished through the incorporation of cultural methods, the deployment of resistant cultivars, and by model-based or phenology-based

fungicide applications. Traditionally, cultural methods have focused on the reduction of primary apple scab inoculum on overwintering leaves. Early studies demonstrated that primary inoculum could be reduced 62 to 84% when fallen leaves were burned, plowed, or removed separately from the orchard (Curtis, 1924; Keitt, 1936; Louw, 1948). Consumer interest in minimal chemical inputs and the increasing number of organic orchards has prompted newer studies for the cultural management of inoculum reduction. The shredding of leaf litter using a flail mower has been utilized in an attempt to increase the rate of leaf degradation by microflora and to redirect ascospore release angle. The success of this method has been variable, however, with reduction in ascopore inoculum and apple scab incidence ranging from 40 to 95% and 45 to 85%, respectively (Sutton et al., 2000). Heavy pruning during the winter and foil coverage of the orchard floor in late winter were also found to significantly reduce apple scab incidence on moderately susceptible cultivars (Holb, 2005; Holb, 2007).

Establishing orchards with cultivars resistant to *Venturia inaequalis* is another management strategy for the control of apple scab. Indeed, cultivars with varying levels of susceptibility to the pathogen have been bred and integrated throughout modern U.S. apple orchards (Biggs et al., 2010). Qualitative, or monogenic, resistance to *V. inaequalis* has been conferred through the introduction of the V_f resistance gene isolated from *Malus floribunda* 821 (Gessler and Pertot, 2012). Unfortunately, after fifty years of durable resistance the V_f resistance gene has been overcome in Europe by virulent pathotypes of *V. inaequalis* (Gessler and Pertot, 2012; Parisi et al., 1993; Parisi et al., 2000) Currently, however, wide-spread breakdown of V_f resistance has yet to be confirmed in the United States (Beckerman et al., 2009; Gessler and Pertot, 2012). Regardless of the potential for host resistance breakdown, the widespread implementation of apple-scab resistant cultivars into commercial production has additional

challenges. In addition to apple scab, diseases such as apple powdery mildew caused by *Podosphaera leucotricha*, and cedar apple rust caused by *Gymnosporangium virginiana* can also result in extensive losses and must also be considered in temperate growing regions (Rosenberger, 2003). The preferential selection of horticultural attributes of fruit desirable to consumers over other traits has also caused setbacks in the development of resistant cultivars (Brown and Maloney, 2008).

The inconsistent success of cultural practices and the absence of durable apple scab resistance in the majority of commercially desired cultivars, mandates the application of fungicides for the management of apple scab. In orchards with a high inoculum potential and susceptible cultivars, the application of fungicides to control apple scab is often initiated at silver tip and targets the primary and secondary infection cycles throughout the spring and summer until harvest in the fall (MacHardy, 1996). Successful management of apple scab during the primary infection period is critical to preventing secondary infections and late-season epidemics. At silver tip, copper compounds are commonly applied for the reduction of conidia overwintering in or on apple buds (Frederick et al, 2015) and also reduce viability of *Erwinia amylovora* populations released from overwintering in cankers (Norelli et al., 2003). Management of apple scab between the phenological stages of green tip and tight-cluster is often accomplished with the multi-site protectant fungicides, captan (FRAC code M4, phthalimides) or mancozeb (FRAC code, dithiocarbamates). Recently, the single-site fungicide dodine has provided an additional alternative for early-season apple scab management, as widespread resistance in populations of *V. inaequalis* is no longer present (Cox et al., 2010).

A combination of the aforementioned protectant fungicides (i.e., mancozeb and captan) and highly effect single-site fungicides such as the sterile demethylation inhibitors (DMIs,

1980s), the quinone-outside inhibitors (QoIs, 1990s), and the recently registered succinate dehydrogenase inhibitors (SDHIs) are currently applied on a seven to ten day interval to manage primary infection by *V. inaequalis* between the phenological stages of pink and second cover. The use of modern single-site fungicide chemistries has enabled apple producers to manage apple scab and many other key fungal diseases prior to and following infection, while minimizing harmful effects on non-target organisms and the environment (Köller, 1999; Köller, 2003; Köller et al., 1997; Köller et al, 2004; Schnabel et al., 2004). Unfortunately, because these fungicides are single-site inhibitors (i.e.,they block a single biochemical pathway in fungi), repetitive use selected for the emergence of resistant *V. inaequalis* populations in the apple production regions east of the Mississippi throughout the last half of the 20th century. An understanding of the relative efficacy of fungicides with similar modes of action against *V. inaequalis*, the prevalence of single site fungicide resistance in populations of *V. inaequalis,* and an understanding the molecular basis for single-site fungicide resistance in *V. inaequalis* are crucial to the development of and preservation of apple scab management programs throughout the northeastern and mid-Atlantic United States.

The Prevalence of Sterile Demethylation Inhibitor Fungicide Resistance in Populations of *Venturia inaequalis.*

The DMI fungicides are a class of broad spectrum, systemic compounds that are highly effective against several plant pathogenic fungi, including yeasts, and the fungi that cause apple scab, rusts, and powdery mildews (Schnabel and Jones, 2001). Members within the DMI fungicide class target the C14-demethylase enzyme encoded by the fungal *CYP51* gene. Specifically, these compounds disrupt ergosterol biosynthesis by inhibiting demethylation at the

14-α carbon of lanosterol, a sterol intermediate of ergosterol, which is essential for hyphal growth (Köller and Scheinpflug, 1987). Although DMI fungicides have activity as protective fungicides, historically they were most effective when used following infection events (Köller and Wilcox, 1999; O'Leary et al., 1987; Schwabe et al., 1984; Szkolnik, 1981) due their ability to suspend hyphal elongation. Indeed, more than 25 years after their introduction for control in commercial apple orchards in the United States, many DMI fungicides continue to demonstrate curative activity up to 96 hours following an infection event (Beckerman et al., 2014).

While DMI fungicides are considered a medium risk for resistance by the fungicide resistance action committee (Stenzel, 2014), the highly specific nature and repetitive use of DMI fungicides has led to their diminished efficacy and widespread resistance in key apple production regions in North America (Braun and McRae, 1992; Hildebrand et al., 1988; Jobin and Carisse, 2007; Köller et al., 1997). Practical resistance, defined as greatly diminished levels of disease control following proper fungicide application under field conditions (Köller, 1991), was first reported for DMIs in populations of *V. inaequalis* collected from a research orchard in Nova Scotia (Hildebrand et al., 1988) and later in a commercial orchard in Michigan (Köller et al., 1997). Since the initial reports, DMI fungicide resistance in populations of *V. inaequalis* has been documented in Virginia (Marine et al., 2007), Pennsylvania (Pfeufer and Ngugi, 2012) and Indiana (Chapman et al., 2011). While individual isolates of *V. inaequalis* collected from NY orchards have previously been found to be resistant to the DMI fungicide fenarimol (Köller and Wilcox, 1999), practical resistance to DMI fungicides in populations of *V. inaequalis* from the state has not been documented.

Since 2004, the increasing inability of the DMI fungicide, myclobutanil, and other DMIs to provide acceptable levels of apple scab control has forced growers to modify their disease

management programs. With limited single-site fungicide alternatives, producers are increasingly relying on multi-site protectant fungicides (i.e., captan and mancozeb) to manage apple scab. Protectant fungicides must be applied more frequently, have little to no post-infection activity, and are relatively ineffective (in comparison to DMIs) in controlling other important apple diseases such as cedar apple rust and powdery mildew (Köller and Wilcox, 1999).

In 2009, several DMI fungicides marketed previously in Europe were registered for management of apple scab in North America. These fungicides included difenoconazole and fenbuconazole, which have been found to have higher intrinsic activity against *V. inaequalis* compared to myclobutanil (Pfeufer and Ngugi, 2012). Because of the increasing occurrences of DMI control failures, in addition to the registration of DMI fungicides for apple scab management, we initiated an investigation into DMI fungicide resistance in *V. inaequalis* populations. Objectives of this research were to (i) determine the extent of myclobutanil resistance in orchard populations of *V. inaequalis* across the mid-Atlantic, the Midwest, and the northeastern U.S. over a ten-year period, (ii) establish baseline in vitro sensitivities for the DMI fungicide difenoconazole in *V. inaequalis*, (iii) evaluate in vitro difenoconazole sensitivity for populations of *V. inaequalis* with practical resistance or sensitivity to myclobutanil, and (iv) determine the effect of myclobutanil or difenoconazole applications in monitored populations of *V. inaequalis* with practical resistance or sensitivity to myclobutanil. This study contributed to the current understanding of DMI fungicide resistance in *V. inaequalis* and provided insight into cross-sensitivity between the DMI fungicides myclobutanil and difenoconazole. Results and conclusions from this study provided insight into the successful deployment of "new generation" DMI fungicides in DMI-resistant populations in *V. inaequalis* and hopefully contributed to preservation of their efficacy in the field.

Molecular mechanisms of resistance to DMI fungicides in *Venturia inaequalis*

Varying degrees of intrinsic activity have historically been observed among the DMI fungicides (Hsiang et al., 1997; Karaoglandis and Thanassoulopoulos et al., 2003; Köller et al., 1991). While cross sensitivity and cross-resistance between DMIs have been extensively documented (Karaoglandis and Thanassoulopoulos et al., 2003; Köller et al., 1991) it is not always guaranteed (Kendall et al., 1993; Peever and Milgroom, 1993; Robbertse et al., 2001). Results to be discussed in a later chapter (Chapter 2), show that cross-sensitivity between myclobutanil and difenoconazole is present in baseline populations, but not myclobutanil-resistant populations of *V. inaequalis* (Villani et al., 2015). The absence of cross-resistance between DMI fungicides could be explained by a number of factors including differences in orchard microclimate, inherent differences in population composition (Xu et al., 2010), type of management programs used, or differences in the genetic components involved in resistance development.

The molecular basis of resistance to the DMI fungicides has been extensively studied in both human and plant fungal pathogens, including *V. inaequalis* (Cools et al., 2013; Hamamoto et al., 2000; Hulvey et al., 2012; Luo and Schnabel, 2008; Lupetti et al., 2002; Ma et al., 2006; Schnabel and Jones, 2001). Common mechanisms of resistance include: i) alterations to the coding sequence of the target gene resulting from point mutations (Asai et al., 1999; Becher et al., 2012; Délye et al., 1997; Stammler et al., 2009) reducing the binding affinity of DMI fungicides; ii) overexpression of energy dependent ABC and MFS transporters encoding drug efflux pumps (Hayashi et al., 2003; Hulvey et al, 2012; Nakaune et al. 1998) and iii) overexpression of the *CYP51A1* gene (Hamamoto et al., 2000; Ma et al., 2006; Schnabel and Jones, 2001). Overexpression of the *CYP51A1* gene in phytopathogenic fungi has been attributed

to insertions and repeated elements acting as transcriptional enhancers or to gene duplications increasing the copy number of the *CYP51A1* gene (Hawkins et al., 2011). In isolates of *V. inaequalis* from Michigan, overexpression of the target gene has been previously determined to be a mechanism of resistance to myclobutanil (Schnabel and Jones, 2001). While a DNA insertion of upstream of the target gene was implicated in promoting overexpression, this insertion was not always present in myclobutanil-resistant and/or high expressing isolates, indicating other mechanisms could be responsible for resistance to the fungicide.

The widespread adaptation of difenoconazole into apple scab management programs in conjunction with a lack of cross-sensitivity between myclobutanil and difenoconazole in isolates of *V. inaequalis* resistant to myclobutanil (Villani et al., 2015) prompted us to reexamine the role of *CYP51A1* in DMI fungicide resistance and determine if different molecular mechanisms for resistance were present. To the knowledge of the coauthors of this study, there have not been any prior reports of a single plant pathogen utilizing different resistant mechanisms for different azole DMI fungicides. Specifically the objectives of this study were to i) evaluate the relative expression of the *V. inaequalis CYP51A1* gene and the presence of mutations within the coding sequence of the *CYP51A1* gene for isolates with varying sensitivity phenotypes to myclobutanil and difenoconazole, and ii) conduct traditional Sanger sequencing and Illumina sequencing with *de novo* assembly to identify upstream anomalies associated with *CYP51A1* overexpression and/or resistance to either DMI fungicide. The results from this study should enable selection of specific DMI fungicides for individual orchard populations of *V. inaequalis* and should provide a rapid molecular screening method for DMI fungicide resistance in the pathogen.

Heteroplasmy of the cytochrome b gene in *Venturia inaequalis* its involvement in QoI fungicide resistance.

The quinone outside inhibitor (QoI) fungicides are another class of single-site fungicides that exhibit high levels of activity against *V. inaequalis.* While the QoIs are labeled for protective and curative control of *V. inaequalis*, their efficacy is generally higher when applied prior to an infection event, because of their ability to inhibit conidial germination. QoI fungicides inhibit mitochondrial respiration by binding to the outer quinone oxidizing pocket of the cytochrome bc_1 enzyme complex III. As a group, the QoIs specifically target the Q_o site within the cytochrome b protein that is encoded by the mitochondrial *cyt b* gene (Zheng and Köller, 1997). This site is integral to electron transport within the mitochondrion and the eventual production of ATP. The binding of a QoI fungicide inhibits electron transfer in the respiratory pathway, which terminates cellular respiration and thus the production of ATP (Bartlett et al., 2002; Gisi et al., 2002; Köller, 1999). Similar to other systemic fungicides, the site-specific nature of QoIs predispose them to rapid resistance development. Shortly after their introduction, isolates of *V. inaequalis* with QoI resistance were discovered in an orchard in Michigan (Köller et al., 2004) and by 2009 widespread resistance was documented in *V. inaequalis* populations from commercial apple orchards in Michigan (Chapman et al., 2011; Lesniak et al., 2011). Recently, an extensive survey of 120 orchard populations of *V. inaequalis* in major apple production regions throughout the northeastern U.S., confirmed resistance to the QoI fungicide, trifloxystrobin in 28% of the populations (Frederick et al., 2014).

The most common mechanism of QoI resistance described for fungal pathogens, including *V. inaequalis*, is a single point mutation at amino acid position 143 within the mitochondrial cytochrome b (*cyt b)* gene (Cox et al., 2008; Fiaccadori et al., 2011; Köller et al.,

2004; Lesniak et al., 2011; Olaya et al., 1998; Zheng and Köller, 1997; Zheng et al., 2000). This mutation results in a change from glycine to alanine (G143A) and imparts a qualitative, vertical, or complete resistance response whereby isolates are highly resistant to QoI fungicides (Köller et al., 2004). Other point mutations within the *cyt b* gene at amino acid position 129 where phenylalanine is exchanged with leucine (F129L) and at position 137 where glycine is replaced by arginine (G137R) have been shown to confer diminished QoI sensitivity in fungal pathosystems (Gisi et al., 2002; Karaoglanidis et al., 2011; Kim et al., 2003; Pasche et al., 2005; Sierotzki et al., 2007; Sierotzki et al., 2000). However, none of these or other mutations have been reported in *V. inaequalis* (Fiaccadori et al., 2011; Lesniak et al., 2011). In addition to the qualitative resistance response, there appears to be a quantitative (polygenic or partial) resistance response to QoI fungicides (Köller et al., 2004) whereby isolates display a range of sensitivity phenotypes when exposed to doses of QoIs below the point at which the alternative respiration pathway becomes activated (Färber et al., 2002; Olaya et al., 1998).

Although quantitative responses are typically explained by the involvement of several genes with minor contributions to an overall phenotype, differences in the ratios of mutant (resistant, A143) to wild type (sensitive, G143) copies of the *cyt b* gene could also account for the way that *V. inaequalis* isolates within a population express quantitative resistance to trifloxystrobin. Populations with varying ratios of A143 to G143 are described as having heteroplasmy of *cyt b*, and this phenomenon has been described for several fungal pathogens, including *V. inaequalis* (Chapman et al., 2003; Fraaije et al., 2002; Kim et al., 2003; Lesemann et al., 2006; Lesniak et al., 2011; Ma and Michailides, 2004). However, none of these studies have addressed the contribution of *cyt b* heteroplasmy to the quantitative resistance response for individual isolates

or attempted to relate the abundance of the A143 allele in a field population to the development of practical resistance to QoI fungicides.

We suspected that quantification of the relative amounts of the *cyt b* A143 allele might help explain the apparent quantitative resistance responses of *V. inaequalis* isolates to QoI fungicides in vitro (Köller et al., 2004) and that quantifying the relative abundance of the A143 allele in an orchard population might improve our understanding of the development of practical resistance, which is defined as the point within the quantitative resistance continuum where fungicide applications result in greatly diminished levels of disease control under field conditions (Köller, 1991). Thus, the overall goal of this study was to develop a better understanding of the role of *cyt b* gene heteroplasmy in resistance to QoI fungicides for isolates and populations of the apple scab pathogen *V. inaequalis*. Specifically, the objectives were to i) use AS-qPCR to detect and quantify *cyt b* heteroplasmy in monoconidial isolates, to determine the contribution of the A143 allele to the quantitative resistance response to trifloxystrobin, and ii) conduct field trials and surveys in conjunction with *cyt b* AS-qPCR to learn how qualitative resistance responses are involved in the development of practical resistance for orchard populations of *V. inaequalis*. Results from this study should provide new approaches for monitoring the development of practical resistance to QoI fungicides in populations of *V. inaequalis* and assist in identifying at risk for practical resistance to QoI fungicides.

Baseline Sensitivity to New Generation SDHI Fungicides and Characterization of the sdhB gene in *V. inaequalis*.

The SDHI fungicides comprise a diverse group of compounds that belong to eight distinct chemical groups. While structurally diverse (Sierotzki and Scalliet, 2013), all target the

succinate dehydrogenase reductase enzyme, a component of complex II in the mitochondrial electron transport chain. The target enzyme contains four nuclear-encoded subunits: SDHA, SDHB, SDHC, and SDHD, encoded by the nuclear *sdhA*, *sdhB*, *sdhC*, and *sdh*D genes, respectively. However all SDHIs currently labeled for crop protection target the ubiquinone-binding pocket, defined by the interface between the SDHB, SDHC, and SDHD subunits (Cecchini, 2003).

As resistance to sterile demethylation inhibitor (DMI) and quinone outside inhibitor (QoI) fungicides in populations of *V. inaequalis* (Köller et al., 1997; Köller et al., 2004) becomes more prevalent throughout major apple production regions in the northeastern and the mid-Atlantic United States (Köller et al., 1997; Köller et al., 2004; Frederick et al, 2014; Villani et al., 2015) integration of new generation SDHI compounds into apple scab management programs will be increasingly important. Similar to the QoI fungicides, (Fernández-Ortuño et al., 2008; Köller et al., 2004), the highly specific mode of action of SDHI fungicides makes them particularly prone to resistance development. Indeed, resistance to one or more SDHI fungicides has been documented in individual field isolates or field populations of several plant pathogenic fungi including *Alternaria alternata* (Avenot et al., 2014), *Botrytis cinerea* (Amiri et al., 2014; Veloukas et al., 2013), *Podosphaera xanthii* (Ishii et al., 2011) and *Didymella bryoniae* (Avenot et al., 2012). Several mutations within the *sdhB, sdhC,* and *sdhD* genes have been associated with losses in SDHI fungicide sensitivity in all of these instances. Most frequently, a mutation of the *sdhB* gene within the fungicide-binding pocket results in the substitution of a highly conserved histidine (H) residue with tyrosine (Y), leucine (L), or arginine (R). Due to the size variation among *sdhB* genes and differences in the substituting residue, this mutation may be denoted differently in fungal species such as H278Y/R (Walker et al., 2011), H272Y/R/L (Amiri et al.,

2014), or H277Y/R/L (Stammler et al., 2011). However other mutations at conserved amino acid sites within the *sdhB* gene have been reported.

Interestingly, substitution of one or more conserved amino acids may only impart resistance or partial resistance to some SDHI fungicides depending on the pathogen, the substituting residue, and the chemical group of the SDHI fungicide (Avenot et al., 2014; Sierotzki and Scalliet, 2013). Because of this highly specific nature of reduced sensitivity or resistance to SDHI fungicides and the growing importance of SDHIs in apple scab management programs, the identification of the target *sdhB* gene in *V. inaequalis* and the evaluation of baseline sensitivities and relative efficacy of SDHI fungicides is warranted. Specifically, the objectives of this study were to i) establish baseline sensitivities of isolates of *V. inaequalis* to SDHI fungicides ii) identify and characterize the *sdhB* gene in *V. inaequalis*, and iii) determine if mutations in the *V. inaequalis sdhB* gene are responsible for apple scab lesion development following applications of different SDHI fungicides. Results from this study provide both an insight into the most efficacious SDHI chemistries for the control of apple scab, and a baseline for monitoring population shifts towards resistance.

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CHAPTER 2

THE PREVALENCE OF MYCLOBUTANIL RESISTANCE AND DIFENOCONAZOLE SENSITIVITY IN POPULATIONS OF *VENTURIA INAEQUALIS*

This chapter has been modified from a publication referenced: Villani, S. M., Biggs, A. L., Cooley, D. R., Raes, J. J., and Cox, K. D. 2015. Prevalence of myclobutanil resistance and difenoconazole insensitivity in populations of *Venturia inaequalis*. Plant Dis. Advance online publication. http://dx.doi.org/10.1094/PDIS-01-15-0002-RE.

ABSTRACT

Demethylation inhibitors (DMI) are a class of single-site fungicides with high levels of protective and curative efficacy against *Venturia inaequalis*, the causal agent of apple scab. To determine the prevalence of resistance to the DMI fungicide myclobutanil, a total of 3,987 single conidial lesion isolates of *V. inaequalis* from 141 commercial, research, and baseline orchard populations were examined throughout New England, the mid-Atlantic, and the Midwest, from 2004 to 2013. Of these orchard populations, 63% had practical resistance, 13% had reduced sensitivity, and 24% were sensitive to myclobutanil. A sensitivity baseline for the recently introduced DMI fungicide, difenoconazole, was established to make comparisons with myclobutanil sensitivity in orchard populations. The mean effective concentration of difenoconazole at which mycelial growth was inhibited by 50% (EC_{50}) was determined to be 0.002 μg ml $^{-1}$ for 44 baseline isolates of *V. inaequalis.* From 2010 to 2013, 1,012 isolates of *V. inaequalis* from 37 of the 141 orchard populations above were screened for sensitivity to

difenoconazole. One orchard population had reduced sensitivity to difenoconazole, while the remaining 36 orchard populations were sensitive to the fungicide. In field experiments, difenoconazole demonstrated high levels of apple scab control on mature apple fruit, despite the fact that the population of *V. inaequalis* had practical resistance to myclobutanil. While our results indicate widespread resistance to myclobutanil, but not difenoconazole, due to the propensity for cross-sensitivity among DMI fungicides, growers with myclobutanil resistance should be cautious when using difenoconazole for disease management.

INTRODUCTION

Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cooke) G. Winter, is among the most prevalent and economically important diseases in commercial apple orchards in regions with temperate climates worldwide (MacHardy, 1996; MacHardy et al., 2001). The absence of durable apple scab resistance in the majority of commercially desired cultivars often mandates more than ten fungicide applications each season to successfully manage the disease (Köller and Wilcox, 2001; Lesniak et al., 2011; MacHardy, 1996). Highly effective single-site fungicides such as the guanidines (1950s), sterile demethylation inhibitors (DMIs, 1980s) and quinone outside inhibitors (QoIs, 1990s) have enhanced our ability to manage a number of phytopathogenic fungi, including *V. inaequalis.* Of these fungicides, the DMIs have been especially attractive to growers because of their strong pre- and post-infection activity. Even ten years after their introduction for use in commercial apple orchards in the United States, DMI fungicides had curative activity up to 96 hours following an infection event (Beckerman et al., 2014).

DMI fungicides are a class of broad spectrum, systemic compounds with high levels of activity against several plant pathogenic fungi, including those that cause apple scab, rusts, and powdery mildews (Schnabel and Jones, 2001). Historically, the DMIs were most effective when used following infection events (Köller and Wilcox, 1999; O'Leary et al., 1987; Schwabe et al., 1984; Szkolnik, 1981), as they arrest fungal growth during and after infection by inhibiting demethylation at the 14-α carbon of lanosterol, a sterol intermediate of ergosterol, which is essential for hyphal growth. While DMI fungicides are considered a medium risk for resistance by the fungicide resistance action committee (Stenzel, 2014), the highly specific nature and repetitive use of DMI fungicides has led to their diminished efficacy and widespread resistance

in key apple production regions in North America (Braun and McRae, 1992; Hildebrand et al., 1988; Jobin and Carisse, 2007; Köller et al., 1997; Villani and Cox, 2011).

In the history of apple scab management in the United States, the loss of one fungicide class due to resistance concerns has led to heavy reliance on the most recently registered alternative single-site fungicide class. For example, resistance to dodine by the 1970s was directly followed by dependence on and subsequent rapid onset of resistance to benzimidazoles (Jones, 1981). Subsequently, the DMI fungicides and the more recently registered QoI fungicides became the industry standard for both protective and post-infection management of apple scab. As early as 1985, roughly ten years after DMI fungicides were first applied commercially, individual *V. inaequalis* isolates were found to have reduced sensitivity to eight DMI fungicides (Stanis and Jones, 1985).

Practical resistance, defined as greatly diminished levels of disease control following proper fungicide application under field conditions (Köller, 1991; Villani and Cox, 2014), was first reported for DMIs in populations of *V. inaequalis* collected from a research orchard in Nova Scotia (Hildebrand et al., 1988) and later in a commercial orchard in Michigan (Köller et al., 1997). Since the initial reports, DMI fungicide resistance in populations of *V. inaequalis* has been documented in Virginia (Marine et al., 2007), Pennsylvania (Pfeufer and Ngugi, 2012) and Indiana (Chapman et al., 2011). While individual isolates of *V. inaequalis* collected from NY orchards have previously been found to be resistant to the DMI fungicide fenarimol (Köller and Wilcox, 1999), practical resistance to more heavily relied upon DMI fungicides in populations of *V. inaequalis* from the state has not been documented.

Since 2004, the authors have received increasing reports of practical resistance to myclobutanil (Nova 40W/Rally 40WSP) and fenarimol (Rubigan/Vintage SC) from apple growers in the mid-Atlantic and Northeastern U.S. The failure of myclobutanil and other DMIs to provide acceptable levels of apple scab control has forced growers to modify their disease management programs. With limited single-site fungicide alternatives, producers are increasingly relying on multi-site protectant fungicides (i.e., captan and mancozeb) to manage apple scab. Protectant fungicides must be applied more frequently, have little to no post-infection activity, and are relatively ineffective (in comparison to DMIs) in controlling apple powdery mildew (Köller et al., 1999) which should be managed during the same time as apple scab in the northeastern U.S. In 2009, several DMI fungicides marketed previously in Europe were registered for management of apple scab in North America. These fungicides included difenoconazole and fenbuconazole, which have been found to have higher in vitro intrinsic activity (Pfeufer and Ngugi, 2012) and greater efficacy in the field (Cox et al., 2013) against *V. inaequalis* compared to myclobutanil.

The early reports of DMI control failures in the mid-Atlantic and the northeastern US apple production regions, accompanied by the advent of newly released DMI fungicides for managing apple scab, prompted us to conduct an investigation of DMI fungicide resistance in *V. inaequalis* populations. The specific objectives of our study were to: (i) determine the extent of myclobutanil resistance in orchard populations of *V. inaequalis* across the mid-Atlantic, the Midwest, and the northeastern U.S. from 2004-2013, (ii) establish baseline in vitro sensitivities for the DMI fungicide difenoconazole in *V. inaequalis*, (iii) evaluate and compare in vitro difenoconazole sensitivity for populations of *V. inaequalis* with practical resistance or sensitivity

to myclobutanil, and (iv) determine the effect of myclobutanil or difenoconazole applications in monitored populations of *V. inaequalis* with practical resistance or sensitivity to myclobutanil.

MATERIALS AND METHODS

Collection of *V. inaequalis* isolates. From 2004 to 2013, isolates of *Venturia inaequalis* were collected from 141 individual apple orchards throughout the northeastern United States. The orchards examined represented the range of DMI fungicide use practices and nearly all of the major apple production operations in the region, which consisted of 14 research, 119 commercial, and eight baseline orchards. Some orchard populations were tested for DMI sensitivity because the grower had reported unsatisfactory control of apple scab that resulted in the appearance of apple scab symptoms following application of a DMI fungicide. However, nearly half of the orchard populations evaluated for sensitivity to myclobutanil were collected prior to local concerns over DMI performance by using bait trees or leaving trees unsprayed. Baseline orchards were designated as such if the orchard had never been exposed to any single-site fungicide classes including the anilinopyrimidines (APs), benzimidazoles, DMIs, guanidines, QoIs, or succinate dehydrogenase inhibitors (SDHIs).

Isolates from each orchard were obtained by arbitrarily collecting 50 to 100 young, fully expanded leaves that contained isolated, young, sporulating primary apple scab lesions throughout the orchard. From each leaf collection, 50 leaves were selected and one individual lesion representing a single-spore infection event (Köller et al., 2004; MacHardy and Gadoury, 1989) from each of those leaves was phenotyped for sensitivity to the DMI fungicide, myclobutanil, using microscopy-aided mycelial relative growth assays as previously described and validated (Frederick et al., 2014; Köller et al., 2004; Villani and Cox, 2014). Briefly, the

single-conidial lesion isolates (referred to as "isolates" throughout the remainder of the manuscript) were removed using a sterile cork borer (5 mm diameter), placed in 1.2 ml of sterile distilled water, and shaken for 60 s to dislodge conidia from the lesion. The leaf discs were promptly removed and 100 µl suspensions of 10^3 to 10^4 *V. inaequalis* conidia ml^{-1} were evenly distributed on potato dextrose agar (PDA; Difco Laboratories) amended with streptomycin sulfate (50 µg ml^{-1}), chloramphenicol (50 µg ml^{-1}), and analytical grade myclobutanil (Sigma Aldrich, St. Louis MO) dissolved in acetone (Sigma Aldrich, St. Louis MO) at a discriminatory dose of 0.1 µg ml^{-1} as suggested by Köller et al. (Köller et al., 1991; Köller et al., 1997). Isolates were incubated at 22°C for six days, and mycelial growth of five randomly selected micro-colonies originating from single spores was determined by measuring micro-colony diameter or germ tube length (for spores with greatly inhibited growth) using a SPOT Idea digital camera (with the SPOT Imaging Basic software package, Diagnostic Instruments Inc., Sterling Heights, MI) attached to an Olympus SZX12 stereoscope (Olympus America Inc., Center Valley, PA).

The in vitro sensitivity response for each isolate in each of the 141 orchard populations was expressed as mean percent relative growth (%RG) as previously described (Frederick et al., 2014; Köller et al., 1991; Smith et al., 1991). Each orchard population was subsequently characterized as having or lacking practical resistance to myclobutanil by comparing the distribution of sensitivity responses (mean %RG) for each orchard population in question to standards in which practical resistance or sensitivity to myclobutanil had already been verified. The practical resistance status of both the standard resistant orchard and the standard sensitive orchard were determined directly by examining fungicide application practices for each standard orchard and assessing the development of apple scab symptoms following application of Rally 40WSP or Nova 40W (myclobutanil; Dow Agrosciences, Indianapolis, IN). In 2011, orchard 61

had 5.8% incidence of apple scab on mature 'Jerseymac' fruit at harvest following four applications of Rally 40WSP and was used as the sensitive standard population. In 2010, orchard 26 had 45.3% incidence of apple scab at harvest on mature 'McIntosh' fruit following four applications of Rally 40WSP and was used as the resistant standard population. For both the sensitive standard and the resistant standard orchard populations, 50 young, fully expanded leaves that contained isolated young, sporulating primary apple scab lesions were collected throughout the orchard and in vitro sensitivity to myclobutanil was determined as described above. Comparisons of isolate sensitivity distributions to the standard orchard populations were made using the nonparametric Kolmogorov-Smirnov (K-S) two sample test in SAS (version 9.3, SAS Institute, Cary, NC).

Using a K-S two-sample test, the practical resistance classification was determined for each of the 141 orchards by comparing the distribution of isolate myclobutanil responses for each orchard population (the "test orchard") to sensitivity distributions of both the resistant and sensitive standards (Frederick et al. 2014). Initially, the probability that the distribution of myclobutanil sensitivity responses for a given test orchard was different from the distribution of the resistant standard orchard ($P_{T=R}$) or different from the distribution in myclobutanil sensitivity of the sensitive standard orchard ($P_{T=S}$) was determined. The outcome of the two probability tests was then used to classify the orchard population in one of four scenarios. For instance, if a given test orchard had $P_{T=R} < 0.05$ and $P_{T=S} \geq 0.05$, the sensitivity distribution of the orchard in question would be significantly different from the resistant standard but not from the sensitive standard, and thus the test orchard would be classified as "sensitive". Alternatively, if a given test orchard had $P_{T=R} \geq 0.05$ and $P_{T=S} < 0.05$, the sensitivity distribution of the orchard in question would be significantly different from the sensitive standard but not from the

resistant standard, and thus the test orchard would be classified as "resistant". In cases where the sensitivity distribution for a test orchard was not significantly different from the standard resistant orchard and the standard sensitive orchard ($P_{T=R} \geq 0.05$ and $P_{T=S} \geq 0.05$) the population in the test orchard would be considered "reduced sensitive" to myclobutanil. Finally, the distribution of a test orchard population may be significantly different from the sensitive standard and the resistant standard. Such a situation may happen when the distribution of the relative growth of isolates in the test orchard population was significantly greater than the resistant standard orchard or when the distribution of the relative growth of isolates in the test orchard population was significantly lower than the sensitive standard orchard. In such situations, an orchard would be classified as "resistant" or "sensitive" respectively. Although sensitivity classifications were based on the distribution of isolate sensitivities within a population of *V. inaequalis*, the mean percent relative growth (%RG) was reported for each orchard population to facilitate comparisons between orchards. Thus, in some instances, sensitivity classifications (i.e., 'resistant' and 'reduced sensitive'), which were determined using the K-S statistical analysis, will have overlapping mean %RG values. In all cases, it is important to understand that classification of a test orchard only implies how its population would behave in regards to that of the standards for the purpose of discussing risk of product failure and the prevalence of resistance to DMI. It is not meant to be used emphatically indicate product failure irrespective of host and environment.

Baseline sensitivity and sensitivity of populations of *V. inaequalis* to difenoconazole.

In order to develop a baseline for difenoconazole sensitivity in *V. inaequalis,* a total of 44 *V. inaequalis* single conidial lesion isolates were collected from two baseline apple orchards located in Geneva, NY, in 2010 and were screened for sensitivity to difenoconazole. For each single

conidial lesion isolate, conidial suspensions were prepared as described above and were evenly distributed on PDA medium that was amended with streptomycin sulfate (50 μg ml^{-1}), chloramphenicol (50 μg ml^{-1}), and analytical grade difenoconazole (Sigma Aldrich, St. Louis, MO) at final concentrations of 0.0, 0.0001, 00005, 0.001, 0.01, 0.05, 0.1, 0.5, and 1 μg ml^{-1}. Following six days of incubation at 22°C, mycelial growth of five randomly selected micro-colonies originating from single spores was determined by measuring micro-colony diameter or germ tube length (for spores with greatly inhibited growth) using a SPOT Idea digital camera attached to an Olympus SZX12 stereoscope as described above. Relative percent inhibition was calculated at each fungicide concentration as previously described (Yoshimura et al., 2004). For each isolate, dose response curves were constructed using relative percent inhibition of colony growth at each log-transformed (log_{10}) concentration for the respective fungicide to determine the value of the effective concentration that inhibited isolate growth by 50% (EC_{50}).

From 2010 to 2013, single conidial lesion isolates that were used for the determination of myclobutanil sensitivity were also screened for sensitivity to analytical grade difenoconazole at a discriminatory concentration of 0.1 μg ml^{-1}. This concentration was chosen on the basis of being able to make comparisons of intrinsic activity with myclobutanil. Difenoconazole sensitivity, expressed as percent relative growth, was determined in an identical manner as the myclobutanil sensitivity assay described above. Orchards were classified as sensitive, reduced sensitive, or resistant using the K-S two-sample test as described above. Since control failures with difenoconazole have not been reported in the region, we expected that the sensitivity responses of *V. inaequalis* isolates to difenoconazole in vitro would have to approach the same level of growth response that allows an isolate or population to achieve resistance to myclobutanil in order for practical resistance to be observed. Thus, the DMI-sensitive and resistant orchards used

for the classification of myclobutanil sensitivity were used to classify orchard sensitivity to difenoconazole using the same sensitive and resistant thresholds.

Myclobutanil and difenoconazole cross sensitivity in a representative baseline (orchard 28) and a myclobutanil-resistant population of *V. inaequalis* (orchard 37) was also evaluated in 2013. Seventeen isolates from a baseline orchard and 35 isolates from a myclobutanil-resistant orchard were assayed for sensitivity to both fungicides. Simple linear regression analysis was used to determine whether there was a relationship between relative growth responses between the two fungicides in vitro (SigmaPlot Version 11.0, Systat Software Inc., San Jose, CA).

Impact of DMI fungicide applications on myclobutanil and difenoconazole sensitivity. From 2010 to 2013, sensitivity to myclobutanil and difenoconazole was evaluated in a research orchard comprised of 15-year-old 'Empire' apples on M.9/M.111 rootstocks. Prior to 2010, myclobutanil had provided acceptable levels of control (i.e., <20% incidence) on mature fruit (Cox et al., 2009), and trees within this research orchard had only received a total of 12 applications of myclobutanil during its production history. To determine the effect of difenoconazole applications on populations of *V. inaequalis* sensitive to DMI fungicides, four applications of Inspire (difenoconazole; Syngenta, Greensboro, NC) were made each year from 2010 to 2013 at the maximum labeled rate of 293 ml Ha^{-1}. Inspire was applied in approximately 2,800 liters of water per hectare using an AA2 GunJet® handgun (TeeJet Technologies, Wheaton, IL) at approximately 2,000 kPA pressure. Treatments were applied on approximately seven to ten day intervals at the phenological timings of pink, bloom, first cover and second cover. Isolated primary apple scab lesions were collected in the spring of 2010 to 2013 and were assayed for sensitivity to myclobutanil and difenoconazole as described above.

To determine the impact of DMI fungicide applications and the degree of stability of myclobutanil resistance, changes in myclobutanil sensitivity were evaluated in a 30-year-old 'McIntosh' and 'Cortland' research apple orchard with a population of *V. inaequalis* previously confirmed to have practical resistance to myclobutanil (Turechek et al., 2005). From 2005 to 2010, Nova 40W or Rally 40WSP was applied at a rate of 333 g Ha^{-1}. Rally 40WSP was applied at the same phenological timings using the same application parameters as described above for the 'Empire' planting. From 2011 to 2013, the myclobutanil selective pressure was removed from the orchard and DMI fungicides were no longer applied during the remainder of the study. In each year, isolated primary apple scab lesions were collected in the spring of 2005 to 2013 and were assayed for sensitivity to myclobutanil as described above.

RESULTS

Sensitivity of *V. inaequalis* orchard populations to myclobutanil. From 2004 to 2013, 141 individual populations of *Venturia inaequalis* throughout the northeastern United States were evaluated for sensitivity to myclobutanil (Table 2.1). A total of 3,987 single conidial lesion isolates of *V. inaequalis* were tested for sensitivity to myclobutanil, with 107 of the orchard populations being represented by 25 or more isolates. The remaining 34 orchard populations were represented by a minimum of 15 isolates.

Throughout the ten-year study, 89 of the 141 (63%) orchard populations of *V. inaequalis* were determined to have practical resistance to myclobutanil on the basis of statistical comparisons with sensitive and resistant population standards. The mean %RG of the orchards with practical resistance ranged from 53.1 to 132.4% with a research orchard population from East Lansing, MI (orchard 108: 53.1 ± 5.6%) and a commercial orchard population from Chazy,

NY (orchard 125: 132.4 ± 12.7%) having the lowest and highest relative growths, respectively. Seventy-nine of the orchard populations exhibiting practical resistance to myclobutanil were commercial orchards. Within these commercial orchards, myclobutanil (Nova 40W or Rally 40WSP) was applied for an average of 13 years with two to four applications of the fungicide per season. The remaining orchard populations classified as having practical resistance to myclobutanil were from research orchards in which DMI fungicides had been applied regularly throughout the planting history.

Only 18 (13%) orchard populations of *V. inaequalis* were found to have reduced sensitivity to myclobutanil. The mean %RGs for these populations ranged from 48.0 to 68.8% with an orchard from Wells, Maine (orchard 104: 48.0 ± 2.5%) and an orchard from Sodus, NY (orchard 171: 68.8 ± 3.3) representing the minimum and maximum endpoints of the 'reduced sensitive' designation. Sixteen orchard populations with reduced sensitivity were from commercial orchards, and in these orchards, applications of myclobutanil were made for an average of 11 years with two to four applications per season. One research orchard (orchard 62) in which DMI fungicides had been applied regularly throughout the history of the orchard and one baseline orchard (orchard 51) that had reportedly never directly been exposed to fungicides were determined to have reduced sensitivity to myclobutanil.

Thirty-four orchard populations of *V. inaequalis* (24%) were found to be sensitive to myclobutanil. The mean %RG of sensitive populations ranged from 9.0 to 54.7% with a baseline population in West Lafayette IN (orchard 9: 9.0 ± 2.1) and a commercial orchard surveyed in 2006 from Milton, NY (orchard 112: 54.7 ± 4.5) representing the minimum and maximum mean %RGs in this classification. Of the 34 sensitive orchard populations, 24 were commercial orchards in which myclobutanil was applied for an average of nine years with approximately one

to two applications per season. Three populations from research orchards that had received applications of DMI fungicides throughout the history of their planting, and seven of the eight total baseline orchard populations were found to be sensitive to myclobutanil.

Table 2.1. Sensitivity classification, percent relative growth, isolation year, and collection location from which *V. inaequalis* populations were surveyed for myclobutanil resistance.

Designation[a]	Classification[b]	n	% RG[c]	Pr>Ksa Resistant[d]	Pr>Ksa Sensitive[d]	Year	State
1	Resistant	30	131.6 ± 13.8	0.0020	<0.0001	2005	NY
3	Resistant	32	96.9 ± 5.9	0.0003	<0.0001	2009	NY
5	Resistant	31	94.5 ± 8.0	<0.0001	<0.0001	2005	NY
6	Resistant	27	58.4 ± 5.9	0.4125	0.0439	2007	MA
8	Resistant	30	67.7 ± 5.7	0.6984	0.0011	2007	NY
9	Resistant	30	111.8 ± 9.0	0.0004	<0.0001	2006	NY
12	Resistant	30	113.7 ± 11.0	0.0003	<0.0001	2007	NY
16	Resistant	30	87.4 ± 6.6	0.0714	<0.0001	2007	NH
18	Resistant	29	86.1 ± 4.7	0.0646	<0.0001	2006	MA
19	Resistant	26	96.4 ± 3.6	<0.0001	<0.0001	2013	VT
20	Resistant	30	95.8 ± 6.9	0.0016	<0.0001	2006	NY
21	Resistant	16	80.0 ± 7.0	0.1268	<0.0001	2007	ME
24	Resistant	30	66.5 ± 3.9	0.6243	0.0006	2007	ME
25	Resistant	28	65.3 ± 5.6	0.5924	0.0016	2006	MA
26	**Resistant**	**36**	**67.7 ± 3.4**	**1.0000**	**0.0055**	**2010**	**NY**
27	Resistant	22	61.0 ± 6.5	0.2757	0.0245	2006	RI
31	Resistant	30	85.0 ± 8.0	0.0456	0.0003	2007	NY
34	Resistant	19	75.1 ± 7.1	0.4401	0.0002	2007	NY
35	Resistant	31	75.0 ± 4.3	0.1869	<0.0001	2005	OH
37	Resistant	35	96.4 ± 2.2	<0.0001	<0.0001	2013	NY
38	Resistant	21	72.7 ± 7.5	0.3866	0.0029	2008	RI
39	Resistant	30	75.7 ± 6.7	0.3701	0.0008	2007	RI
42	Resistant	30	69.3 ± 5.1	0.0015	0.5806	2006	MA
43	Resistant	31	64.1 ± 5.8	0.069	0.002	2012	ME

45	Resistant	30	66.0 ± 3.6	0.3415	<0.0001	2006	NY
47	Resistant	38	113.5 ± 13.1	0.0006	<0.0001	2008	RI
48	Resistant	47	89.7 ± 3.7	0.0010	<0.0001	2008	WV
49	Resistant	30	83.7 ± 3.8	0.0308	<0.0001	2006	NY
53	Resistant	30	108.2 ± 11.3	0.0105	<0.0001	2007	WV
54	Resistant	17	109.5 ± 8.8	0.0017	<0.0001	2008	NY
55	Resistant	30	84.0 ± 8.8	0.2610	0.0003	2006	MA
56	Resistant	30	79.6 ± 5.8	0.0701	<0.0001	2006	MA
57	Resistant	30	76.2 ± 4.9	0.2849	<0.0001	2007	NY
59	Resistant	30	94.8 ± 7.1	0.0078	<0.0001	2007	CT
60	Resistant	30	77.8 ± 3.1	0.0701	<0.0001	2007	NY
63	Resistant	22	82.9 ± 5.0	0.0471	<0.0001	2007	NY
64	Resistant	30	92.4 ± 8.2	0.0123	<0.0001	2006	NY
67	Resistant	30	69.8 ± 6.4	0.4642	0.0010	2007	MA
68	Resistant	30	74.9 ± 5.1	0.1926	<0.0001	2005	NY
70	Resistant	30	73.7 ± 5.2	0.4746	0.0008	2006	WV
72	Resistant	31	85.7 ± 4.0	0.0111	<0.0001	2005	OH
74	Resistant	30	66.4 ± 4.1	0.7860	0.0005	2005	NY
75	Resistant	30	80.1 ± 5.2	0.0625	0.0003	2007	MA
77	Resistant	31	82.2 ± 8.2	0.4104	<0.0001	2005	WV
78	Resistant	30	97.0 ± 8.4	0.0048	<0.0001	2005	NY
80	Resistant	30	105.6 ± 5.9	0.0002	<0.0001	2005	NY
82	Resistant	30	73.9 ± 3.6	0.1363	<0.0001	2005	OH
83	Resistant	30	63.0 ± 4.7	0.4261	0.0050	2007	NY
84	Resistant	31	67.6 ± 6.7	0.3556	0.0050	2008	RI
87	Resistant	30	68.7 ± 5.0	0.6056	<0.0001	2005	OH
88	Resistant	37	87.8 ± 4.7	0.0008	<0.0001	2013	NY
90	Resistant	34	80.7 ± 4.8	0.0295	<0.0001	2009	PA
94	Resistant	26	83.8 ± 13.3	0.8396	0.0015	2006	MA
96	Resistant	30	82.9 ± 4.8	0.0244	<0.0001	2005	WV
97	Resistant	30	90.7 ± 3.7	0.0020	<0.0001	2007	RI

98	Resistant	25	84.6 ± 8.5	0.1736	0.0001	2008	NY
99	Resistant	35	94.2 ± 7.6	0.0105	<0.0001	2008	NY
100	Resistant	30	61.8 ± 4.4	0.0877	0.0010	2006	NY
102	Resistant	34	61.6 ± 5.0	0.9072	0.0097	2008	MA
103	Resistant	27	82.0 ± 11.1	0.0273	<0.0001	2008	RI
105	Resistant	32	114.8 ± 13.8	<0.0001	<0.0001	2005	OH
106	Resistant	30	65.9 ± 7.6	0.2317	0.0092	2007	NY
107	Resistant	33	69.1 ± 6.1	0.0885	0.0004	2009	NY
108	Resistant	41	53.1 ± 5.6	0.5169	0.0347	2009	MI
109	Resistant	30	73.6 ± 7.4	0.2973	0.0050	2007	NY
111	Resistant	30	79.4 ± 11.3	0.4754	<0.0001	2007	RI
113	Resistant	30	93.9 ± 8.1	0.1050	<0.0001	2007	NY
117	Resistant	30	78.7 ± 5.5	0.1387	<0.0001	2004	NY
118	Resistant	29	60.0 ± 4.5	0.3550	0.0040	2007	NY
120	Resistant	30	84.0 ± 6.9	0.0625	<0.0001	2005	WV
121	Resistant	29	80.3 ± 7.9	0.4495	<0.0001	2006	RI
122	Resistant	30	64.4 ± 4.4	0.6984	0.0008	2005	NY
123	Resistant	30	99.1 ± 11.0	0.0053	<0.0001	2007	NY
124	Resistant	30	116.4 ± 11.1	<0.0001	<0.0001	2006	MA
125	Resistant	30	132.4 ± 12.7	<0.0001	<0.0001	2005	NY
127	Resistant	30	94.4 ± 4.3	0.0004	<0.0001	2005	WI
141	Resistant	21	73.4 ± 7.3	0.8552	0.0002	2010	ME
148	Resistant	21	77.9 ± 2.3	0.0008	<0.0001	2011	NY
149	Resistant	21	94.9 ± 3.0	<0.0001	0.0004	2009	MA
150	Resistant	18	93.4 ± 5.6	0.0115	<0.0001	2011	VA
154	Resistant	16	98.4 ± 5.2	0.0033	<0.0001	2011	NC
157	Resistant	31	82.0 ± 3.5	0.8639	<0.0001	2012	VT
158	Resistant	24	68.8 ± 4.5	0.2249	0.0015	2011	ME
162	Resistant	24	60.3 ± 4.2	0.1778	0.0096	2012	MI
163	Resistant	36	78.0 ± 3.5	0.7264	<0.0001	2012	ME
170	Resistant	33	76.3 ± 3.9	0.7767	<0.0001	2012	NY

172	Resistant	35	70.0 ± 2.6	0.7497	<0.0001	2012	NY
173	Resistant	17	87.2 ± 8.1	0.0448	0.0020	2013	VT
175	Resistant	26	99.2 ± 3.6	<0.0001	<0.0001	2013	NY
14	Reduced Sensitive	32	49.9 ± 8.3	0.0868	0.1888	2009	MI
22	Reduced Sensitive	30	51.3 ± 3.2	0.0055	0.0043	2005	MA
30	Reduced Sensitive	24	55.8 ± 7.9	0.2423	0.3291	2007	MA
33	Reduced Sensitive	30	50.2 ± 4.0	0.0055	0.0215	2007	NH
44	Reduced Sensitive	30	60.6 ± 13.8	0.4261	0.0751	2007	RI
51	Reduced Sensitive	30	57.5 ± 4.2	0.0429	0.0007	2006	NY
62	Reduced Sensitive	30	59.5 ± 6.7	0.0877	0.0751	2006	NY
76	Reduced Sensitive	30	54.3 ± 5.0	0.0484	0.0076	2006	VT
89	Reduced Sensitive	30	49.2 ± 2.8	0.0063	0.0119	2005	NH
104	Reduced Sensitive	24	48.0 ± 2.5	<0.0001	0.0068	2011	ME
110	Reduced Sensitive	34	55.3 ± 3.2	0.0001	0.0053	2012	VT
142	Reduced Sensitive	35	48.3 ± 1.8	<0.0001	0.0183	2013	MI
147	Reduced Sensitive	23	62.3 ± 3.8	0.0158	0.0004	2012	NY
153	Reduced Sensitive	20	52.5 ± 3.4	<0.0001	0.0068	2011	ME

164	Reduced Sensitive	37	58.9 ± 3.4	0.0006	0.0027	2012	NY
165	Reduced Sensitive	19	62.6 ± 3.4	0.0117	0.0014	2012	MA
171	Reduced Sensitive	34	68.8 ± 3.3	0.024	<0.0001	2012	NY
174	Reduced Sensitive	37	49.5 ± 3.3	0.0046	0.0158	2013	ME
4	Sensitive	30	50.0 ± 6.7	0.0025	0.1600	2007	NH
11	Sensitive	30	50.8 ± 4.4	0.0356	0.0842	2007	RI
13	Sensitive	26	41.2 ± 13.8	<0.0001	0.2924	2011	NY
23	Sensitive	30	27.8 ± 2.1	<0.0001	0.0842	2007	NY
28	Sensitive	17	12.9 ± 3.4	<0.0001	0.0088	2010	NY
29	Sensitive	36	30.9 ± 2.1	<0.0001	0.1243	2008	RI
32	Sensitive	17	23.5 ± 7.5	0.0004	0.0494	2009	MI
41	Sensitive	30	37.5 ± 4.9	<0.0001	0.4260	2007	RI
46	Sensitive	22	13.7 ± 5.2	<0.0001	0.0004	2009	MI
50	Sensitive	33	36.4 ± 6.7	0.0023	0.1945	2009	NY
52	Sensitive	15	29.4 ± 6.9	0.0183	0.9048	2009	OH
58	Sensitive	20	29.3 ± 6.5	0.0017	0.3446	2009	MI
61	**Sensitive**	**36**	**38.4 ± 3.6**	**0.0007**	**1.0000**	**2011**	**NY**
69	Sensitive	19	21.9 ± 6.2	0.0009	0.1575	2005	NY
71	Sensitive	16	33.6 ± 2.9	0.0002	0.4930	2004	NY
73	Sensitive	30	43.9 ± 3.2	0.0006	0.0751	2006	WV
85	Sensitive	27	23.8 ± 5.3	<0.0001	0.1573	2009	RI
86	Sensitive	17	33.0 ± 6.6	0.0054	0.4576	2013	ME
92	Sensitive	34	9.0 ± 2.1	<0.0001	0.0002	2009	IN
93	Sensitive	20	25.7 ± 9.3	<0.0001	0.0096	2009	ME
101	Sensitive	22	30.0 ± 3.5	<0.0001	0.5401	2012	NY
112	Sensitive	30	54.7 ± 4.5	0.0066	0.1445	2006	NY

[a] Orchard designations in bold designate the resistant standard population and the sensitive standard population used for determining the myclobutanil sensitivity classification according to the Kolmogorov-Smirnov two-sample test (K-S) test. Both the resistant and sensitive standard are designated in bold text.

[b] Orchard classification was determined by comparing the distribution of myclobutanil sensitivity responses (isolate percent mean relative growth values) for a given *V. inaequalis* orchard population to standard orchard populations with and without practical resistance to myclobutanil using the K-S two-sample test in SAS (Version 9.3, SAS Institute, Cary, NC).

[c] The orchard population mean percent growth of *V. inaequalis* isolates on medium amended with 0.1 µg ml^{-1} analytical grade myclobutanil relative to that on non-fungicide medium (%RG). Values are means and standard errors of *n* isolates for each population with five single-conidium colonies for each isolate.

[d] Probability that the distribution of sensitivity responses for the orchard population in question is not significantly different from the standard orchard population with (Pr>Ksa Resistant) or without (Pr>Ksa Sensitive) practical resistance to myclobutanil.

For the purpose of examining broader implications of myclobutanil resistance, orchard populations were sorted by their myclobutanil sensitivity classification according to the state and region from which they were collected (Table 2.2). Overall, 57, 69, and 15 populations of *V. inaequalis* were examined from the New England region (CT, MA, ME, NH, RI, VT), the mid-Atlantic region (NC, NY, PA, VA, WV), and the Midwest region (IN, MI, OH, WI), respectively. While the majority of populations from each region were resistant to myclobutanil, the mid-Atlantic had the highest percentage of resistant populations (71%) followed by New England (56%) and the Midwest (53%). The Midwest had the greatest percentage of populations that were sensitive to myclobutanil (33%). Of the states (MA, ME, NY and RI) in which more than ten populations of *V. inaequalis* were investigated, only Maine had more populations that were sensitive to myclobutanil (45%) than were resistant to myclobutanil (35%).

Table 2.2: Summary of *V. inaequalis* orchard populations by region and state classified on the basis of in vitro sensitivity to the DMI fungicides myclobutanil and difenoconazole.

Practical resistance classification[a]	Region	State	# of orchard populations (myclobutanil)	Percentage of orchard population for state (myclobutanil)	# of orchard populations (difenoconazole)	Percentage of orchard population for state (difenoconazole)
		CT	1	100%	.	.
		MA	12	75%	0	0%
		ME	6	35%	0	0%
		NH	1	25%	.	.
		RI	9	64%	.	.
	New England	VT	3	60%	0	0%
		MD	0	0%	0	0%
		NC	1	100%	.	.
		NY	40	70%	0	0%
		PA	1	100%	.	.
		VA	1	100%	0	0%
	Mid-Atlantic	WV	6	75%	0	0%
		IN	0	0%	.	.
		OH	5	83%	.	.
		MI	2	29%	0	0%
Resistant	Midwest	WI	1	100%	.	.
Total Resistant			89	63%	0	0%
		CT	0	0%	.	.
		MA	3	19%	0	0%
		ME	3	18%	0	0%
		NH	2	50%	.	.
		RI	1	7%	.	.
	New England	VT	2	40%	0	0%
		MD	0	0%	0	0%
Reduced		NC	0	0%	.	.
Sensitive		NY	5	9%	1	7%

	Mid-Atlantic	PA	0	0%	.	.
		VA	0	0%	0	0%
		WV	0	0%	0	0%
		IN	0	0%	.	.
		MI	2	29%	0	0%
		OH	0	0%	.	.
	Midwest	WI	0	0%	.	.
Total Reduced Sensitive			18	13%	1	3%
		CT	0	0%	.	.
		MA	1	6%	1	100%
		ME	9	45%	13	100%
		NH	1	25%	.	.
		RI	4	29%	.	.
	New England	VT	0	0%	4	100%
		MD	1	100%	1	100%
		NC	0	0%	.	.
		NY	12	21%	13	93%
		PA	0	0%	.	.
		VA	0	0%	1	100%
	Mid-Atlantic	WV	2	25%	1	100%
		IN	1	100%	.	.
		MI	3	43%	2	100%
		OH	1	16%	.	.
Sensitive	Midwest	WI	0	0%	.	.
Total Sensitive			34	24%	36	97%

[a] Orchard classification was determined by comparing the distribution of myclobutanil or difenoconazole sensitivity responses (isolate percent mean relative growth values) for a given *V. inaequalis* orchard population to standard orchard populations with and without practical resistance to myclobutanil using the K-S two-sample test in SAS (Version 9.3, SAS Institute, Cary, NC).

Baseline sensitivity and sensitivity of populations of *V. inaequalis* to difenoconazole.

The difenoconazole EC_{50} values for baseline isolates of *V. inaequalis* ranged from 0.0002 to 0.009 μg ml^{-1} (mean EC_{50} = 0.002 μg ml^{-1}) (Figure 2.1). The majority (39 of 44) of isolates screened for baseline sensitivity had EC_{50} values below 0.004 μg ml^{-1}. While a few isolates demonstrated a slight reduction in sensitivity to difenoconazole, no isolates tested had EC_{50} values that were either less than or greater than 10x the mean.

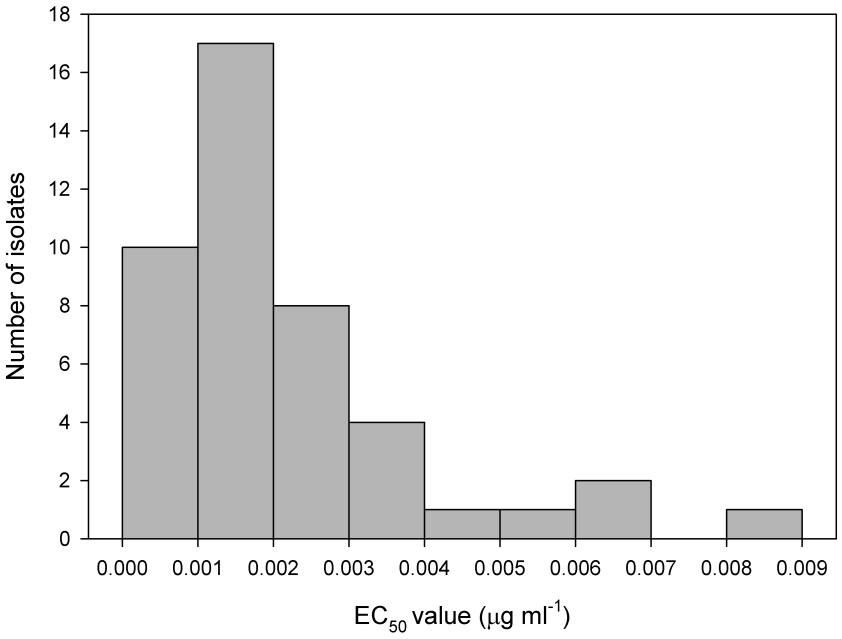

Figure 2.1. Distribution of difenoconazole EC50 values for 44 baseline isolates of *V. inaequalis*. Baseline isolates were collected from apple orchards in New York that have never been exposed to single-site fungicide.

From 2010 to 2013, 37 orchard populations of *Venturia inaequalis* throughout the northeastern United States were evaluated for sensitivity to difenoconazole (Table 2.3). Of these populations, three were from baseline orchards, six were from research orchards, and 28 were from commercial orchards. A total of 1,012 single-conidial lesion isolates of *V. inaequalis* were tested for sensitivity to difenoconazole, with 20 of the orchard populations being represented by

25 or more isolates. The remaining 17 orchard populations were represented by a minimum of 15 isolates.

Table 2.3. Sensitivity classification, percent relative growth, isolation year, and collection location from which *V. inaequalis* populations were surveyed for difenoconazole resistance.

Designation[a]	Classification[b]	n	% RG[c]	Pr>Ksa Resistant[d]	Pr>Ksa Sensitive[d]	Year	State
148	Reduced Sensitive	21	42.0 ± 6.1	0.0750	0.8135	2011	NY
13	Sensitive	25	11.3 ± 1.5	<0.0001	<0.0001	2011	NY
19	Sensitive	26	24.8 ± 3.0	<0.0001	0.0863	2013	VT
26	Sensitive	35	35.0 ± 3.5	<0.0001	0.5187	2010	NY
28	Sensitive	17	16.0 ± 2.7	<0.0001	0.0006	2011	NY
37	Sensitive	35	39.1 ± 4.5	0.0011	0.2714	2013	NY
43	Sensitive	31	13.1 ± 2.1	<0.0001	0.0005	2012	ME
61	Sensitive	36	21.9 ± 3.5	<0.0001	0.1131	2011	NY
86	Sensitive	16	3.2 ± 1.2	<0.0001	<0.0001	2013	ME
88	Sensitive	35	22.3 ± 3.3	<0.0001	0.0023	2013	NY
101	Sensitive	22	15.1 ± 3.1	<0.0001	0.0071	2012	NY
104	Sensitive	23	5.7 ± 0.6	<0.0001	<0.0001	2011	ME
110	Sensitive	34	29.7 ± 3.1	<0.0001	0.3625	2012	VT
119	Sensitive	19	11.1 ± 4.6	<0.0001	0.0003	2010	WV
141	Sensitive	21	23.6 ± 6.1	0.0010	0.0911	2010	ME
142	Sensitive	36	12.6 ± 1.8	<0.0001	<0.0001	2013	MI
147	Sensitive	23	37.6 ± 3.9	0.0013	0.8305	2012	NY
150	Sensitive	17	12.6 ± 2.7	<0.0001	0.0009	2011	VA
151	Sensitive	23	12.6 ± 2.4	<0.0001	0.0001	2011	ME
152	Sensitive	25	24.8 ± 4.3	<0.0001	0.1736	2011	ME
153	Sensitive	19	16.3 ± 3.5	<0.0001	0.0016	2011	ME
157	Sensitive	32	27.3 ± 3.2	<0.0001	0.1464	2012	VT
158	Sensitive	24	10.1 ± 1.5	<0.0001	<0.0001	2011	ME

161	Sensitive	15	20.1 ± 7.4	0.0002	0.0675	2010	NY
162	Sensitive	48	24.5 ± 2.8	<0.0001	0.0510	2012	MI
163	Sensitive	32	28.4 ± 3.6	<0.0001	0.3728	2012	ME
164	Sensitive	37	41.1 ± 2.9	<0.0001	0.1042	2012	NY
165	Sensitive	19	15.8 ± 2.9	<0.0001	0.0032	2012	MA
166	Sensitive	36	4.26 ± 1.9	<0.0001	<0.0001	2012	ME
167	Sensitive	35	10.0 ± 1.4	<0.0001	<0.0001	2012	ME
168	Sensitive	23	4.5 ± 1.1	<0.0001	<0.0001	2012	ME
169	Sensitive	25	13.7 ± 2.7	<0.0001	0.0002	2012	MD
170	Sensitive	33	29.6 ± 3.5	<0.0001	0.2034	2012	NY
171	Sensitive	35	37.8 ± 3.0	0.0002	0.5294	2012	NY
173	Sensitive	16	8.6 ± 3.3	<0.0001	0.0002	2013	VT
174	Sensitive	37	13.5 ± 2.9	<0.0001	<0.0001	2013	ME
175	Sensitive	26	28.3 ± 5.1	0.0003	0.2565	2013	NY

[a] Designation number represents the identical *V. inaequalis* population screened for resistance to myclobutanil

[b] Orchard classification was determined by comparing the distribution of difenoconazole sensitivity responses (isolate percent mean relative growth values) for a given *V. inaequalis* orchard population to standard orchard populations with and without practical resistance to myclobutanil using the K-S two-sample test in SAS (Version 9.3, SAS Institute, Cary, NC).

[c] The orchard population mean percent growth of *V. inaequalis* isolates on medium amended with 0.1 μg ml^{-1} analytical grade difenoconazole relative to that on non-fungicide medium (%RG). Values are means and standard errors of *n* isolates for each population with five single-conidium colonies for each isolate.

[d] Probability that the distribution of sensitivity responses for the orchard population in question is not significantly different from the standard orchard population with (Pr>Ksa Resistant) or without (Pr>Ksa Sensitive) practical resistance to myclobutanil.

None of the orchard populations were found to have practical resistance to difenoconazole, and only one population (orchard 148) was found to have reduced sensitivity to difenoconazole. The population with reduced sensitivity to difenoconazole was collected from a commercial orchard in which DMI fungicides had been used extensively and had a history of

practical resistance to myclobutanil. This reduced-sensitive population had a mean %RG on difenoconazole-amended medium of 42.0 ± 6.1%. Thirty-six remaining orchard populations were classified as being sensitive to difenoconazole. The mean %RG of sensitive orchard populations ranged from 3.2% to 41.1% with a commercial orchard population in Sweden, ME (orchard 86: 3.2 ± 1.2%) and a commercial orchard population in Geneva, NY (orchard 164: 41.1 ± 2.9%) having the lowest and highest relative growths, respectively. Within all 29 commercial orchard populations, myclobutanil (Nova 40W or Rally 40WSP) was applied for an average of 13 years with two to four applications per season. Because difenoconazole in the form of Inspire Super (difenoconazole + cyprodinil; Syngenta, Greensboro, NC) was first registered for use in NY in 2009, the product had only been available for use a maximum of four seasons, and most growers had only made one to two applications per season during those four seasons.

With the exception of a baseline orchard (orchard 28), all orchard populations were more sensitive to difenoconazole compared to myclobutanil and most individual isolates were more sensitive to difenoconazole than myclobutanil (data not shown). Out of the 37 orchard populations surveyed for sensitivity to both fungicides, four distinct phenotypic combinations were observed. Fourteen orchard populations were found to be resistant to myclobutanil but sensitive to difenoconazole, ten orchard populations had reduced sensitivity to myclobutanil but were sensitive to difenoconazole, one orchard population was resistant to myclobutanil and had reduced sensitivity to difenoconazole, and twelve orchard populations were sensitive to both fungicides (Tables 2.1 and 2.3). In a representative baseline population (orchard 28), some cross sensitivity was observed between difenoconazole and myclobutanil (R = 0.61) (Figure 2.2a**).** However, there was little to no cross sensitivity between the two fungicides in a representative population with myclobutanil resistance (orchard 37) (R = 0.07) (Figure 2.2b).

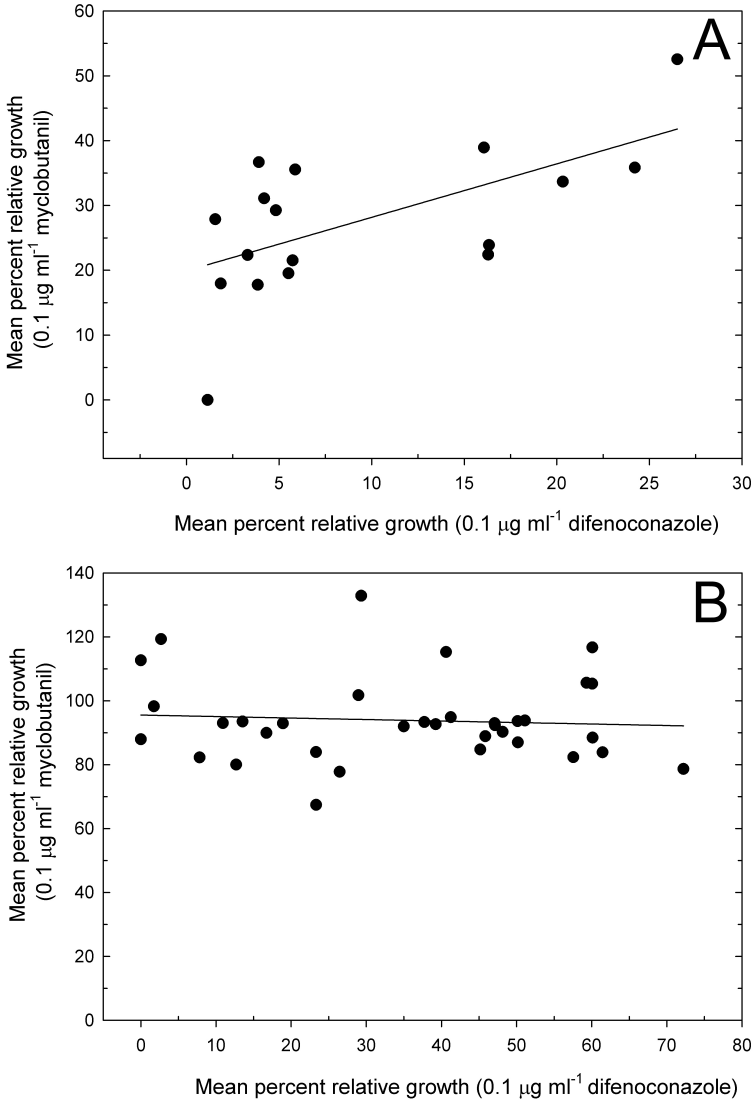

Figure 2.2. Mean percent relative growth values of *V. inaequalis* isolates on 0.1 μg/ml difenoconazole plotted against mean percent relative growth of those isolates on 0.1 μg/ml myclobutanil. Isolates were collected from A) a baseline orchard or B) a research orchard resistant to myclobutanil. A linear distribution function [y = yo + ax)] was fitted to the isolate mean percent relative growth values for myclobutanil and difenoconazole. For the baseline orchard, the *R* value was 0.610 (n = 17; P = 0.0123). For the myclobutanil resistant orchard, the *R* value was 0.073 (n = 35; P = 0.667).

Impact of difenoconazole or myclobutanil fungicide applications on DMI fungicide sensitivity. In the 'Empire' research orchard, Inspire (difenoconazole) was applied a total of 16 times from 2010 to 2013. A decrease in sensitivity ("increase in resistance") to both

myclobutanil and difenoconazole was observed in the population of *V. inaequalis* that was exposed to Inspire during this four-year period (Figure 2.3). Losses in sensitivity to myclobutanil increased steadily at a rate of 25% RG per year (Figure 2.3). In 2010, the mean %RG of this population on medium amended with myclobutanil was 23.8 ± 6.8%. By 2013, the population had developed practical resistance to myclobutanil with a mean %RG of 96.4 ± 2.2%. While a loss of sensitivity to difenoconazole was also observed, percent changes in sensitivity during the four-year period were not nearly as high as those for myclobutanil. In 2010, the isolate mean %RG on difenoconazole-amended medium was 19.0 ± 8.9% and by 2013 the population was still sensitive to the fungicide with an isolate mean %RG of 39.1 ± 4.5% (Figure 2.3).

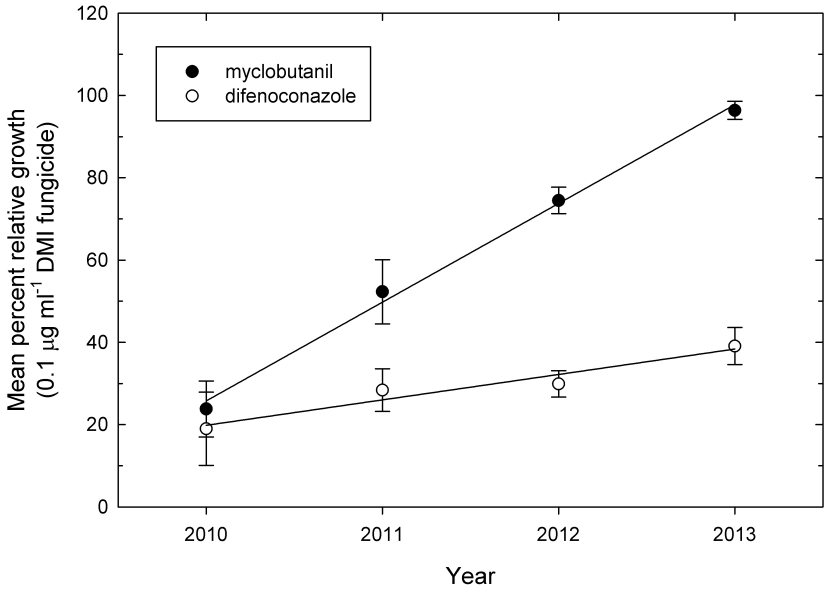

Figure 2.3. Mean percent relative growth and standard errors of *V. inaequalis* isolates on 0.1 µg/ml myclobutanil (closed circles) or difenoconazole (open circles) collected annually from a research orchard from 2010 to 2013. During each year, a minimum of 20 isolates of *V. inaequalis* were evaluated for sensitivity to each DMI fungicide. Points where error bars did not overlap had significant differences in sensitivity between the two fungicides.

In vitro sensitivity to myclobutanil and difenoconazole was also determined for an orchard population with previously confirmed practical resistance to myclobutanil (Turechek et al., 2005). In the 'McIntosh'/'Cortland' orchard from 2005 to 2010, myclobutanil (Rally 40WSP or Nova 40W) was applied 24 times. Interestingly, no significant difference in myclobutanil sensitivity was observed between the population evaluated in 2005 (82.6 ± 8.1%) and that evaluated in 2010 (88.5 ± 5.0%) (Figure 2.4).

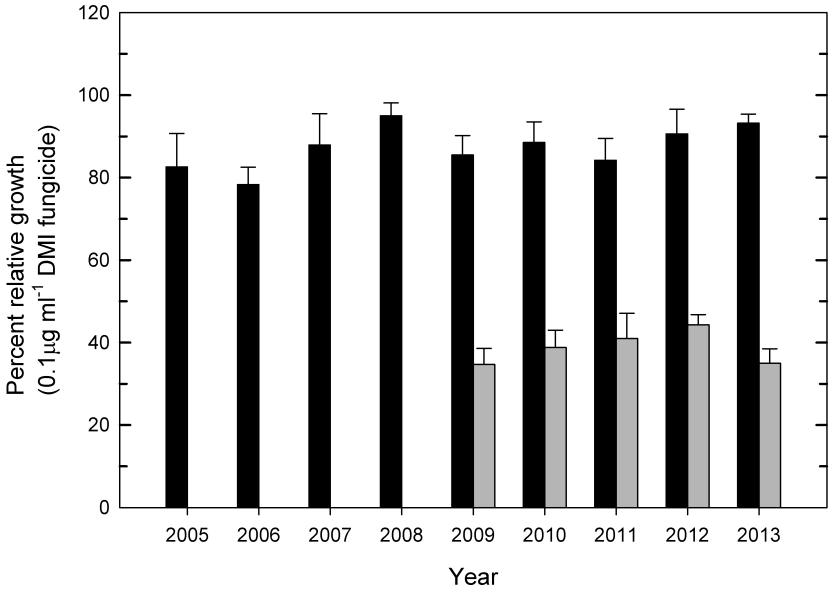

Figure 2.4. Mean percent relative growth of *V. inaequalis* isolates collected from a research orchard from 2005 to 2013 to the DMI fungicides myclobutanil (black bars) and difenoconazole (gray bars). Difenoconazole sensitivity was not evaluated from 2005 through 2008. From 2005 to 2010 myclobutanil was applied four times per season, and applications were discontinued in 2011. The number of isolates (n) tested for sensitivity to myclobutanil and/or sensitivity to difenoconazole were 2005: $n = 30$; 2006: $n = 30$; 2007: $n = 30$; 2008: $n = 50$; 2009: $n = 32$; 2010: $n = 29$; 2011: $n = 29$; 2012: $n = 35$; and 2013: $n = 35$.

In addition, when myclobutanil applications were terminated in the orchard (2011 to 2013), no significant difference in sensitivity to myclobutanil was observed compared to the years in which myclobutanil was applied in the orchard (2005 to 2010). Surprisingly, sensitivity to myclobutanil actually decreased from 2011 to 2013 with isolate mean %RGs on myclobutanil-

amended medium during those years increasing from 84.2 ± 5.3% to 93.2 ± 2.2%. In vitro sensitivity to difenoconazole was lower than that of myclobutanil ranging from 34.7 ± 4.1% to 44.3 ± 2.2% between 2009 and 2013 with a similar lack of fluctuation between years (Figure 2.4).

DISCUSSION

One hundred and forty-one individual orchard populations of *Venturia inaequalis* from 16 states representing three major geographic regions were tested for sensitivity to the DMI fungicide myclobutanil over a period of ten years. The majority of orchard populations were found to have practical resistance to the fungicide. Many of these orchards were commercial operations with an average of 13 applications of myclobutanil throughout the history of the orchard. While myclobutanil may no longer be a reliable apple scab management option in orchards east of the Mississippi River, the use of newly released DMI fungicides such as difenoconazole may still be an effective alternative in management of apple scab. Indeed, nearly all populations of *Venturia inaequalis* tested for sensitivity to difenoconazole were found to be sensitive despite their phenotypic responses on myclobutanil-amended medium.

Resistance to myclobutanil and other DMI fungicides began appearing in commercial *V. inaequalis* populations as early as 1991 (Köller et al., 1997) and is now prevalent in major apple production regions including those in Michigan, Virginia, Pennsylvania, Indiana, and Nova Scotia (Chapman et al., 2011; Köller et al., 1997; Marine et al., 2007; Pfeufer and Ngugi, 2012). Although resistance to myclobutanil was previously detected in individual isolates of *V. inaequalis* from New York (Köller et al., 1997), this is the first report of widespread myclobutanil resistance in the state and the New England region. Overall, 89 of 141 orchard

populations across 16 states were confirmed to have practical resistance to myclobutanil. Because the distribution of myclobutanil sensitivity of isolates within each population was directly compared to a standard orchard population in which myclobutanil failed to adequately control apple scab, it is possible that myclobutanil could be ineffective in these 89 orchards dependent on the susceptibility of the cultivar and weather during subsequent growing seasons.

While the majority of orchard populations screened for myclobutanil sensitivity in this study were collected from orchards in which myclobutanil failed to provide adequate levels of apple scab control in the field, there were a few instances in which a field failure was reported but the population tested sensitive or reduced sensitive to myclobutanil in vitro. This was especially the case in 2009, in which there was low apple scab infection pressure through bloom throughout the northeastern U.S., followed by favorable secondary apple scab conditions throughout the summer months. Dry conditions through bloom resulted in longer than average application intervals and as a result, minor primary apple scab infection events may have been missed. The weather across the region following petal fall and continuing throughout the summer months was wet and accompanied by below average temperatures (Rosenberger and Cox, 2010). Usually during the summer, secondary apple scab infection slows because of a reduction of conidial production when temperatures surpass 26 to 29°C (MacHardy, 1996). Because primary infections may have become established during the pre-bloom period, and because of the lack of consecutive days with warm temperatures to retard conidial production, the appearance of apple scab symptoms may have been mistaken for a fungicide control failure. Interestingly, several orchard populations evaluated for sensitivity in 2009 arrived for screening following petal fall. In other years of the survey, poor coverage or sprayer calibration may have resulted in fungicide control failures in the field rather than fungicide resistance.

In general, the number of historical myclobutanil applications was directly related to the sensitivity classification of the orchard. Orchards classified as sensitive in this study only had on average 9 to 18 applications of myclobutanil, while many of those classified as resistant had 52 or more applications (data not shown). For orchards in which accurate fungicide application records were obtained, those with more than 25 total myclobutanil applications had orchard populations of *V. inaequalis* that were significantly less sensitive to myclobutanil than the sensitive standard used in this study. This number is in good agreement with Gao et al. in which it was predicted that 36 total applications of myclobutanil were required before isolates (or populations) of *V. inaequalis* transitioned to an insensitive phenotype (Gao et al., 2009). However, other factors, including application rate, application timing (protective vs. curative), inoculum potential, and the inclusion of fungicide mix- partners, may influence minimum number of total myclobutanil applications prior to the onset of resistance (Beckerman et al., 2014; Gao et al., 2009; Köller et al., 2005; Kunz et al., 1997; Pfeufer and Ngugi, 2012).

Surprisingly one baseline orchard, without any historical applications of DMI fungicides was classified as reduced sensitive in this study. Although there are considerable numbers of wild apple trees in the region, the baseline orchard was fairly isolated from commercial operations. Because the spatial spread of isolates of *V. inaequalis* with reduced sensitivity to DMI fungicides have been found previously to occur over a limited distance (Gao et al., 2009), the arrival of reduced sensitive isolates of *V. inaequalis* most likely took a number of years. Given the number of wild hosts and the age of the baseline orchard (greater than 15 years), it is plausible that isolates with reduced sensitivity originated from a commercial orchard managed with DMI fungicides several kilometers away.

Maine was the only state surveyed in which the majority of its orchard populations of *V. inaequalis* were sensitive to myclobutanil. While fungicide use practices were no different than orchards in NY and other states in New England, other factors such as climate may have contributed to the preservation of myclobutanil efficacy in Maine. While little is known about the effect of extended low temperatures on pseudothecia survival and viable ascospore emergence, cessation of pseudothecia and ascopore development has been observed at or below temperatures of 0°C. (Gadoury and MacHardy, 1982). Indeed, extended sub-freezing temperatures in Maine could result in reduced primary inoculum and consequently a lower absolute number of myclobutanil-resistant ascospores could have been released in the following spring. Another factor that may contribute to the greater number of sensitive populations in Maine is the relatively small orchard size and low cultivar diversity within orchards (personal communication). Indeed, Pfeufer and Ngugi (2012) found that orchards smaller than ten hectares and those planted with less than ten cultivars were significantly more sensitive to DMI fungicides.

In this study, the mean difenoconazole EC_{50} value for 44 NY baseline isolates of *V. inaequalis* was determined to be 0.002 µg ml^{-1}. This concentration was considerably lower than previously reported EC_{50} values for *V. inaequalis* baseline populations from Germany and Chile, which were 0.09 and 0.017 µg ml^{-1}, respectively (Henríquez et al., 2011; Kunz et al., 1997). Similarly, the wild-type *V. inaequalis* isolates used in other DMI baseline studies in North America also had a relatively higher level of variability in DMI sensitivity (Henríquez et al., 2011; Smith et al., 1991) than the baseline population selected for this study. In the current study, minimum and maximum EC_{50} values of *V. inaequalis* isolates were separated by a factor of 45 with a corresponding resistance factor of 4.5 (data not shown). These factors are much lower

than those reported for other DMI baseline studies on *V. inaequalis* and other fungal pathogens (Henríquez et al., 2011; Kunz et al., 1997; Smith et al.,1991; Spolti et al., 2014). The low frequency of isolates with reduced sensitivity to difenoconazole in our baseline population could have implications for the rate of development of resistance to difenoconazole. Within a given population, isolates with an EC_{50} value above the mean for the population should have a greater likelihood for survival when difenoconazole is applied, compared to isolates more sensitive to the fungicide. In the presence of a DMI fungicide, selection for such isolates with reduced sensitivity could ultimately lead to practical resistance over time. Thus the paucity of isolates with reduced sensitivity to difenoconazole in our NY baseline population may favor a delayed onset of resistance to difenoconazole.

Of the 36 orchard populations that were confirmed to be sensitive to difenoconazole, 24 of these were either resistant or had reduced sensitivity to myclobutanil. Similarly in our research orchard with stable practical resistance to myclobutanil, we found that isolates of *V. inaequalis* were sensitive to difenoconazole in each of five years tested. These observations were in agreement with previous studies that found difenoconazole demonstrated greater intrinsic activity compared to other DMI fungicides (Henríquez et al., 2011; Kunz et al., 1997; Pfeufer and Ngugi, 2012; Thomas et al., 2012). While varying degrees of intrinsic activity among the DMI fungicides have been widely documented for a number of plant pathogenic fungi (Köller et al., 1991; Wong and Midland, 2007; Ypema et al., 1997), generally cross sensitivity within this class has historically been observed in baseline populations (Hsiang et al., 1997; Karaoglandis and Thanassoulopoulos et al., 2003; Köller et al., 1991). In the current study, a positive correlation in the responses of *V. inaequalis* isolates to myclobutanil and difenoconazole was observed when the isolates were collected from a baseline orchard. However, this degree of cross

sensitivity was not evident in a population that previously had extensive exposure and was resistant to myclobutanil. A similar inconsistency in DMI cross sensitivity had been observed in *Pyrenophora teres* for populations collected from different locations (Peever and Milgroom, 1993). A lack of cross sensitivity between two populations could be explained by differences in orchard microclimate, inherent differences in population composition (Xu et al., 2010), or type of management programs used. The absence of DMI cross-sensitivity for isolates in a myclobutanil-resistant population of *V. inaequalis* could also result from different genetic components being involved in resistance development for the two fungicides. Indeed, several mechanisms including overexpression of the target *CYP51A1* gene (Schnabel and Jones, 2001), point mutations within the *CYP51A1* gene (Délye et al., 1997), and overexpression of energy dependent drug efflux pumps (Nakaune, et al., 1998) have all been previously implicated in DMI fungicide resistance. In depth examination into the molecular biology of *V. inaequalis* isolates collected from the same population with varying levels of sensitivity to myclobutanil and difenoconazole would be needed to test for the involvement of different genetic mechanisms of resistance.

Applications of myclobutanil or difenoconazole were made to populations of *V. inaequalis* displaying practical resistance or sensitivity to myclobutanil to examine changes in the distribution of isolate sensitivities to DMI fungicides. Twenty-four applications of myclobutanil applied to a *V. inaequalis* population over six seasons did not further shift the distribution of isolates towards greater levels of myclobutanil resistance. Moreover, the population did not shift toward myclobutanil sensitivity following the cessation of myclobutanil applications. In other fungal pathogens of fruit, including *Monilinia fructicola*, a fitness penalty has been associated with isolates that have reduced sensitivity to DMI fungicides (Cox et al.,

2007). Although fitness penalties for DMI resistance have not been documented in *V. inaequalis,* we still suspect that periods without myclobutanil use (>20 years) could shift a population toward sensitivity. Indeed, populations of *V. inaequalis* formally resistant to dodine, were recently found to be sensitive to the chemistry following the curtailment of dodine use within an orchard for more than 20 years (Cox et al., 2010).

In our research orchard of 'Empire' apples with a myclobutanil-sensitive population of *V. inaequalis*, 16 applications of difenoconazole over a four-year period resulted in increased population shifts toward myclobutanil and difenoconazole resistance. Interestingly, the level of resistance to myclobutanil in terms of the population mean increased at a steady rate of 25% RG per year compared to 6.7% RG for difenoconazole. Despite the discussion above about a lack of complete cross sensitivity between myclobutanil and difenoconazole, we suspect that the field rate of difenoconazole may have quickly selected for isolates that were highly resistant to myclobutanil. Indeed, as a population becomes resistant to DMI fungicides, higher rates of a fungicide or applications of a more intrinsically active fungicide at an effectively similar rate may select for the most resistant members of a pathogen population (Lalancette et al., 1987). While the population in our research orchard of 'Empire' apples was sensitive to myclobutanil in 2010, 20% of isolates tested in the orchard had RG values greater than 60% (data not shown). Because individual isolates of *V. inaequalis* with resistance to myclobutanil were already present in the orchard, even a single season of applications from the more intrinsically active difenoconazole may have quickly selected for highly resistant members.

Overall, results of this study have shown that practical resistance to myclobutanil is present in populations of *Venturia inaequalis* throughout the major apple production regions in the northeastern United States. Unlike other fungicide classes (i.e. benzimidazoles and QoIs)

where qualitative resistance resulted in a relatively rapid emergence of resistance (<10 years of use) in the region, DMI fungicides like myclobutanil had been an essential component of apple scab management programs in the northeastern U.S. for more than fifteen years. While myclobutanil may no longer provide acceptable levels of apple scab control in most of the commercial orchards evaluated in this study, no orchard populations of *V. inaequalis* have practical resistance to difenoconazole. Still, caution should be exercised when applying difenoconazole in populations with resistance to myclobutanil; as such populations tend to have isolates with reduced sensitivity to difenoconazole.

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CHAPTER 3

OVEREXPRESSION OF THE *CYP51A1* GENE AND REPEATED ELEMENTS MEDIATE DIFFERENTIAL SENSITIVITY TO DMI FUNGICIDES IN *VENTURIA INAEQUALIS*

This chapter has been modified from work submitted for publication: Villani, S. M., Hulvey, J., Hily, J.-M., and Cox, K. D. Overexpression of the CYP51A1 gene and repeated elements mediate differential sensitivity to DMI fungicides in *Venturia inaequalis.* Phytopathology.

ABSTRACT

The involvement of overexpression of the target *CYP51A1* gene in *Venturia inaequalis* was investigated in isolates of *Venturia inaequalis* exhibiting differential sensitivity to the DMI fungicides myclobutanil and difenoconazole. Relative expression (R.E.) of the *CYP51A1* gene was significantly greater ($P < 0.0001$) for isolates with resistance to both fungicides (M_RD_R phenotype) or with resistance to difenoconazole only (M_SD_R phenotype) compared to isolates that were resistant only to myclobutanil (M_RD_s phenotype) or sensitive to both fungicides (M_SD_S phenotype). An average of nine-fold and 13-fold increases in *CYP51A1* R.E. were observed in isolates resistant to difenoconazole compared to isolates with M_RD_s and M_sD_s phenotypes, respectively. Linear regression analysis of isolate relative growth on myclobutanil-amended medium and $\log_{10}$ R.E. revealed that little to no variability in isolate sensitivity to myclobutanil could be explained by target gene overexpression ($R^2 = 0.078$). To investigate *CYP51A1* upstream anomalies associated with *CYP51A1* overexpression and/or resistance to difenoconazole, Illumina sequencing was conducted for three isolates with resistance to

difenoconazole and one baseline isolate of *V. inaequalis*. A repeated element, "EL 3,1,2", with the properties of a transcriptional enhancer was identified two to four times upstream of *CYP51A1* in difenoconazole resistant isolates, but was not found in isolates with the M_RD_s phenotype. These results suggest that different mechanisms govern resistance to two azole DMI fungicides.

INTRODUCTION

Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cooke) G. Winter, is among the most prevalent and economically important diseases of apple in the northeastern United States (MacHardy, 1996; MacHardy et al., 2001). In the U.S., disease management relies on the application of multi- and single-site fungicides due to the paucity of apple cultivars that have both durable apple scab resistance and desired horticultural traits (Köller and Wilcox, 2001; Lesniak et al., 2011; MacHardy 1996). Introduced in the 1980s, the sterile demethylation inhibitors (DMIs) are a class of broad spectrum, single-site fungicides that have consistently demonstrated high pre- and post-infection activity against a number of apple fungal pathogens including *V. inaequalis* (Köller and Wilcox, 1999; Szkolnik, 1981*;* Villani et al., 2015), *Podosphaera leucotricha* (Yoder, 2000), and *Gymnosporangium juniper-virginianae* (Yoder et al., 2014). Indeed, even ten years after their introduction for use in commercial apple orchards in the United States, DMIs provided curative activity up to 96 hours following infection by *V. inaequalis* (Beckerman et al., 2014).

The ability of DMI fungicides to arrest fungal growth during the infection process contributes to the success of this fungicide class as a potent curative fungicide. Specifically, DMI fungicides interrupt ergosterol biosynthesis by inhibiting demethylation at the 14-α carbon of lanosterol, a sterol intermediate of ergosterol, which is essential for hyphal growth (Köller and Scheinpflug, 1987). This highly specific mode of action, in addition to their repetitive use as a post-infection fungicide, has gradually led to diminished efficacy and resistance in major apple production regions throughout the United States and Canada (Braun and McRae, 1992; Hildebrand et al., 1988; Jobin and Carisse, 2007; Köller et al., 1997; Villani and Cox, 2011, Villani et al., 2015).

Unfortunately, growers have been increasingly reporting instances of suspected and/or confirmed resistance to older DMI fungicide products including Rally 40WSP/Nova 40W (myclobutanil) and Rubigan/Vintage SC (fenarimol). An extensive ten-year study on myclobutanil resistance in populations of *V. inaequalis* throughout the Northeastern and Mid-Atlantic United States concluded that 63% of the populations screened had practical resistance to the fungicide (Villani et. al., 2015). During the same study, practical resistance to the more intrinsically active DMI fungicide, difenoconazole (Inspire), was not observed in any populations with confirmed resistance to myclobutanil (Villani et al., 2015). While cross sensitivity within this class has historically been observed in other plant pathogens (Hsiang et al., 1997; Karaoglandis and Thanassoulopoulos, 2003), cross-sensitivity between myclobutanil and difenoconazole was only apparent in DMI-sensitive populations and not in DMI-resistant populations of *V. inaequalis* from NY. This lack of cross-sensitivity between myclobutanil and difenoconazole in a population of *V. inaequalis* with practical resistance to myclobutanil, suggests that different genetic mechanisms may be involved in resistance development for the two fungicides (Hawkins et al., 2011; Villani et al., 2015).

The molecular basis of resistance to the DMI fungicides has been extensively studied in both human and plant fungal pathogens, including *V. inaequalis* (Cools et al., 2013; Hamamoto et al., 2000; Hulvey et al., 2012; Luo and Schnabel, 2008; Lupetti et al., 2002; Ma et al., 2006; Schnabel and Jones, 2001). The more common mechanisms of resistance include: i) alterations to the coding sequence of the target gene resulting from point mutations (Asai et al., 1999; Becher et al., 2012; Délye et al., 1997; Stammler et al., 2009) reducing the binding affinity of DMI fungicides; ii) overexpression of energy dependent ABC and MFS transporters encoding drug efflux pumps (Hayashi et al., 2003; Hulvey et al, 2012; Nakaune et al. 1998) and iii)

overexpression of the *CYP51A1* gene (Hamamoto et al., 2000; Ma et al., 2006; Schnabel and Jones, 2001). Overexpression of the *CYP51A1* gene in phytopathogenic fungi has been attributed to insertions and repeated elements acting as transcriptional enhancers or to gene duplications increasing the copy number of the *CYP51A1* gene (Hawkins et al., 2011). In isolates of *V. inaequalis* from Michigan, overexpression of the target gene has been previously determined to be a mechanism of resistance to myclobutanil (Schnabel and Jones, 2001). An insertion of 553 bp upstream of the *CYP51A1* gene was found to contain three predicted promoters and coincided with higher expression values in myclobutanil-resistant isolates. This insertion was not always present in myclobutanil-resistant and/or high expressing isolates, and overexpression of *CYP51A1* was not always present in isolates of *V. inaequalis* resistant to myclobutanil indicating other mechanisms could be responsible for resistance to that fungicide.

The widespread adaptation of difenoconazole into apple scab management programs in conjunction with a lack of cross-sensitivity between myclobutanil and difenoconazole in isolates of *V. inaequalis* resistant to myclobutanil (Villani et al., 2015) prompted us to reexamine the role of *CYP51A1* in DMI fungicide resistance and determine if different molecular mechanisms for resistance were present. Specifically our objectives were to i) evaluate the relative expression of the *V. inaequalis CYP51A1* gene and the presence of mutations within the coding sequence of the *CYP51A1* gene for isolates with varying sensitivity phenotypes to myclobutanil and difenoconazole, and ii) conduct traditional Sanger sequencing and Illumina sequencing with *de novo* assembly to identify upstream anomalies associated with *CYP51A1* overexpression and/or resistance to either DMI fungicide.

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MATERIALS AND METHODS

Collection of *V. inaequalis* isolates. The contribution of the *Venturia inaequalis* *CYP51A1* gene in resistance to the DMI fungicides myclobutanil and/or difenoconazole was evaluated for 50 isolates of *V. inaequalis.* Isolates were selected during the 2010 and 2011 seasons from four research and commercial apple orchards located in western NY, Maine, and West Virginia, and two western NY baseline orchards. According to records submitted by growers and research personnel, there were no fewer than 20 historical DMI fungicide applications in the research and commercial orchards surveyed for this study. Isolates collected from baseline orchards were never exposed to any single-site fungicide classes including the anilinopyrimidines (APs), benzimidazoles, DMIs, guanidines, quinone outside inhibitors (QoIs), or succinate dehydrogenase inhibitors (SDHIs).

All isolates selected for this study were obtained from isolated, young, sporulating apple scab lesions originating from infections on young, fully expanded leaves. Prior to selecting a single conidium for molecular characterization of the *CYP51A1* gene, each single clonal conidial lesion isolate was phenotyped for sensitivity to the DMI fungicides myclobutanil and difenoconazole using microscopy-aided mycelial relative growth assays as previously described and validated (Frederick et al., 2014; Köller et al., 2004; Villani et al., 2015). Briefly, individual sporulating clonal conidial lesions were removed using a sterile cork borer (5 mm diameter), placed in 1.2 ml of sterile distilled water, and shaken for 60 s to dislodge conidia from the lesion. The leaf discs were promptly removed and 100 µl suspensions of 10^3 to 10^4 *V. inaequalis* conidia ml^{-1} were evenly distributed on potato dextrose agar (PDA; Difco Laboratories) amended with streptomycin sulfate (50 µg ml^{-1}), chloramphenicol (50 µg ml^{-1}), and either analytical grade myclobutanil or difenoconazole (Sigma Aldrich, St. Louis MO) dissolved in acetone (Sigma

Aldrich, St. Louis MO) at a discriminatory dose of 0.1 μg ml $^{-1}$ (Villani et al., 2015) or no fungicide. Single-conidial lesion isolates were incubated at 22°C for six days, and mycelial growth of five randomly selected micro-colonies originating from single spores was determined by measuring micro-colony diameter or germ tube length (for spores with greatly inhibited growth) using a SPOT Idea digital camera with the SPOT Imaging Basic software package (Diagnostic Instruments Inc., Sterling Heights, MI) attached to an Olympus SZX12 stereoscope (Olympus America Inc., Center Valley, PA). The in vitro sensitivity response for each single conidial isolate was expressed as mean percent relative growth (%RG) as previously described (Frederick et al., 2014; Smith et al., 1991; Villani et al., 2015).

Each clonal conidial lesion isolate was characterized as being sensitive to or having practical resistance to myclobutanil or difenoconazole based on previously validated thresholds for practical resistance (Villani et al., 2015). Isolates that had RG values greater than 60% on either myclobutanil or difenoconazole amended medium were characterized as resistant to the respective fungicide, whereas any isolate with RG values less than 60% on either DMI amended medium was considered sensitive since labeled rates of Rally 40WSP or Inspire (difenoconazole) could still effectively control isolates of *V. inaequalis* with such phenotypes under field conditions (Villani et al., 2015).

Extraction of RNA. To maintain phenotypes with varying sensitivities to DMIs, isolates of *V. inaequalis* with practical resistance (RG>60%) to myclobutanil, difenoconazole, or both DMI fungicides were maintained on PDA amended with a low concentration (0.01 μg ml^{-1}) of myclobutanil, difenoconazole, or both fungicides respectively. Alternatively, isolates sensitive to both DMI fungicides were maintained on PDA in the absence of either fungicide. Following eight weeks of incubation at 22°C, 50 to 100 mg of mycelium was harvested from the agar

surface and ground in liquid nitrogen using a sterile mortar and pestle. Total RNA was extracted from the 50 isolates of *V. inaequalis* using the Omega Bio-Tek E.Z.N.A.® Plant RNA Kit (Omega Bio-Tek, Norcross, GA). DNA was removed with the TURBO DNA-*free* Kit (Ambion® by Life Technologies, Carlsbad, CA). For all isolates, RNA was standardized to a concentration of 5 ng ml^{-1} prior to expression analysis of the *CYP51A1* gene.

cDNA synthesis and reverse transcription quantitative PCR. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to determine the relative expression of the *CYP51A1* gene for the 50 selected isolates of *V. inaequalis.* The iScript™ One-Step RT-PCR with SYBR Green Kit (Bio-Rad Laboratories Inc., Hercules, CA) utilizing the SYBR Green I reporter dye, was used for first strand DNA synthesis and all subsequent RT-qPCR reactions. To normalize the concentration of RNA, a 162-bp fragment of the *V. inaequalis* actin housekeeping gene (Accession No. AF269254) (Schnabel and Jones, 2001) was amplified with primers ViActin-F and ViActin-R (Table 3.1). Primers ViCyp51-F and ViCyp51-R (Table 3.1) were designed to amplify a 161-bp region within the *CYP51A1* gene (Accession No. AF227916). Reactions were carried out in 25 µl volumes and contained 12.5 µl 2x SYBR Green RT-PCR reaction mix (final concentration 1x), 300 nM each primer, 0.5 µl iScript reverse transcriptase for one-step RT-PCR, and 12.5 ng of RNA. Amplifications were performed in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) with the following parameters: 10 min at 50°C; 5 min at 95°C; followed by 40 cycles of 10 s at 95°C and 30 s at 57°C. For each isolate, three technical replicates were included and the experiment was repeated. A final melt curve analysis was also included to ensure proper amplification of a single amplicon. For three isolates (baseline, myclobutanil-R/difenoconazole-S, and myclobutanil-S/difenoconazole-R), induced expression of the *CYP51A1* gene was evaluated by applying a mixture of myclobutanil

and difenoconazole at their maximum labeled field rates of 333 g Ha^{-1} and 293 ml Ha^{-1}, respectively, using an airbrush sprayer (Aztek 320A, Testor). The fungicides were applied for 5 s over two *V. inaequalis* colonies (~40 mm diameter) and RNA was extracted 24 hours following application.

The comparative threshold cycle (C_T) method (Livak and Schmittgen, 2001) was used to determine *CYP51A1* expression of test isolates relative to the expression of calibrator isolate 26A-22-11, the isolate with the greatest mean difference in C_T values between *CYP51A1* and the *actin* gene (ΔC_T). Data was calibrated by determining $\Delta\Delta C_T$, the difference in ΔC_T values between the test isolate and the calibrator isolate, and relative expression of the *CYP51A1* gene for each isolate was calculated using the formula $2^{-\Delta\Delta CT}$ as described previously (Livak and Schmittgen, 2001; Schnabel and Jones, 2001). A validation experiment was performed with dilutions of the calibrator isolate prior to utilizing the C_T method, to ensure that the efficiency of both *CYP51A1* amplification and *actin* amplification were approximately equal (slope=0.01).

The effect of *CYP51A1* relative expression on DMI sensitivity phenotype (resistance to neither, both, or one DMI fungicide) was determined using the Generalized Linear Mixed Models with the GLIMMIX procedure of SAS v9.4 (version 9.4; SAS Institute Inc., Cary, NC). Differences in relative expression of isolates exhibiting different sensitivity phenotypes was determined using the 'lsmeans' statement of GLIMMIX at the 5% level of significance. Simple linear regression analysis was conducted to determine the relationship between isolate relative growth for each respective fungicide and mean relative expression ($\log_{10}$ transformed) (SigmaPlot Version 11.0, Systat Software Inc., San Jose, CA).

Extraction of genomic DNA. Prior to extraction of genomic DNA (gDNA), isolates were grown and maintained in a manner identical to that described above for RNA extraction. Following eight weeks of incubation at 22°C, 50 to 100 mg of mycelium from each isolate was harvested from the growth medium and ground in liquid nitrogen using a sterile mortar and pestle. Extraction of gDNA was accomplished using the Omega Bio-Tek E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek) in accordance with the manufacturer's instructions.

PCR amplification of the *CYP51A1* gene in *V. inaequalis*. In order to determine if mutations altering the coding sequence of the *CYP51A1* gene were present in isolates resistant to the DMI fungicide difenoconazole, the entire *CYP51A1* gene in *V. inaequalis* was sequenced for five isolates representing different difenoconazole sensitivity phenotypes. Primers were designed to amplify the *CYP51A1* gene based on the sequence of *V. inaequalis* isolate Ent23 (Accession No. AF227916) (Schnabel and Jones, 2001). Using the primer sets SVcyp-38-F/SVcyp400-R, SVcyp300-F/SVcyp1200-R, and SVcyp1080-F/SVcyp1720-R (Table 3.1), the 1,674 bp *CYP51A1* gene was amplified for a baseline isolate (3a-27-10), a myclobutanil-sensitive/difenoconazole sensitive isolate (26a-45-11), a myclobutanil-resistant/difenoconazole-resistant isolate (38b-45-11), a myclobutanil-sensitive/difenoconazole resistant isolate (40b-40-11), and a myclobutanil resistant/difenoconazole sensitive isolate (40b-28-11). For each primer set, PCR reactions were performed in 25 µl reaction volumes and contained 1X PCR buffer, 0.4 µM each primer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 0.625 U of GoTaq Flexi DNA Polymerase (Promega Corporation, Madison, WI) and 5 to 10 ng of genomic DNA. All amplifications were performed in a T100 Thermal Cycler (Bio-Rad Laboratories Inc.) with the following program: 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C; followed by a final extension of 10 min at 72°C.

PCR products were separated on a 2% agarose gel (Bio-Rad Laboratories Inc.) stained with ethidium bromide, in 1X Tris-acetate-EDTA buffer at 100 V for 1 h. Photographs of the gel were taken on a KODAK Gel Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY). Using the QiaQuick PCR Purification Kit (Qiagen, Valencia, CA), PCR products were purified and were sequenced with internal primers in both directions for each primer set (Table 3.1) using an Applied BioSystems Automated 3730xl DNA Analyzer at the Cornell University DNA Sequencing facility in Ithaca, NY. For each isolate, sequences of *CYP51A1* were assembled with using CLC Main Workbench (Version 6.8.2, CLCBio, Boston, MA). After removing two introns previously characterized by Schnabel and Jones (2001), the resulting sequences were translated and aligned to identify any anomalies in amino acid sequence among the isolates.

Cloning of the *CYP51A1* upstream promoter region. To obtain the gDNA sequence upstream of the *V. inaequalis CYP51A1* coding region, 5' rapid amplification of genomic ends (RAGE) was carried out on baseline isolate 3a-27-10 in accordance to Liu and Baird (2001). Following the addition of a poly(C) tail with terminal transferase (TdT), polymerase chain reaction was performed using primer Vicyp22R (Table 3.1), which was designed to anneal to the 5' end of the *V. inaequalis CYP51A1* coding region, and a selective anchored primer (SAP) complimentary to the cytosine tail (Liu and Baird, 2001). To improve specificity and improve DNA concentration, PCR using the product from the first reaction was conducted with universal amplification primer (UAP) (Liu and Baird, 2001) and Vicyp11R (Table 3.1). The purified product was cloned into PGEM-T easy (Promega) according to the manufacturer's instructions and ten clones were subjected to Sanger sequencing as described above.

Amplification and sequencing of the *CYP51A1* upstream promoter region for DMI resistant isolates of *V. inaequalis*. To determine putative upstream anomalies associated with resistance to difenoconazole and/or myclobutanil, PCR and Sanger sequencing were conducted for all 50 isolates of *V. inaequalis* with varying sensitivity to the two fungicides. Based on the upstream sequences obtained by RAGE and subsequent cloning, primer set Vi23F and Vi-134R (Table 3.1) was designed and used to amplify the region -738 bp to -135 bp upstream of the *V. inaequalis CYP51A1* gene (relative to baseline isolate 3a-27-10). PCR reactions were performed in 25 µl volumes as described above. Amplifications were performed in a T100 Thermal Cycler (Bio-Rad Laboratories Inc.) with the following program: 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 68°C; followed by a final extension of 7 min at 68°C. PCR products were separated on a 2% agarose gel (Bio-Rad Laboratories Inc.) stained with ethidium bromide, in 1X Tris-acetate-EDTA buffer at 100 V for 1 h. Photographs of the gel were taken on a KODAK Gel Logic 200 Imaging System (Eastman Kodak Company). PCR products were purified and sequenced as described above using primer Vi-185R (Table 3.1).

To determine the presence of anomalies in the region immediately upstream of the *CYP51A1* gene not amplified by Vi23F and Vi-134R (i.e. -134 to the first 5' adenine relative to the start), PCR was performed using primers AJ468F (Schnabel and Jones, 2001) and SV395R (Table 3.1). PCR reactions were performed in 25 µl volumes as described above. Amplifications were performed in a T100 Thermal Cycler (Bio-Rad Laboratories Inc.) using the following program: 2 min at 94°C; 35 cycles of 30 s at 94°C, 45 s at 53°C, and 2 min at 72°C; followed by a final extension of 7 min at 72°C. PCR products were separated on a 1% agarose gel (Bio-Rad Laboratories Inc.) stained with ethidium bromide, in 1X Tris-acetate-EDTA buffer

at 100 V for 1 h. Photographs of the gel were taken on a KODAK Gel Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY). PCR products were purified and sequenced as described above using primer AJ414 (Schnabel and Jones, 2001) (Table 3.1).

Upstream sequence assembly and prediction of promoter sequences. For isolates in which upstream fragments were successfully amplified for both sets of primers (Vi23F and Vi-134R; AJ468F and SV395R), assembly and alignments were made using CLC Main Workbench (Version 6.8.2, CLCBio, Boston, MA). For all isolates, the prediction of promoters within the upstream sequence of *CYP51A1* was carried out using the Berkeley Drosophila Genome Project's Neural Network Promoter Prediction software with a standard cutoff score of 0.8.

Illumina sequencing, de novo assembly, and annotation of *CYP51A1* upstream sequences. Illumina sequencing was conducted to elucidate the upstream region of *CYP51A1* for three isolates in which no fragment was generated with primer pair AJ468F/SV395R. Illumina sequencing of isolates 40b-40-11 (M_SD_R), 41b-14-11 (M_SD_R), and 43b-40-11 (M_RD_R) was accomplished using the Illumina MiSeq v3 600bp kit. Additionally, a baseline isolate, 3a-27-10, in which the 738 bp upstream sequence of *CYP51A1* had already been assembled using PCR and Sanger sequencing was included for comparison. Genomic DNA from each of the four isolates was extracted using the Omega Bio-Tek E.Z.N.A.® Plant DNA Kit as previously described. Sample preparation, including library construction was completed at the Cornell University Genomic Facility (Ithaca, NY) using the TruSeq DNA Library Prep Kit (Illumina, San Diego, CA). Digital PCR was performed at the Cornell University Genomic Facility (Ithaca, NY) on all samples to ensure optimum DNA loading for proper clustering on the Illumina sequencer. A single MiSeq run with 300 bp paired-end reads was preformed on the four pooled isolate samples expected to provide 15GB of sequence data. After sequencing, low-quality Illumina MiSeq

data were filtered and raw reads were assembled for each isolate using CLC Genomics Workbench with default settings (Version 8.0.1, CLC Bio, Aarhus, Denmark). Additional assembles were also made on the combined read data from the three difenoconazole resistant isolates to maximize coverage of the *CYP51A1* upstream region.

Table 3.1. Oligonucleotide primers used in this study and description of their amplification target.

Primer	Sequence (5' to 3')	Amplification targets
ViActin-F ViActin-R	GAT GGA GCA AAA GAA GTT CGT CA GAA TTG AGG GTA GCG TTT GGT ATA G	Quantitative PCR primers for determining the expression level of the actin gene in *V. inaequalis*
ViCyp51-F ViCyp51-R	GAT GGA GCA AAA GAA GTT CGT CA GAA TTG AGG GTA GCG TTT GGT ATA G	Quantitative PCR primers for determining the expression level of the *CYP51A1* gene in *V. inaequalis*
Vicyp22R Vicyp11R	GCAAAGGAGAGAGGAGTCCCAT AGGAGTCCCATTGTTGGTGGTGAG	Primers used during RAGE cloning to elucidate the far upstream/promoter region of the *CYP51A1* gene in *V. inaequalis*
AJ468F SV395R	ATC CCT GAA TTT GAG AGC ACT ACA TAG GAT TTG AGA GCT TCG GTG GTG AGA C	Amplification of the region -290 to +578 relative to the *CYP51A1* upstream sequence of *V. inaequalis* isolate 3a-27-10
AJ414	TGC CAA GAA GAA TGA AGG TAA A	Sequencing of the PCR product generated by primers AJ468F and SV395R Amplification of EL 3,1,2 in immediate *CYP51A1* upstream region
Vi23F Vi-134R	GAA ACC TAG GTG CAA GAG CAA TTA CCC TGG CCC TGG CTT TGG CTT T	Amplification of the region -738 to -134 relative to the *CYP51A1* upstream sequence of *V. inaequalis* isolate 3a-27-10
VI-185R	TGG CAT GGC CCA CCA ATC ACA TGC T	Sequencing of the PCR product generated by primers Vi23F and Vi-134R
SVcyp-38-F SVcyp400-R	ATA CAG ACG AGC AAC ACC ACA CT TCC TCT GCG TTG ACA TGG CTC T	Amplification of the region -38 to +400 relative to the first 5' adenosine of *CYP51A1*
SVcyp300-F SVcyp1200-R	TTT ACC TTC ATT CTT CTT GGC A ATG CAT GCG AAG AGT TTC TTT G	Amplification of the region +300 to +1200 relative to the first 5' adenosine of *CYP51A1*
SVcyp1080-F SVcyp1720-R	GGA AGA ACT ATA CCA AGA ACA A TCC CAG GCT GTT CAC CAA ACG T	Amplification of the region +1080 to +1720 relative to the first 5' adenosine of *CYP51A1*

seqSVcyp160-F seqSVcyp260-R	CAC TGG TTT CCC TTC TTC GGC A TTG CAT TCT CAT TGT CGC ATT AC	Sequencing of the PCR product generated by primers SVcyp-38-F and SVcyp400-R
seqSVcyp620-F seqSVcyp720-R	ACC CTC AAT TCA AAG GCG AAA AAC CTC CTT GCC TTG TAG TGA G	Sequencing of the PCR product generated by primers SVcyp300-F and SVcyp1200-R
seqSVcyp1340-F seqSVcyp1440-R	AGA AGT GGG AGC CTC ACC GTT G GAG TCC ATA GCC GTA ATC CTC T	Sequencing of the PCR product generated by primers SVcyp1080-F and SVcyp1720-R
EL3,1-for	TGA GCC GTG TCC GAG GTG AGT C	Amplification of EL 3,1,2 in immediate *CYP51A1* upstream region
VI-185F 7535hiup-R	AGC ATG TGA TTG GTG GGC C GTC GGT GGT AGA CTC ACC ACC AT	Amplification of EL 3,1,2 in Vi-134^{+59} $^{TO\ 228}$ *CYP51A1* upstream region

RESULTS

DMI fungicide sensitivity and expression of the *CYP51A1* gene. DMI fungicide sensitivity and expression of the *V. inaequalis CYP51A1* gene was evaluated for 50 isolates that were collected from six orchard populations in 2010 and 2011. The mean %RG of isolates on medium amended with a discriminatory concentration of myclobutanil and difenoconazole ranged from 0 to 131.2% and from 0 to 138.3%, respectively. Mean relative expression (R.E.) of the *CYP51A1* gene using the comparative C_T method ranged from 1.00 to 179.21, with the calibrator isolate, 26A-22-11, and an isolate resistant to difenoconazole, 7a-48-10, having the lowest and highest R.E., respectively (Table 3.2). Application of difenoconazole and/or myclobutanil to mature colonies of three *V. inaequalis* isolates 24 h prior to RNA extraction in an attempt to induce target gene expression did not impact the level of *CYP51A1* relative expression compared to copies of the isolates that did not receive the fungicide application prior to extraction (data not shown). Hence, induced and non-induced R.E. data for was pooled for individual isolates. Isolates resistant to difenoconazole had a mean *CYP51A1* R.E. of 45.91 ± 8.3, which was significantly higher than isolates sensitive to difenoconazole (mean R.E. = 4.19 ± 0.6;

$P < 0.0001$). For isolates resistant to myclobutanil, mean *CYP51A1* R.E. was slightly greater than isolates sensitive to myclobutanil (mean 27.68 ± 5.9 compared to 19.08 ± 7.6), but these differences were not significant ($P = 0.275$).

To understand the extent of the contribution of *CYP51A1* expression in resistance to myclobutanil and difenoconazole, linear regression analysis of isolate mean %RG on difenoconazole or myclobutanil amended medium and $\log_{10}$ R.E. values was performed. The regression analysis revealed significant relationships between isolate %RG on either difenoconazole or myclobutanil amended medium and $\log_{10}$ R. E., ($P \leq 0.0001$ and $P = 0.0489$, respectively). However, while *CYP51A1* expression explained some of the variability in isolate sensitivity to difenoconazole ($R^2 = 0.630$) (Figure 3.1a), little to none of the variability in isolate sensitivity to myclobutanil could be explained by *CYP51A1* R.E ($R^2 = 0.078$) (Figure 3.1b).

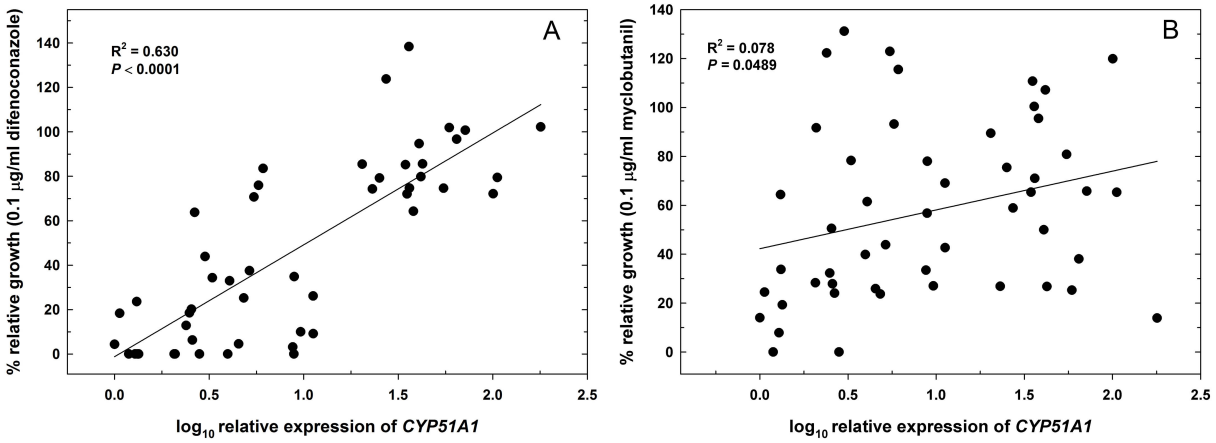

Figure 3.1. Log_{10} R.E. values of *CYP51A1* plotted against mean percent relative growth of 50 isolates of *V. inaequalis* on **(A)** difenoconazole amended medium and **(B)** myclobutanil amended medium at a discriminatory concentration of 0.1 µg/ml. A linear distribution function [y = yo + ax)] was fitted to the $\log_{10}$ R.E. values of *CYP51A1* and mean percent relative growth values for each respective fungicide

In order to gain further insight into the role of *CYP51A1* overexpression in difenoconazole resistance and/or myclobutanil resistance, isolates were further characterized into four groups

based on their in vitro sensitivity to myclobutanil and difenoconazole. Of the 50 isolates evaluated in this study, 15 were resistant to myclobutanil and difenoconazole (M_RD_R), nine were resistant to myclobutanil but sensitive to difenoconazole (M_RD_S), eight were sensitive to myclobutanil but resistant to difenoconazole (M_SD_R), and 18 were sensitive to both fungicides (M_SD_S) (Table 3.2).

Table 3.2. DMI sensitivity classification, *CYP51A1* relative expression, size of insertions present in the region -738 bp to -135 bp upstream of *CYP51A1*, amplification success of the region -134 bp upstream of *CYP51A1*, and the frequency of EL 3,1,2 in 50 isolates of *Venturia inaequalis* with differing sensitivity to the DMI fungicides myclobutanil and difenoconazole.

Collection year	County (state)	Isolate	DMI sensitivity classification[a]	*CYP51A1* relative expression [b]	Insertion size (Vi23F/ Vi-134R)[c]	Amplification with AJ468-F/SV395RC [d]	Frequency of Element 3,1,2 in *CYP51A1* promoter region
2011	Ontario (NY)	38b-45-11	M_RD_R	34.53	0	-	2
2011	Ontario (NY)	38b-24-11	M_RD_R	38.05	0	-	2
2011	Ontario (NY)	40a-35-11	M_RD_R	36.00	0	-	2
2011	Ontario (NY)	40a-45-11	M_RD_R	41.64	0	-	2
2011	Ontario (NY)	40b-25-11	M_RD_R	5.77	0	-	4
2011	Ontario (NY)	40b-33-11	M_RD_R	5.46	499	+	0
2011	Ontario (NY)	40b-7-11	M_RD_R	100.42	0	-	2
2011	Ontario (NY)	41a-37-11	M_RD_R	53.94	0	-	2
2011	Ontario (NY)	41b-37-11	M_RD_R	71.50	0	-	2
2011	Ontario (NY)	42a-1-11	M_RD_R	36.25	0	-	2
2011	Ontario (NY)	43b-40-11	M_RD_R	20.39	0	-	2
2011	Ontario (NY)	45b-24-11	M_RD_R	6.10	0	-	2
2010	Ontario (NY)	7a-15-10	M_RD_R	105.73	0	-	2
2010	Ontario (NY)	7a-27-10	M_RD_R	25.16	0	+	0

2010	Ontario (NY)	7a-7-10	M_RD_R	35.22	0	-	2
2011	Ontario (NY)	40b-40-11	M_SD_R	27.28	0	-	2
2011	Ontario (NY)	41b-14-11	M_SD_R	64.44	0	-	2
2011	Ontario (NY)	41b-36-11	M_SD_R	58.89	0	-	2
2011	Ontario (NY)	42a-10-11	M_SD_R	23.10	0	+	0
2011	Ontario (NY)	42a-2-11	M_SD_R	42.51	0	+	0
2011	Ontario (NY)	42a-40-11	M_SD_R	2.65	0	-	4
2011	Ontario (NY)	45b-4-11	M_SD_R	40.78	0	-	2
2011	Ontario (NY)	7a-48-11	M_SD_R	179.21	0	-	2
2011	Ontario (NY)	40b-28-11	M_RD_S	2.39	352	+	0
2010	Ontario (NY)	7a-1-10	M_RD_S	8.90	0	+	0
2010	Ontario (NY)	7a-4-10	M_RD_S	3.00	0	-	0
2011	Ontario (NY)	45b-33-11	M_SD_S	11.23	0	+	0
2011	Ontario (NY)	43a-20-11	M_SD_S	8.75	0	+	0
2011	Ontario (NY)	44a-23-11	M_SD_S	4.82	0	+	0
2011	Ontario (NY)	42a-30-11	M_SD_S	9.64	0	+	0
2010	Ontario (NY)	7a-6-10	M_SD_S	8.88	499	+	0
2010	Tompkins (NY)	6a-21-10	M_RD_S	1.31	0	-	0
2010	Tompkins (NY)	6a-44-10	M_RD_S	3.29	0	-	0
2010	Tompkins (NY)	6a-29-10	M_RD_S	2.09	0	+	0
2010	Tompkins (NY)	6a-37-10	M_RD_S	4.06	0	+	0
2010	Tompkins (NY)	6a-23-10	M_SD_S	3.96	0	-	0
2010	Jefferson (WV)	1a-32-10	M_RD_S	11.28	499	+	0
2010	Jefferson (WV)	1a-22-10	M_SD_S	2.55	499	+	0
2010	Jefferson (WV)	1a-31-10	M_SD_S	5.17	0	-	0
2011	Penobscot (ME)	26a-15-11	M_SD_S	4.53	224	+	0
2011	Penobscot (ME)	26a-22-11	M_SD_S	1.00	224	+	0
2011	Penobscot (ME)	26a-27-11	M_SD_S	2.49	0	+	0

2011	Penobscot (ME)	26a-45-11	M_SD_S	2.58	499	+	0
2010	Ontario (NY)	3a-18-10	M_SD_S	1.19	0	+	0
2010	Ontario (NY)	3a-19-10	M_SD_S	1.28	0	+	0
2010	Ontario (NY)	3a-27-10	M_SD_S	2.81	0	+	0
2010	Ontario (NY)	3a-41-10	M_SD_S	1.06	0	+	0
2010	Ontario (NY)	3a-50-10	M_SD_S	1.32	0	+	0
2010	Ontario (NY)	4a-37-10	M_SD_S	2.07	0	+	0
2010	Ontario (NY)	4a-45-10	M_SD_S	1.34	193	+	0

[a]DMI sensitivity classification for isolates of *V. inaequalis* based on phenotypic response of the isolate on PDA amended with 0.1 mg ml^{-1} myclobutanil or difenoconazole. Isolates in which mean %RG was ≥ 60% were characterized as resistant to myclobutanil (M_R) or to difenoconazole (D_R). Isolates with mean %RG values below 60% on myclobutanil or difenoconazole amended medium were classified as sensitive and were characterized as M_S or D_S, respectively.

[b]Relative expression of the *CYP51A1* gene is the relative amount of mRNA of the "test" isolate *CYP51A1* gene compared to the calibrator isolate 26a-22-11. The comparative threshold cycle (C_T) method was used to determine *CYP51A1* expression of test isolates relative to the expression of calibrator isolate.

[c] Size (bp) of insertions present in the region -738 bp to -135 bp upstream of the *V. inaequalis CYP51A1* gene amplified with the primer pair Vi23F and Vi-134R.

[d] Successful (+) or unsuccessful (-) amplification of the region -134 bp upstream of *CYP51A1* using primer pair AJ468F and SV395R.

When sensitivity to myclobutanil was accounted for in isolates resistant to difenoconazole (M_RD_R and M_SD_R) relative expression was significantly greater ($P \leq 0.0001$) than for isolates sensitive to difenoconazole (M_RD_S and M_SD_S) (Figure 3.2). Conversely, when the difenoconazole sensitivity classification remained constant (i.e., M_RD_S vs. M_SD_S), no significant differences in relative expression of *CYP51A1* were observed between isolates resistant or sensitive to myclobutanil (Figure 3.2).

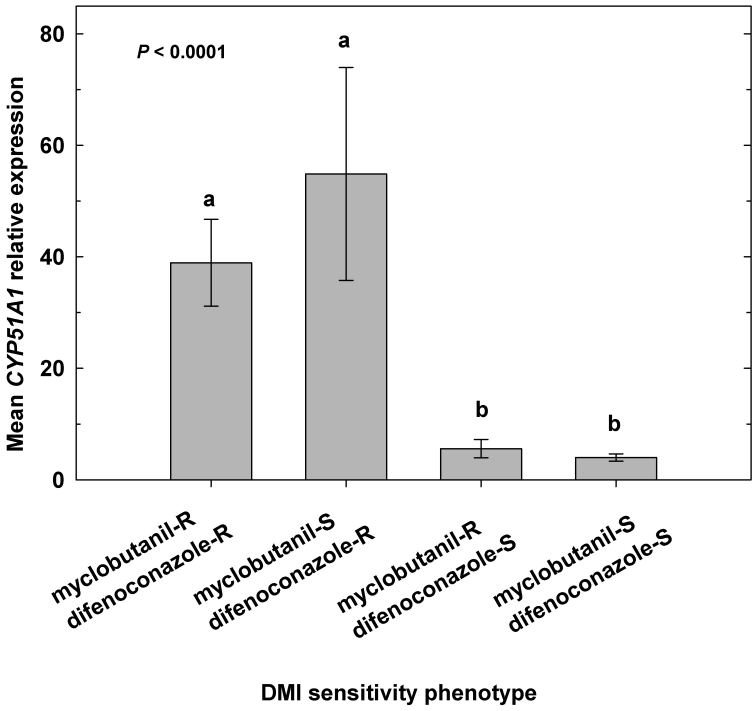

Figure 3.2. Mean *CYP51A1* R.E. according to DMI sensitivity phenotypes: myclobutanil-R/difenoconazole-R (M_RD_R, n = 15); myclobutanil-S/difenoconazole-R(M_SD_R, n = 8); myclobutanil-R/difenoconazole-S (M_RD_S, n = 9); and myclobutanil-S/difenoconazole-S (M_SD_S, n = 18). Bars denoted by the same letter are not significantly different as denoted by the 'lsmeans' statement of the GLIMMIX procedure in SAS v9.4 at the 5% level of significance.

PCR amplification of the *CYP51A1* gene in *V. inaequalis.* The 1,674 bp *CYP51A1* gene was sequenced for four isolates of *V. inaequalis* representing each the four DMI-sensitivity classifications and baseline isolate 3a-27-10. The sequence of the *CYP51A1* gene for the five isolates had 100% identity to *V. inaequalis* isolate Ent54 (Accession no. AF262756). Following identification of two previously characterized introns (Schnabel and Jones, 2001), translation revealed no missense mutations in the coding region of the gene (data not shown).

Cloning of the *CYP51A1* upstream promoter region. 5' RAGE was used to clone the upstream region of the *V. inaequalis CYP51A1* gene for baseline isolate 3a-27-10 (GenBank

Accession No. KT694303). A 738-bp region was isolated upstream of the *CYP51A1* open reading frame (ORF) and had 99% nucleotide identity to the upstream region of *V. inaequalis* isolate Ent23 (Accession No. AF227916, Schnabel and Jones, 2001). A novel 387-bp region of DNA was discovered in the region directly upstream of the previously characterized upstream region of the *V. inaequalis CYP51A1* gene (Schnabel and Jones, 2001). Given that none of the previously described upstream insertions (Schnabel and Jones, 2001) were detected in the upstream region of the baseline isolate, all insertions or deletions in the promoter region of *CYP51A1* will be presented in the context of isolate 3a-27-10 unless otherwise noted.

Amplification of the region -738-bp to -135-bp upstream of the *CYP51A1* gene was accomplished using primer set Vi23F and Vi-134R and yielded fragments of 593, 604, 797, 828 or 1,103 bp. The anticipated 604 bp fragment was amplified in 40 of the 50 *V. inaequalis* isolates and was present across all DMI sensitivity classifications. For nine of the 50 isolates, sequence analysis of the three larger fragments revealed individual insertions of 193, 224, or 499 bp, respectively. The 224 and 499 bp insertions upstream of *CYP51A,* were identical to those reported by Schnabel and Jones (2001). The 193 bp insert was located in a single isolate -321 bp upstream of the *CYP51A1* gene (4a-45-11; GenBank Accession No. KT694304), while the 224 bp insertion and 499 bp insertions were both located at position-323. In one isolate, 40b-28-11 (GenBank Accession No. KT694305), a 363 bp region located between positions -686 to -324 upstream of *CYP51A1* was deleted and replaced by a 352 bp sequence. This substituted region had 279 continuous nucleotides in common with the 499 bp insertion. The entire sequences of both the 193 bp insertion and the 224 bp insertion were embedded within the 499 bp insertion, however,

were disrupted by a 304 bp sequence and a 275 bp sequence, respectively, containing a predicted promoter (see below).

Using a cutoff score of 0.8, two promoters, designated Promoter 1 (5'-AGGCCCGATTGATATATTACGCCGAGACGGCAATTCCCGTGGCAACCCGC-3'; score = 0.96) and Promoter 2 (5'-ATCGTAACCATAATAAAGGCGTAGGATTGGAAGTTTAATAAGTGGAATC-3'; score = 0.93), were predicted within the 499 bp and 352 bp insertions. Promoter 2 was the only promoter predicted within the 193 and 224 bp insertions. These promoters were identical to those identified within the 225 bp and 500 bp insertions characterized previously (Schnabel and Jones, 2001). The presence of either or both promoters in the region -738 bp to -135-bp upstream of *CYP51A1* did not impact expression of the *CYP51A1* gene. Relative expression of isolates with anomalies in this region ranged from 2.55 to 11.28, while the R.E. of isolates that contained no insertions (604 bp fragment), in this upstream region ranged from 1.06 to 179.21. In addition, the 499 bp insertion, containing Promoters 1 and 2, was detected in isolates that were resistant or sensitive to both DMI fungicides (Table 3.2).

Amplification of the region -134 bp upstream of the *CYP51A1* gene using primers AJ468F and SV395R produced an 868 bp fragment in 25 of 50 isolates and a 1,051 bp fragment for a single *V. inaequalis* isolate. Sequence analysis of PCR products revealed an insertion of 183 bp in a single isolate (1A-22-10) with a M_SD_S phenotype (GenBank Accession No. KT694306). The insertion was located -65 bp relative to the first 5' adenine of *CYP51A1* and contained a single promoter (5'-GACGAAACTTATTTATAGAAGGTCTGTGCGTAGGTGGGCCGACGTTTAGC-3') with a

score of 0.94. Upstream sequences for the other 25 isolates with the 868 bp fragment were identical sequences and no insertions were identified.

For 24 of the 50 isolates, no amplification of the region -134bp upstream of the *CYP51A1* gene was achieved using the AJ48F/SV395R primer set. Several other primer combinations located within 200 bp of the AJ48F/SV395R primer sites also failed to amplify (data not shown). Relative expression was significantly greater ($P = 0.0005$) for isolates in which amplification of the immediate upstream region was not obtained (mean R.E. = 39.28 ± 8.3) compared to isolates in which amplification was achieved (mean R.E. = 7.48 ± 1.9). In addition, the majority (79%) of these isolates were characterized as resistant to difenoconazole. Within the four DMI sensitivity characterizations, no amplification of the region -134 bp upstream of *CYP51A1* was achieved for 87% and 75% of isolates with the M_RD_R or M_SD_R phenotype, respectively, compared to 33% and 11% for those with the M_RD_S or M_SD_S phenotypes, respectively.

Illumina sequencing and bioinformatics. Illumina MiSeq sequence reads for one baseline isolate and three isolates resistant to difenoconazole yielded an average of 9 million paired-end reads of Illumina sequence data. For each isolate, an average of 10,000 contigs of approximately 300 bp in length were assembled. To obtain maximum coverage of the *CYP51A1* upstream region, genomic reads were combined and assembled for all three difenoconazole resistant isolates. The combined assembled genome of the three difenoconazole resistant isolates revealed that the region -134bp upstream of *CYP51A1* was split between two contigs. Alternatively, the -738 bp upstream of *CYP51A1*, which had previously been sequenced for isolate 3a-27-10, was found on a single contig for the baseline isolate. Due to the number of repeated elements (see below) and presumed distance between the two upstream contigs (≥46

kbp) assembly of the two contigs upstream of *CYP51A1* could not be accomplished for the difenoconazole resistant isolates.

In the combined genome assembly for difenoconazole resistanct isolates, a novel sequence of 474 bp (Figure 3.3) was identified upstream of *CYP51A1* on the contig containing the *CYP51A1* gene (GenBank Accession No. KT694307). In this sequence, a unique 169 bp repeatable element, designated "EL 3,1,2", was detected 379 bp from the 5' end of *CYP51A1* and again 59 bp downstream of primer Vi-134 primer binding site (Vi-134$^{+59\ TO\ 228}$) (Figure 3.3). This element was not located anywhere in the genome of the baseline isolate, but was repeated four times throughout the genome of isolate 40b-40-11 and twice throughout the genomes of isolates 41b-14-11 and 43b-40-11. A BLAST database search of EL 3,1,2 identified no similarity to any known sequences.

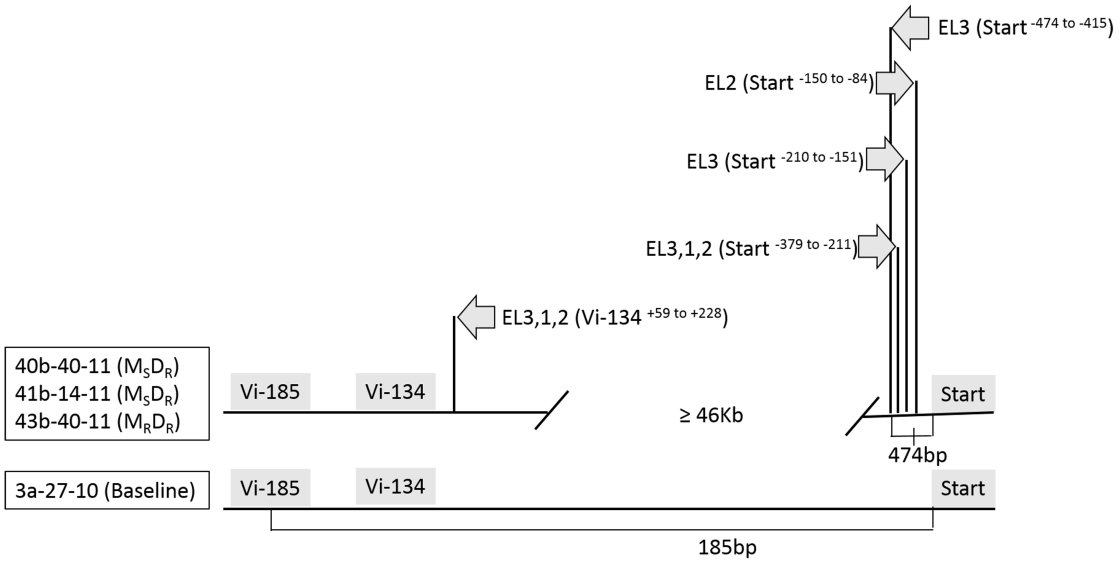

Figure 3.3. Illustration of the upstream region of the *V. inaequalis CYP51A1* gene based on the combined assembly of difenoconazole-resistant isolates 40b-40-11, 41b-14-11, and 43b-40-11. Positions of repeated elements mapped downstream of the ~46 kb region between Vi-134 and the *CYP51A1* gene are relative to

the start of the gene (i.e. Start$^{-150 \text{ to } -84}$), whereas those mapped prior to the ~46 kb region are relative to primer Vi-134R (i.e. Vi-134$^{+59 \text{ to } +228}$). An illustration of a baseline isolate is included to provide comparison of element insertion positions. Positions of elements and insertions are meant to demonstrate relative position and are not drawn to scale.

Three shorter repeated elements were identified within EL 3,1,2 and throughout the genome of the combined assembly for the three difenoconazole resistant isolates. The first, designated "EL1", was a 42 bp sequence that was observed twice upstream of *CYP51A1* in EL 3,1,2 in all three resistant isolates (Figure 3.3). Throughout the assembled genome sequence data, EL1 was found once in the baseline isolate and as many as six times in the assembled genome of the difenoconazole resistant isolates. The second element, "EL2", was a 67 bp sequence found three times upstream of *CYP51A1:* twice in EL 3,1,2 and once by itself (Figure 3.3). Throughout the assembled genomes, EL2 was found once in the baseline isolate and up to ten times in the assembled genome of the difenoconazole resistant isolates. Element "EL3", a 60 bp sequence, was identified four times upstream of *CYP51A1* in the assembled genome of the difenoconazole resistant isolates (Figure 3.3). Throughout the assembled genomes, it was found six times in the assembled genome of the difenoconazole resistant isolates, but not found in the baseline isolate.

Based on the combined assembled genome of the three *V. inaequalis* isolates, primers were designed to verify the correct assembly of the *CYP51A1* upstream region and to also detect the presence of EL 3,1,2 in isolates of *V. inaequalis* in which amplification of the region -134 bp upstream of *CYP51A1* was unsuccessful. Primer pair EL3,1-for/AJ414 (Table 3.1) amplified a 907 bp fragment directly upstream of *CYP51A1* that contained EL 3,1,2 (Figure 3.4). To detect the presence of EL 3,1,2 in the region downstream of the Vi-134 primer binding site (Vi-134$^{+59 \text{ TO } 228}$), primers Vi-185F and 7535hiup-R (Table 3.1) amplified a 450 bp for 17 of 23 isolates with difenoconazole resistance in which a single insertion of EL 3,1,2 was present (GenBank

Accession No. KT694308). In two isolates, 42a-40-11 and 40b-25-11, a 957 bp fragment was produced (Figure 3.4). Sequence analysis of these two isolates revealed that EL 3,1,2 was repeated in tandem four times (GenBank Accession No. KT694309), however EL 3,1,2 was not present in the region directly upstream of *CYP51A1* for these two isolates. EL 3,1,2 was repeated a minimum of two times upstream of *CYP51A1* in 19 of 23 (83%) isolates resistant to difenoconazole and was not observed in the *CYP51A1* region for any isolates sensitive to difenoconazole (Table 3.2).

Determination of putative *CYP51A1* transcriptional enhancers. No promoters with a score of 0.8 or greater were identified within EL 3,1,2. In addition, no promoters were detected within the 474 bp sequence (see above) characterized upstream of *CYP51A1* for isolates with EL 3,1,2. Interestingly, analysis of transcription factor (TF) binding sites within 474 bp upstream of *CYP51A1* revealed differences in TF binding sites relevant to *CYP51A1* expression and/or DMI fungicide resistance. Within the 474 bp upstream of *CYP51A1* for isolate 40b-40-11, there were 15 binding sites for Stb5p, a TF activator for *ERG11* (*CYP51A1*) in yeast compared to only six in the baseline isolate (3a-27-10). Additionally, isolate 40b-40-11 had fewer TF binding sites 474 bp upstream of *CYP51A1* for the ergosterol biosynthesis repressor Mot3p (Montañés et al., 2011) in comparison to the baseline isolate (3a-27-10) with 3 and 5 binding sites, respectively.

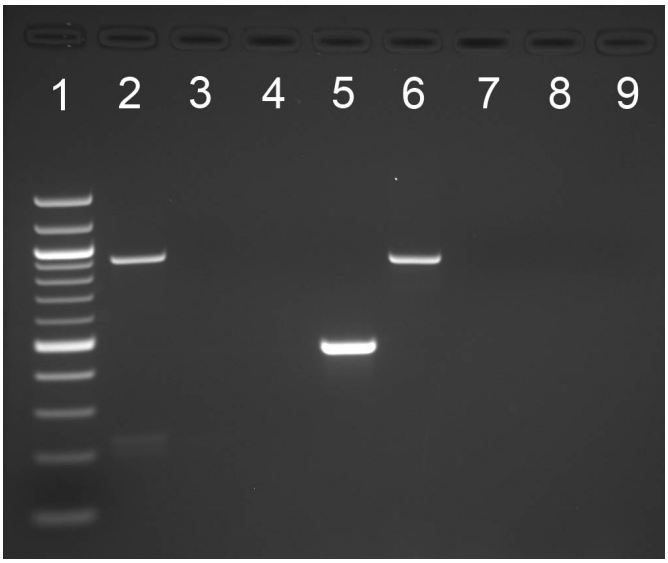

Figure 3.4. Amplification of a 907 bp fragment containing EL 3,1,2 in the region directly upstream of *CYP51A1* (primers EL3,1-for/SV395R; lanes 2-5,8) or a 450 bp or 957 bp fragment containing EL 3,1,2 at downstream of position Vi -134$^{+59\ TO\ 228}$ (primers Vi-185F/7535hiup-R lanes 5-7,9). EL 3,1,2 was repeated in tandem four times in isolates that produced the 957 bp fragment. Lane 1, Quick-Load 100-bp DNA Ladder (New England Bio-Labs); Lanes 2 and 5, isolate 38b-24-11; lanes 3 and 6, isolate 40b-25-11; lanes 4 and 7, isolate 1a-31-10; lanes 8 and 9, non-template control.

DISCUSSION

Sterol demethylation inhibitor fungicides target the 14α-demethylase enzyme, an essential precursor to ergosterol biosynthesis encoded by the *CYP51A1* gene. Resistance to DMIs has been attributed to a number of mechanisms including amino acid alterations in the target enzyme, overexpression of genes encoding drug efflux pumps, and overexpression of the *CYP51A1* gene. While several studies have previously focused on elucidating the molecular mechanisms of resistance for a single DMI fungicide (Cools et al., 2013), in the present study we found that the molecular mechanism for resistance to difenoconazole was not responsible for resistance to myclobutanil in isolates of the apple scab pathogen, *Venturia inaequalis.* To our

knowledge, this is the first report of a plant pathogen exhibiting different mechanisms of resistance to two azole DMI fungicides.

Overexpression of the *CYP51A1* gene has been implicated in DMI fungicide resistance for several phytopathogenic fungi including *Penicillium digitatum* (Hamamoto et al., 2000; Sun et al., 2013), *Blumeriella jaapii* (Ma et al., 2006), *Monilinia fructicola* (Luo and Schnabel, 2008), *Puccinia triticina* (Stammler et al., 2009), and *Mycospharella graminicola* (Cools et al., 2010). In this study, relative expression of *CYP51A1* was evaluated for 50 isolates of *V. inaequalis* exhibiting varying levels of sensitivity to difenoconazole and myclobutanil. Isolates resistant to difenoconazole (those with the M_RD_R and M_SD_R phenotype) had a nine-fold increase in *CYP51A1* relative expression compared to isolates with an M_RD_S phenotype and a 13-fold increase in relative expression compared to isolates an M_sD_s phenotype. This level of expression was similar to other phytopathogenic fungi resistant to DMI fungicides where expression values were observed to be more than five-fold greater when target gene overexpression was the primary mechanism of resistance (Hamamoto et al., 2000; Luo et al., 2008; Ma et al., 2006).

CYP51A1 overexpression and subsequent DMI fungicide resistance can result from a number of factors. In the human pathogen *Candida glabrata* (Marichal et al., 1997) and the plant pathogen *Fusarium graminearum* (Liu et al., 2011) multiple copies of *CYP51* were found to be the driving force of target gene overexpression. Genomic sequencing of a baseline isolate of *V. inaequalis* and of three difenoconazole-resistant isolates found no paralogous *CYP51* genes, but a unique 169-repeatable element upstream of *CYP51A1* was identified. This element, EL 3,1,2, was repeated two to four times upstream of *CYP51A1* in 83% of difenoconazole-resistant isolates but not in isolates sensitive to difenoconazole.

Although EL 3,1,2 is highly associated with difenoconazole-resistance and *CYP51A1* overexpression, the repeatable element was not found in the upstream region of *CYP51A1* for two isolates with M_SD_R phenotypes and for two isolates with M_RD_R phenotypes. The absence of EL 3,1,2 in these four isolates further illustrates the complexity of DMI fungicide resistance in *V. inaequalis* because resistance to DMIs may be governed not only by different mechanisms,, but the molecular basis promoting those mechanisms may differ as well. For example, in myclobutanil-resistant isolates from Michigan, the presence of a 553 bp insertion upstream of *CYP51A1* was linked to overexpression of the *V. inaequalis CYP51A1* gene (Schnabel and Jones, 2001). However, this insertion was only present in 23% of myclobutanil-resistant isolates and in isolates with a *CYP51A1* relative expression value of 5.0 or greater. Furthermore, we previously identified the 553 bp insertion in two DMI-sensitive isolates of *V. inaequalis* from Virginia (Cox et al., 2008). In the present study, the correlation of EL 3,1,2 and difenoconazole resistance was more apparent in isolates with overexpression of *CYP51A1* (Schnabel and Jones, 2001), being identified in the majority (83%) of isolates with difenoconazole-resistance.

The presence of EL 3,1,2 solely in isolates with difenoconazole resistance strongly supports its association with resistance to difenoconazole and as a driving factor of *CYP51A1* overexpression. Similar relationships between repeated elements in the upstream region of *CYP51A1* and overexpression of the gene have previously been characterized. In *P. digitatum*, a 126-bp element acting as a transcriptional enhancer was tandemly repeated five times upstream of *CYP51* in DMI resistant strains and only once in DMI-sensitive strains. While no promoters were predicted within the 126 bp tandem repeat, it was suggested that the presence of known fungal and vertebrate transcription factor (TF) binding sites within the sequence might serve as a mechanism for *CYP51* transcriptional enhancement (Hamamoto et al, 2000).

Similar to the 126 bp tandemly repeated element found in *P. digitatum,* TF binding sites, but no predicted promoters, were found in El 3,1,2. Although, there were only 15 TF binding sites present, eight had the capacity to bind the transcriptional enhancer zinc cluster protein TF Stb5p. Like many zinc cluster proteins (Macpherson et al., 2006; Rank et al., 1975; Saunders and Bank, 1982; Wehrschutz-Sigl et al., 2004) Stb5p has been implicated as a positive regulator of multi-drug resistance genes and also as a direct activator of *ERG11* (*CYP51A1*) (Larochelle et al., 2006; Macpherson et al., 2006) in yeast. The introduction of eight Stb5p binding sites within each EL 3,1,2 insertion upstream of *CYP51A1* may have contributed to the observed higher *CYP51A1* expression values in difenoconazole-resistant *V. inaequalis* isolates. Due to the paucity of predicted promoters in the upstream region of *CYP51A1*, we also searched for TF binding sites 474 bp upstream of the first 5' adenine of *CYP51A1* for both baseline isolate 3a-27-10 and M_SD_R isolate 40b-40-11. Compared to the baseline isolate, which had six Stb5p binding sites within the queried region, 15 Stb5p binding sites were identified for the difenoconazole resistant isolate, which included the eight from El 3,1,2.

In addition, two additional binding sites for the TF Mot3p were confirmed in the *CYP51A1* upstream region of the baseline isolate compared to the difenoconazole resistant isolate. Unlike Stb5p, Mot3p represses the transcription of ergosterol biosynthesis genes (Montañés et al., 2011). The presence of these two transcription regulators, which appear to function antagonistically, raises questions regarding the importance of their ratio (Kim and Forger, 2012) in *CYP51A1* expression and subsequent difenoconazole resistance. In baseline isolates, the ratio of Mot3p (repressor) to Stb5p (activator) was 5:7, compared to 3:15 in the difenoconazole-resistant isolates. Thus, it may not simply be the introduction of Stb5p binding

sites into the immediate upstream region of *CYP51A1*, but also the lower ratio of the TFs repressing ergosterol biosynthesis.

The absence of EL 3,1,2 and the relatively low levels of *CYP51A1* relative expression in isolates of *V. inaequalis* with the M_RD_S phenotype, suggests that the molecular basis for resistance is different between myclobutanil and difenoconazole. Indeed, mean relative *CYP51A1* expression for isolates of *V. inaequalis* with resistance to myclobutanil only (M_RD_S) was not significantly different than isolates exhibiting the M_SD_S phenotype indicating that the contribution of target gene overexpression in myclobutanil resistance is minor. Regression analysis of isolate %RG on myclobutanil or difenoconazole amended medium and $\log_{10}$ *CYP51A1* relative expression values further supported the observation that target gene overexpression was not a primary genetic determinant of resistance to myclobutanil. Indeed, a similar relationship was observed for propiconazole-resistant isolates of *Sclerotinia homoeocarpa* and the *S. homoeocarpa* efflux transporter, *ShatrD*, which led to the conclusion that *ShatrD* overexpression and not *CYP51* overexpression was the driving force behind propiconazole resistance in that pathosystem (Hulvey et al., 2012). The low levels of *CYP51A1* expression for isolates with M_RD_S phenotypes in this study are in stark contrast to *CYP51A1* expression values for myclobutanil-resistant isolates of *V. inaequalis* from Michigan, which had an 18-fold increase in *CYP51A1* relative expression (Schnabel and Jones, 2001). While difficult to speculate the reasons for this discrepancy between the two studies, it is not beyond the realm of possibility that the isolates screened in the 2001 study could have had a multiple DMI resistance phenotype that was mediated by overexpression of the target gene.

Resistance to DMI fungicides has also been conferred in fungi by the presence of point mutations leading to a change in amino acid sequence of *CYP51A1* (Becher and Wirsel, 2012;

Délye et al., 1997; Gadoury et al., 2012; Wyand and Brown, 2005). Depending on the location of an amino acid substitution, the binding affinity of DMI fungicides can be significantly reduced. Unlike the target genes of QoI fungicides where amino acid substitutions are generally conserved across all fungal genera (Fernández-Ortuño et al., 2008) *CYP51* point mutations conferring DMI resistance are not frequently conserved in phytopathogenic fungi (Becher and Wirsel, 2012; Cools et al., 2013).-Upon-verifying the predicted *CYP51A1* amino acid sequence (Schnabel and Jones, 2001), we found no alterations within the coding sequence among any of the isolates, or any of the mutations that have previously been associated with DMI resistance in other phytopathogens (Cools et el., 2013).

Overall, the results of this study have shown that the *CYP51A1* gene is overexpressed in isolates of *V. inaequalis* with practical resistance to difenoconazole (M_SD_R) or to difenoconazole and myclobutanil (M_RD_R), but not in isolates with practical resistance to myclobutanil only. This finding suggests that resistance between myclobutanil and difenoconazole may develop independently and helps to explain the apparent lack of cross-sensitivity in several isolates with practical resistance to myclobutanil. The extent that overexpression of *CYP51A1* contributes to resistance of other DMI fungicides in *V. inaequalis* is still undetermined. Although we were unable to elucidate the mechanism of myclobutanil resistance, the involvement of overexpressed genes encoding drug efflux transporters (Hulvey et al., 2012; Hayashi et al, 2002; Nakaune et al., 1998) has yet to be evaluated for *V. inaequalis*. Identification and expression analysis of undiscovered drug efflux pumps and the mechanisms promoting their overexpression, might not only provide explanation for myclobutanil resistance in *V. inaequalis* but could also provide insight into multiple-fungicide resistance for this pathogen.

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CHAPTER 4

HETEROPLASMY OF THE CYTOCHROME B GENE IN *VENTURIA INAEQUALIS* AND ITS INVOLVMENT IN QUANTITATIVE AND PRACTICAL RESISTANCE

This chapter has been modified from a publication referenced: Villani, S. M., and Cox, K. D. 2012. Heteroplasmy of the cytochrome b gene in *Venturia inaequalis* and its involvement in quantitative and practical resistance to trifloxystrobin. Phytopathology 104:945-953.

ABSTRACT

Quantitative (partial) and qualitative (complete) resistance responses to quinone outside inhibitor (QoI) fungicides have been documented for the apple scab pathogen *Venturia inaequalis*. Resistance monitoring efforts have traditionally focused on the detection of qualitative resistance based on a single point mutation, G143A, within the cytochrome b (*cyt b*) gene. In order to better understand the role of heteroplasmy of the *cyt b* gene in the QoI resistance response for isolates and populations of *V. inaequalis*, an allele-specific quantitative PCR (AS-qPCR) was developed to quantify the relative abundance of the A143 (resistant) allele in 45 isolates of *V. inaequalis* with differing in vitro resistance responses to the QoI fungicide trifloxystrobin. Although a high relative abundance of the A143 allele (>62%) was associated with isolates with high resistance responses (50 to 100% relative growth on trifloxystrobin amended medium), heteroplasmy of the *cyt b* gene was not the primary factor involved in isolates with moderate resistance responses (29 to 49% relative growth). The relative abundance of the A143 allele in isolates with moderate resistance to trifloxystrobin rarely exceeded 8% suggesting that other resistance mechanisms are involved in moderate resistance and that the QoI resistance response is therefore polygenic. In

research orchards where QoI fungicides failed to control apple scab (practical resistance), field trials were conducted to demonstrate the link between practical resistance and the abundance of the A143 allele. Relative abundance of the A143 allele in these orchard populations exceeded 20% in 2011 and 2012. Similarly, of the eight additional commercial orchards screened in 2011, the relative abundance of the A143 allele always exceeded 20% in those with QoI practical resistance. While heteroplasmy of the *cyt b* gene did not entirely explain the response of isolates with moderate resistance to QoIs, the relative abundance of A143 in orchard populations of *V. inaequalis* helps to explain the point of emergence for practical resistance to trifloxystrobin across several orchard populations with differing production histories.

INTRODUCTION

Apple scab, caused by the pathogen *Venturia inaequalis* (Cooke) G. Winter, is one of the most devastating diseases of apple in the northeastern United States (34,35). In the absence of durable resistance in most of the commercially desirable apple cultivars, fungicides provide the only means for controlling apple diseases (34,36). Because of the polycyclic nature of apple scab coupled with the temperate climate of the northeastern United States, which results in cool, humid weather during spring and early summer, apple growers must apply fungicides 10 or more times per season to control the disease (24,35). The use of modern site-specific fungicide chemistries such as sterol demethylation inhibitors (DMIs, 1980s) and quinone outside inhibitors (QoIs, late 1990s) has enabled apple producers to manage apple scab and many other key fungal diseases (i.e. cedar apple rust and powdery mildew) while minimizing harmful effects on non-target organisms and to the environment (21-23,25,27,43). Unfortunately, because these fungicides are single-site inhibitors (i.e., they block a single biochemical pathway in fungi), repetitive use has selected for the emergence of resistant *V. inaequalis* populations in the apple production regions east of the Mississippi throughout the last half of the 20th century. Such was the case for dodine resistance in the late 1960s (15,47), benzimidazole resistance in the 1970s (15,16,19), and DMI resistance in the early 1990s (25). Since the turn of the 21st century, concerns of widespread resistance to the DMI fungicides have led to greater reliance on QoI fungicides to manage apple scab in this region. Not long after their introduction, isolates of *V. inaequalis* with QoI resistance were discovered in an orchard in Michigan (23) and by 2009 widespread resistance to QoI fungicides was documented in *V. inaequalis* populations from commercial apple orchards in Michigan (3,27). Although QoI resistance in *V. inaequalis*

developed rapidly in Michigan (3,27), there have been few reports of resistance to QoIs in orchards from apple producing regions in the eastern United States (4,12).

QoI fungicides inhibit mitochondrial respiration by binding to the outer quinone oxidizing pocket of the cytochrome bc_1 enzyme complex III. This binding inhibits electron transfer in the respiratory pathway, which terminates cellular respiration and thus the production of ATP (2,13,21). One of the most common mechanisms of QoI resistance described for fungal pathogens is a single point mutation at amino acid position 143 within the cytochrome b (*cyt b)* gene. This mutation results in a change from glycine to alanine (G143A) and seems to impart a qualitative, vertical, or complete resistance response whereby isolates are highly resistant to QoI fungicides (23). The presence of this single nucleotide polymorphism (SNP) in *cyt b* has been consistently associated with qualitative resistance responses to QoI fungicides in a number of fungal pathogens (2,7,10,17,48) including *V. inaequalis* (4,8,23,27,40,49,50). For the remainder of the manuscript, we will use the terms 'qualitative resistance' to refer to an isolate with a complete resistance phenotype and 'qualitative resistance response(s)' to refer to any response(s) imparted by the presence of one or more mitochondria with the G143A *cyt b* gene mutation. Other point mutations within the *cyt b* gene at amino acid position 129 where phenylalanine is exchanged with leucine (F129L) and at position 137 where glycine is replaced by arginine (G137R) have been shown to confer diminished QoI sensitivity in fungal pathosystems (13,17,18,41,44,45). However, none of these or other mutations have been reported in *V. inaequalis* (8,27,37).

In addition to the qualitative resistance response, there appears to be a quantitative (polygenic or partial) resistance response to QoI fungicides (23) whereby isolates display a range of sensitivity phenotypes when exposed to doses of QoIs below the point at which the alternative

respiration pathway becomes activated (6,40). Such in vitro responses are regarded as quantitative for an individual isolate. For the remainder of the manuscript, we will use the term 'quantitative resistance' and 'quantitative resistance response(s)' to refer to the collection of factors working in concert that are responsible for an isolate's response to QoI fungicides. We will also use these terms to refer to an isolate's overall phenotypic response to QoI fungicides in vitro.

Although quantitative responses are typically explained by the involvement of several genes with minor contributions to an overall phenotype, differences in the ratios of mutant (resistant, A143) to wild type (sensitive, G143) copies of the *cyt b* gene could also account for the way that *V. inaequalis* isolates within a population express quantitative resistance to trifloxystrobin. Because each single *cyt b* gene is encoded by a single mitochondrion, and there are usually more than 10 mitochondria per cell (34) there are several *cyt b* genes within just a single cell of *V. inaequalis.* Each copy of *cyt b* might either be resistant to QoI fungicides and thus have the A143 allele, or may be sensitive to QoIs and thus have the G143 allele. The nature of *cyt b* heteroplasmy has been well documented (26,31,32) however its role in quantitative practical resistance to QoI fungicides had not been investigated. Populations with varying ratios of A143 to G143 are described as having heteroplasmy of the *cyt b*, and this phenomenon has been described for several fungal pathogens, including *V. inaequalis* (3,10,18,26,27,30,37,50). However, none of these studies have addressed the contribution of *cyt b* heteroplasmy to the quantitative resistance response for individual isolates or attempted to relate the abundance of the A143 allele in a field population to the development of practical resistance to QoI fungicides. Practical resistance is defined as the point within the quantitative resistance continuum where applications of the fungicide result in greatly diminished levels of disease control under field

conditions (20). It has been suggested that practical resistance to QoI fungicides in orchard populations of *V. inaequalis* develops when the population transitions from isolates with quantitative resistance to isolates with qualitative (complete) resistance (23). However, we suspect that practical resistance may appear when the relative abundance or frequency of A143 (resistant) allele for a given field population of *V. inaequalis* exceeds some undefined threshold for the QoI fungicide in question.

A quantitative approach such as allele-specific quantitative PCR (AS-qPCR) could be useful for investigating the role of heteroplasmy of the *cyt b* gene in quantitative resistance responses for individual isolates and the subsequent development of practical resistance in field populations. AS-qPCR has already been used for detecting and quantifying QoI resistance in *Blumeria graminis* f.sp. *tritici* in wheat (10), *Alternaria* species infecting pistachio (29,31-33), and even *V. inaequalis* (37). Quantification of the relative amounts of the *cyt b* A143 allele may help explain the apparent quantitative resistance responses of *V. inaequalis* isolates to QoI fungicides in vitro (23). In addition, quantifying the relative abundance of the A143 allele in an orchard population may improve our understanding of the development of practical resistance. The goal of this study was to develop a better understanding of the role of *cyt b* gene heteroplasmy in resistance to QoI fungicides for isolates and populations of the apple scab pathogen *V. inaequalis*. Specifically we wished to use AS-qPCR to detect and quantify *cyt b* heteroplasmy in monoconidial isolates, thereby allowing us to examine the contribution of the A143 allele to the quantitative resistance response to trifloxystrobin. We also conducted field trials and surveys in conjunction with *cyt b* AS-qPCR to learn how qualitative resistance responses are involved in the development of practical resistance for orchard populations of *V. inaequalis*.

MATERIALS AND METHODS

Collection of *V. inaequalis* isolates and the determination of quantitative resistance to trifloxystrobin in vitro. A total of 45 monoconidial isolates of *V. inaequalis* that represented the scope of typical QoI fungicide sensitivity responses were selected to examine heteroplasmy of the *cyt b* gene in regards to the G143A mutation. These isolates were collected from 12 commercial apple orchards in the northeastern United States, and from two baseline orchards in western NY that had never been directly exposed to QoIs or other modern fungicide chemistries (e.g. DMIs, guanidines, and benzimidazoles). The selected isolates were obtained from apple scab lesions originating from infections on young fully-expanded leaves and were phenotyped for quantitative resistance to the QoI fungicide trifloxystrobin using a modified microscopy-aided mycelial relative growth assay previously described and validated (23). Briefly, individual sporulating lesions were removed using a sterile cork borer (5 mm diameter) and stored in a dry, sterile 1.5 ml microcentrifuge tube at room temperature for no longer than three months. Prior to fungicide sensitivity evaluation, conidia were separated from the lesion by shaking the disc in 1 ml of sterile distilled water for 60 s. The leaf discs were promptly removed and 100 µl suspensions of 10^3 to 10^4 *V. inaequalis* conidia ml^{-1} were evenly distributed on potato dextrose agar (PDA) (Difco Laboratories) amended with streptomycin sulfate (50 µg ml^{-1}), chloramphenicol (50 µg ml^{-1}), and analytical grade trifloxystrobin (TR medium) (Sigma Aldrich, St. Louis MO) at a discriminatory dose of 0.02 µg ml^{-1} as suggested by Köller et al. (23). Additionally, 100 µl conidial suspensions were distributed on PDA amended only with streptomycin sulfate and chloramphenicol (PDA++) as a control. Isolates were incubated at 22°C for six days, and mycelial growth of five randomly selected micro-colonies was determined by measuring micro-colony diameter or germ tube length using a SPOT Idea digital camera (with

the SPOT Imaging Basic software package, Diagnostic Instruments Inc., Sterling Heights, MI) attached to an Olympus SZX12 stereoscope (Olympus America Inc., Center Valley, PA). The quantitative resistance response for each isolate was expressed as mean percent relative growth (%RG) as previously described (23). To determine a qualitative resistance phenotype, conidia from each isolate were also placed on a medium amended with trifloxystrobin in the presence of salicylhydroxamic acid (SHAM) (0.2 μg ml^{-1} trifloxystrobin, 100 μg ml^{-1} SHAM) (TR+S medium) and were evaluated for the emergence of *V. inaequalis* micro-colonies following 14 days of incubation as previously described (23).

Extraction of genomic DNA and molecular detection of the G143A *cyt b* gene mutation by direct sequencing. To preserve their QoI resistance phenotypes prior to DNA extraction, monoconidial isolates with mean RGs >29% on TR medium were maintained as colonies on PDA amended with a low dose of trifloxystrobin (0.02 μg ml^{-1}) while baseline and sensitive isolates were maintained on PDA. To assess whether or not the low dose of trifloxystrobin (0.02 μg ml^{-1}) in the medium greatly influenced the relative copy number of isolates of with RGs >29%, nine representative isolates in the category were also grown on un-amended PDA. Following incubation for eight weeks at 22°C on PDA medium, approximately 200 mg of mycelium from each isolate was ground in liquid nitrogen using a mortar and pestle. DNA extraction was accomplished using the Omega Bio-Tek Plant DNA Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's instructions.

To determine the presence of the *cyt b* G143A mutation in each isolate, *V. inaequalis cyt b* specific primers, ViCytB-5F (5'-GGA CCA AGT AAT CAC TGG TGT ATG G-3') and ViCytB-6071R (5'-TTG AAA GCT AGG CTA GGG CGA ACA-3') (4) were used to generate a 938 bp amplicon that included codon 143, and these were used for direct sequencing. PCR

reactions were performed in 25 µl reaction volumes and contained 1x PCR buffer, 0.4 µM each primer, 2.5mM $MgCl_2$, 200 µM each dNTP, 0.625 units of GoTaq Flexi DNA polymerase (Promega Corporation, Madison, Wisconsin), and 5 ng genomic DNA. Amplifications were performed using an iCycler thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA) with the following program: 3 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C, followed by a final extension step of 5 min at 72°C. PCR products were separated on a 2% agarose gel (Bio-Rad Inc., Hercules, CA) stained with ethidium bromide, in 1x TAE buffer at 100 V for 1 h. Photographs of the gel were taken on a KODAK Gel Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY). PCR products were purified using the QiaQuick PCR Purification Kit (Qiagen, Valencia, CA) and were sequenced with primer ViCytB-1F (5'-AGA GCA ACG AGT AGA CGG TAG T-3') (4) using an Applied BioSystems Automated 3730xl DNA Analyzer at the Cornell University DNA Sequencing facility in Ithaca, NY.

Allele-specific quantitative PCR amplification. The abundance of A143 allele (resistant) copies relative to the total *cyt b* abundance (A143 + G143 alleles) was determined by performing quantitative PCR (qPCR) using two primer sets in a manner similar to that described by Ma et al. (32). Primers VIcytbF (5'-GAG TAG ACG GTA GTT AAT GTA TTC AT-3') and VIcytbINT-R (5'-ACG GTA TAG CAC TCA TAA GGT TTG-3') were designed using the published sequence of the *V. inaequalis cyt b* gene (GenBank accession number AF004559) to obtain an optimized fragment for qPCR that would detect both the G143 and A143 alleles in *V. inaequalis* isolates. An optimized fragment for detecting only the A143 allele in *V. inaequalis* isolates was created using forward primer VIR-F (5'-GAC TAT CGC GTA TCT AAA TCA TAT A-3') and reverse primer G143AMM4 (5'-GGT TTG TGA TGA CAG TTG GAG-3'), the latter having been designed for allele specific-PCR for the detection of QoI-resistant *V. inaequalis* isolates in

France (9). Amplifications for qPCR were performed with the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Inc., Hercules, CA), using a SYBR Green I fluorescent dye detection system (Bio-Rad Inc.). Amplifications were conducted in a 25 µl reaction volume and contained 12.5 µl iQ SYBR Green Supermix (1x final concentration), 1 µl each of primers VIcytb-F and VIcytbINT-R or of primers VIR-F and G143AMM4 (0.4 µM final concentration), and 1 µl (5 ng) DNA template using the following program: 3 min at 94°C, 40 cycles of 94°C for 15 s, 64°C for 25 s, and 68°C for 30 s. Three technical replicates were included for each isolate and the experiment was repeated.

As previously suggested by Ma et al. (32), abundance of the A143 allele relative to the abundance of both G143 + A143 alleles in each sample was determined from standard curves using a set of serial dilutions of DNA from a highly resistant isolate as a quantitative comparison standard (positive control). In this instance, a standard curve was generated for the two qPCR primer pairs VIcytb-F/VIcytbINT-R and VIR-F/G143AMM4 using 10-fold serial dilutions (5.0 to 5.0 x 10^{-4} ng μl^{-1}) of DNA from *V. inaequalis* isolate #11-1-10, which was highly resistant to trifloxystrobin in vitro (no inhibition of growth on 0.02 or 0.2 µg μl^{-1} trifloxystrobin). Additionally, a non-template control and a trifloxystrobin sensitive baseline isolate (#3-50-10) were included to detect any false-positive amplification by primer pair VIR-F and G143AMM4. Data was analyzed with Bio-Rad CFX Manager™ software (version 2.1). The mean abundance of the resistant allele for each isolate was determined from three (technical) replicate qPCR reactions for the resistant allele (A143) relative to corresponding replicate qPCR reactions for the sensitive and resistant alleles (G143 + A143) (32).

Determination of thresholds for practical resistance to trifloxystrobin and quantification of A143 alleles for an orchard with QoI resistance in *V. inaequalis*. To verify

and establish standards for populations with trifloxystrobin practical resistance, field trials were performed in a research orchard at the New York State Agricultural Experiment Station in Geneva, NY in 2011 and 2012. The orchard contains 15-year-old 'Empire' apples on M.9/M.111 interstem rootstocks and has a long history of DMI and QoI fungicide use. Treatment programs used to verify practical resistance consisted of either (1) four applications of Flint 50WG (trifloxystrobin; Bayer CropScience, Cary, NC) at the maximum labeled rate of 140 g/Ha; (2) four applications of Inspire (difenoconazole; Syngenta, Greensboro, NC) at the maximum labeled rate of 293 ml/Ha; or (3) no fungicide applications ('untreated'). All fungicides were applied in approximately 2,800 liters of water per hectare using an AA2 GunJet® handgun (TeeJet Technologies, Wheaton, IL) at approximately 2,000 kPA pressure. The four applications for each fungicide treatment were made at seven to ten day intervals at the phenological stages of pink, bloom, 1st cover and 2nd cover. Plots sprayed with trifloxystrobin or difenoconazole also received additional applications of protectant (non-selective) fungicides when trees were at green tip, half-inch green, tight cluster, and petal fall. These additional applications consisted of captan (2.8 kg/Ha) (Captan 80 WDG, Arysta LifeScience Company, Cary, NC) mixed with mancozeb (3.3 kg/Ha) (Penncozeb 75DF, United Phosphorus Inc, King of Prussia, PA). All plots received a standard insecticide and weed management program, but did not receive applications of fruit thinners or growth regulators for retaining apples. Treatments were arranged in a randomized block design with four replications. Apple scab inoculum consisted of natural leaf litter on the orchard floor. Incidence of mature 'Empire' fruit with apple scab lesions was determined at harvest in 2011 and 2012 by evaluating 10 subsamples of five fruit from each of the four replicates per treatment. All incidence data was subject to arcsine square root transformation to stabilize treatment variances. Transformed incidence data was then subjected to analysis of

variance for a randomized block design using generalized linear mixed models with the GLIMMIX procedure of SAS (version 9.3; SAS Institute Inc., Cary, NC).

The abundance of individual *V. inaequalis* isolates and the overall population abundance of qualitative resistance responses as determined by the *cyt b* A143 allele were evaluated for the research orchard population in both years of the field trial. Each year, 50 fully expanded leaves with young sporulating apple scab lesions were collected, and two *V. inaequalis* apple scab lesions from each of these leaves were excised as described previously (23). From each of the 50 leaves, one of the two leaf lesions was evaluated for QoI sensitivity using the TR+S conidial emergence assay as described above (23). To assess qualitative resistance responses using AS-qPCR, we also employed a pooled sampling scheme similar to that of other protocols for monitoring orchard population sensitivity in *V. inaequalis* (32,37,44). Specifically, the remaining 50 excised lesions (one lesion from each leaf) were pooled and then divided randomly into two replicate composite samples (25 lesions per sample). Each sample was ground in approximately 25 ml liquid nitrogen prior to DNA extraction, and AS-qPCR was performed on the resulting samples as describe above. The relative abundance of the A143 allele for the orchard population was determined for three replicate AS-qPCR reactions and the two replicate composite leaf samples as described above.

Quantification of the QoI qualitative resistance response in orchard populations of *V. inaequalis*. A total of eight orchard populations from NY and New England were selected for evaluation. Three of the orchards were reported to have populations with practical resistance to QoI fungicides (Cox et al. *unpublished*), while the remaining five populations included two baseline populations and three from orchards where apple scab has been successfully managed with QoI fungicides. The two baseline populations were from old, isolated plantings that have

never been exposed to QoIs or to other modern fungicides (e.g. DMIs, benzimidazoles). The QoI qualitative resistance responses for orchard populations of *V. inaequalis* demonstrating practical resistance were assessed using the conidial germination assay and AS-qPCR as described above. Relationships between the frequencies of isolates determined to possess qualitative resistance by the conidial germination on TR+S medium and the relative abundance of the A143 allele in the composite samples were examined by regression analysis using SAS (version 9.3; SAS Institute, Inc., Cary, NC).

RESULTS

Evaluation of quantitative and qualitative resistance to trifloxystrobin for 45 monoconidial isolates of *V. inaequalis* from the northeastern U.S. Across all isolates selected to represent the range of QoI sensitivity phenotypes, the quantitative resistance response, expressed as mean percent relative growth (%RG) on media amended with 0.02 μg ml^{-1} trifloxystrobin, ranged from 0.0 to 134.8%. Baseline isolates, as expected, failed to grow at this discriminatory concentration. Amplification using PCR primers ViCytB-5F and ViCytB-6071R yielded an anticipated 938 bp fragment for all *V. inaequalis* isolates regardless of their in vitro sensitivity to trifloxystrobin (Figure 4.1).

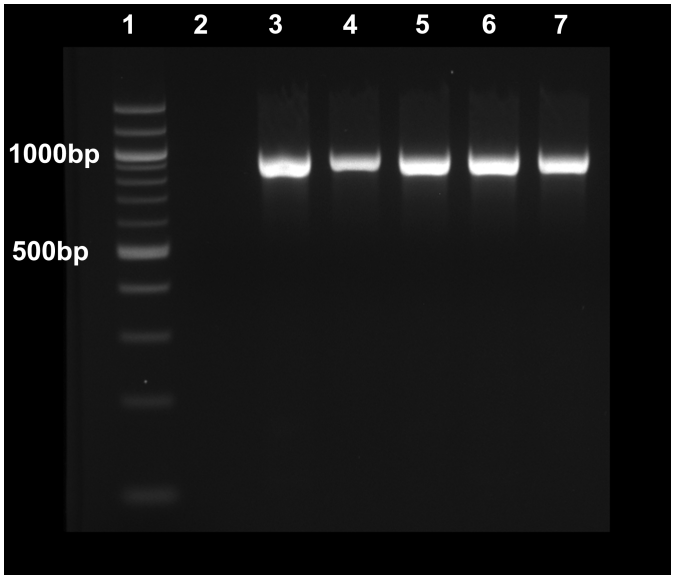

Figure 4.1. Results from amplification of a 938 bp fragment in the *V. inaequalis* cytochrome b gene for which the amplified product contained the region of the G143A mutation. Lane 1, Quick-Load 100 bp DNA Ladder (New England BioLabs); lane 2, non-template control; lanes 3-7, *V. inaequalis* isolates with increasing quantitative resistance responses to trifloxystrobin (Isolate number 3-50-10, 11-19-10, 37-23-10, 11-10a-11, and 14-38-10, respectively).

Sequencing of PCR products with primer ViCytB-1F revealed a single point mutation at amino acid position 143 of the *V. inaequalis cyt b* gene (GGT/glycine to GCT/alanine) in 24 isolates (Table 4.1). In some isolates with the QoI-resistant (QoI-R) genotype, evidence of heteroplasmy could be observed in sequencing electropherograms (Figure 4.2).

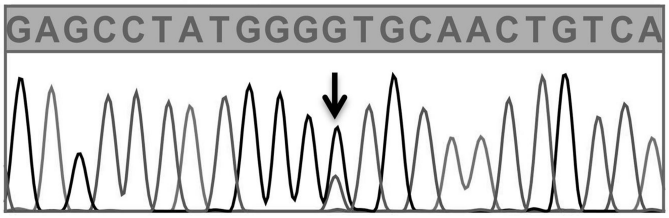

Figure 4.2. Electropherogram illustrating heteroplasmy at amino acid position 143 of the *V. inaequalis cyt b* gene. Guanine (G), represented by the higher peak immediately below the arrow, is the most abundant nucleotide at position 143; however, cytosine (C) is also detected at the same position.

As expected, there was concordance between the direct sequencing method and the conidia emergence assay (TR+S) for the determination of qualitative resistance (data not shown). For the 24 isolates with the QoI-R genotype, the quantitative resistance response (%RG) ranged from 40.3 to 134.8% with a mean of 90.23 ± 4.6%, while those with the QoI-sensitive (QoI-S) genotype had %RG values ranging from 0.0 to 48.5% with a mean of 23.44 ± 4.6%.

Table 4.1. In vitro sensitivity to the QoI fungicide, trifloxystrobin, molecular detection of the G143A mutation, and quantification of the resistant A143 allele in northeastern United States *V. inaequalis* isolates collected in 2010 and 2011.

Year	County (state)	Orchard Designation	Isolate	Mean % relative growth on 0.02 μg ml^{-1} trifloxystrobin [a]	Cytochrome b gene genotype at codon 143[b]	Relative abundance of the resistant allele (%) (A143)[c]
2010	Ontario (NY)	RS10	14-38-10	101.19 ± 19.1	A143	100.0
2010	Ontario (NY)	RS10	14-37-10	84.46 ± 7.6	A143	100.0
2010	Ontario (NY)	RS10	14-4-10	107.94 ± 3.5	A143	83.94
2010	Ontario (NY)	DA10	11-27-10	39.81 ± 1.9	G143	0.01
2010	Ontario (NY)	DA10	11-48-10	41.93 ± 5.3	G143	0.00
2010	Ontario (NY)	DA10	11-45-10	65.33 ± 3.1	A143	82.59
2010	Ontario (NY)	DA10	11-19-10	28.83 ± 3.1	G143	0.11
2010	Ontario (NY)	DA10	11-39-10	0.00 ± 0.0	G143	0.08
2010	Ontario (NY)	DA10	11-28-10	90.13 ± 0.0	A143	100
2010	Ontario (NY)	DA10	12-19-10	84.41 ± 7.0	A143	71.07
2010	Ontario (NY)	DA10	12-26-10	33.60 ± 2.0	G143	0.17
2010	Ontario	DA10	12-5-10	45.00 ± 27.8	G143	0.11

	(NY)					
2010	Ontario (NY)	DA10	8-33-10	96.27 ± 18.7	A143	62.71
2010	Ontario (NY)	DA10	27-35-10	98.75 ± 8.4	A143	98.28
2010	Ulster (NY)	HVL-Pond	28-16-10	0.00 ± 0.0	G143	0.00
2010	York (ME)	McDSan	37-23-10	38.07 ± 2.7	G143	0.01
2010	York (ME)	McDSan	37-20-10	0.00 ± 0.0	G143	0.00
2010	Ontario (NY)	Lakeview	41-9-10	45.58 ± 8.1	G143	7.75
2010	Ontario (NY)	Lakeview	41-3-10	90.18 ± 4.5	A143	88.27
2010	Ontario (NY)	Lakeview	41-2-10	81.21 ± 12.1	A143	100.0
2010	Ontario (NY)	Lakeview	41-11-10	101.53 ± 3.9	A143	90.94
2010	Adams (PA)	DocPA2	47-18-10	134.82 ± 10.4	A143	100.0
2010	Tompkins (NY)	LT2	6-47-10	92.81 ± 11.2	A143	93.12
2010	Tompkins (NY)	LT2	6-12-10	92.26 ± 5.7	A143	100.0
2010	Tompkins (NY)	LT2	6-40-10	90.50 ± 7.3	A143	93.30
2010	Ulster (NY)	HVL-M9	29-23-10	38.28 ± 21.7	G143	0.03
2010	Tompkins (NY)	LT1	5-1-10	81.79 ± 11.4	A143	100.0
2010	Ontario (NY)	GFB-BL	3-17-10	0.00 ± 0.0	G143	0.04
2011	Franklin (MA)	VV	11-10a-11	50.88 ± 2.6	A143	87.4
2011	Franklin (MA)	VV	11-11a-11	83.86 ± 14.2	A143	71.43
2011	Franklin (MA)	VV	11-13-11	100.63 ± 6.5	A143	98.27
2011	Franklin (MA)	VV	11-17-11	72.80 ± 3.3	A143	98.60
2011	Franklin (MA)	VV	11-1-11	132.65 ± 14.8	A143	88.34
2011	Franklin (MA)	VV	11-22-11	46.93 ± 4.7	G143	0.02
2011	Franklin (MA)	VV	11-20-11	66.50 ± 4.7	A143	87.65
2011	Franklin (MA)	VV	11-25-11	40.27± 5.4	A143	40.26
2011	Franklin (MA)	VV	11-8a-11	48.45± 3.2	G143	0.01
2011	Oxford (ME)	King	28-17-11	41.46 ± 17.1	G143	0.20

2011	Penobscot (ME)	GAMac	37-22-11	44.36 ± 16.2	G143	1.68
2011	Tompkins (NY)	LT	5-44-11	124.23 ± 0.0	A143	100.0
2011	Ontario (NY)	Traver	32-11-11	0.00 ± 0.0	G143	0.02
2011	Ontario (NY)	GFB-BL	3-27-10	0.00 ± 0.0	G143	0.00
2010	Ontario (NY)	GFB-BL	3-28-10	0.00 ± 0.0	G143	0.00
2010	Ontario (NY)	GFB-BL	3-50-10	0.00 ± 0.0	G143	0.00
2010	Ontario (NY)	GFB-BL	3-35-10	0.00 ± 0.0	G143	0.01

[a] Phenotypic or quantitative QoI resistance response was expressed as percent micro-colony growth on medium amended with analytical grade trifloxystrobin relative to that on non-fungicide medium. Values are means and standard errors of five randomly selected single conidium micro-colonies for each isolate.
[b] Genotype of the cytochrome b gene at codon 143 as determined by direct PCR and sequencing.
[c] Relative abundance of the resistant allele of isolates determined by AS-qPCR from three technical replications and two biological replications.

Quantification of the A143 allele in monoconidial isolates of *V. inaequalis* using allele-specific qPCR. Primers VIcytb-F and VIcytbINT-R designed for qPCR amplified a 216 bp fragment for *V. inaequalis* isolates with either the (QoI-S) or QoI-R genotype. Sequencing of PCR products generated by this primer set yielded results at amino acid position 143 that were identical to those achieved using primers ViCytB-5F and ViCytB-6071R in traditional PCR amplification and direct sequencing (data not shown). Allele-specific primers VIR-F and G143AMM4 produced a 244 bp fragment for *V. inaequalis* isolates confirmed to have the QoI-R genotype (Figure 4.3). These primers failed to amplify a product for isolates with the QoI-S genotype (Figure 4.3), and those isolates had little to no growth on TR amended medium or conidia emergence on TR+S amended medium.

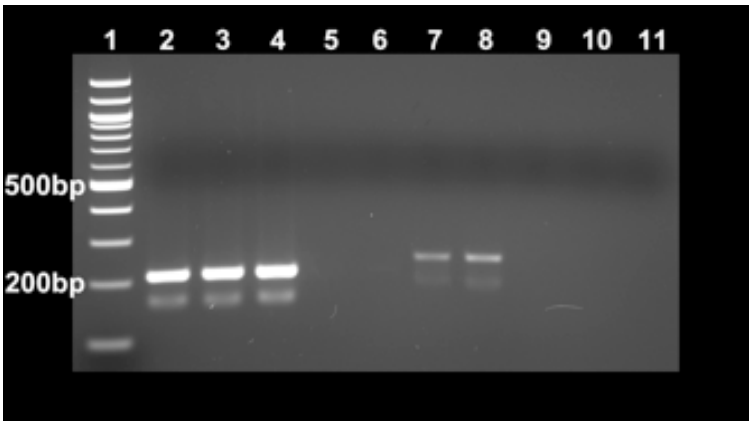

Figure 4.3. Allele-specific PCR amplification of the cytochrome b G143A allele for *V. inaequalis* QoI-resistant and QoI-sensitive isolates with primers VIcytb-F and VIcytbINT-R and VIR-F and G143AMM4 in lanes 2-6 and 7-11, respectively. Lane 1, Quick-Load 100 bp DNA Ladder (New England BioLabs); lanes 2 and 7, trifloxystrobin-resistant isolate #14-38-10; lanes 3 and 8, trifloxystrobin-resistant isolate #6-47-10; lanes 4 and 9, trifloxystrobin-sensitive isolate #3-50-10; lanes 5 and 10, *P. expansum*; and lanes 6 and 11 non-template control.

All primer sets failed to amplify *Penicillium expansum*, an out-group fungus often isolated on apple scab lesions in addition to *V. inaequalis* (Figure 4.3). Independent standard curves were generated using Bio-Rad CFX Manager™ software (version 2.1), for qPCR primer sets VIcytb-F/VIcytbINT-R and VIR-F/G143AMM4. For both primer sets, detection of the targeted region in the *cyt b* gene was achieved for *V. inaequalis* concentrations of 5 x 10^{-4} ng μl^{-1} or greater.

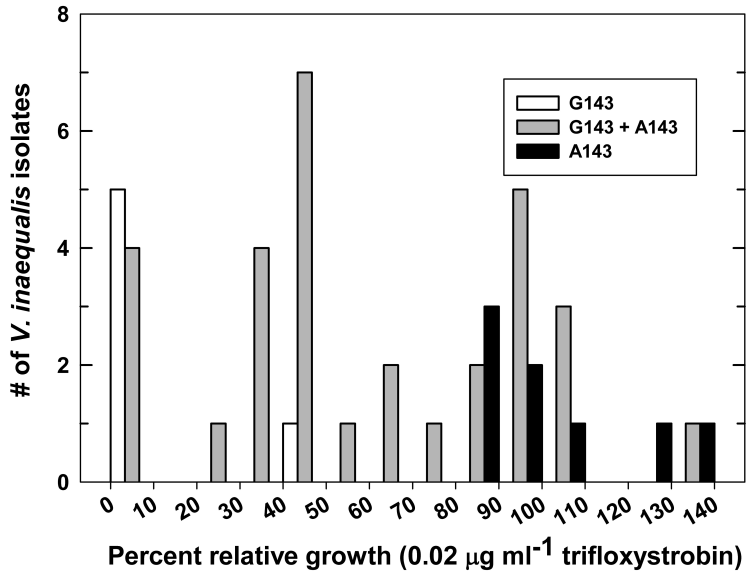

Figure 4.4. Distribution of quantitative resistance responses to trifloxystrobin for 45 *V. inaequalis* isolates for each cytochrome b gene genotype at codon 143 as determined by direct PCR and sequencing. Bars represent the total number of isolates with only the G143 genotype (white bars), only the A143 genotype (black bars), or isolates with G143A heteroplasmy (gray bars). Bars are differentially positioned within a bin to allow for visual discrimination, not to imply a specific subset of %RG values within a bin.

The relative abundance of the resistant allele (A143) in monoconidial *V. inaequalis* isolates ranged from 0 to 100% (Table 4.1). Isolates which only possessed the sensitive G143 allele failed to grow on medium amended with trifloxystrobin with the exception of isolate #11-48-10, which had a mean %RG value of 41.9%. By comparison, isolates homoplastic for the resistant A143 allele had qualitative resistance and displayed a high quantitative resistance response in vitro as evidenced by %RG values ranging from 81.2 to 134.8% (Figure 4). Approximately two-thirds of isolates tested (31 of 45) demonstrated heteroplasmy of the *cyt b* gene, having copies of both resistant and sensitive alleles. Amongst the isolates demonstrating heteroplasmy, there was a wide range in quantitative resistance responses ranging from completely sensitive (0% RG) to completely resistant (≥100% RG) (Figure 4.4). Moreover, the relative abundance for isolates with mean RGs > 29% on TR medium that were cultured initially on both TR medium and un-amended PDA were not significantly different from on another ($P > 0.05$) (data not shown).

Overall, there was no clear relationship between the relative abundance of the A143 allele and the quantitative resistance response to trifloxystrobin expressed as %RG (Figure 4.5). For example, even when relative growth of isolates on TR amended medium was as high as 40%, the relative abundance of the A143 allele ranged from 0.0 to 0.17% and thus likely did not contribute to higher quantitative resistance responses. For the majority of isolates examined, it wasn't until the quantitative resistance response exceeded 50% RG that the relative abundance of the A143

allele was observed to exceed 60% (Figure 5). Similarly, nearly all of the isolates with > 50%RG were found to contain the G143A mutation using direct PCR and sequencing (Table 4.1).

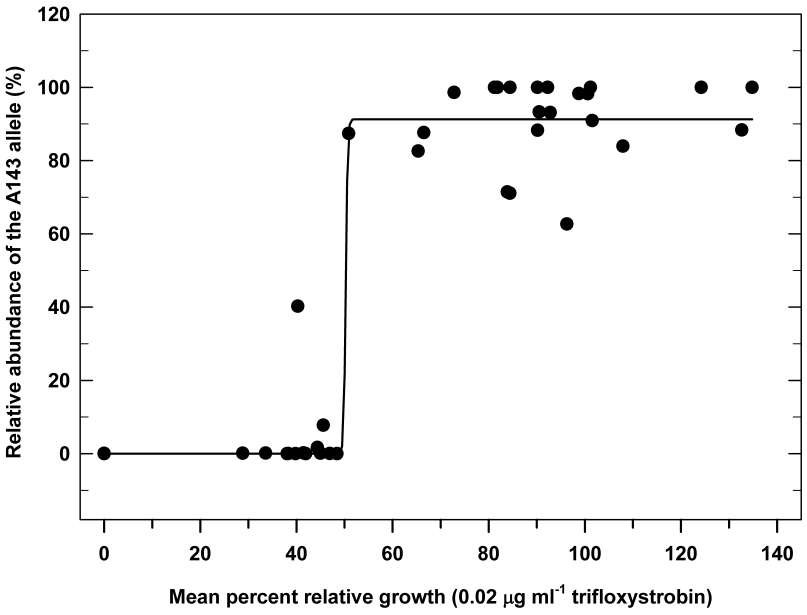

Figure 4.5. Relative abundance of the resistant allele determined by AS-qPCR for 45 *V. inaequalis* isolates with a range in quantitative resistance responses to trifloxystrobin. The presence of the line is to illustrate the shape of the relationship, not to imply a regression analysis.

Determination of thresholds for practical resistance to trifloxystrobin and quantification of A143 alleles for an orchard with QoI resistance to *V. inaequalis*. In field trials to verify and establish standards for populations with practical resistance, trifloxystrobin failed to provide a level of apple scab control comparable to the difenoconazole standard. Both trifloxystrobin and difenoconazole were applied at maximum labeled rates and identical timings, were subject to identical infection periods, and received identical fungicide cover programs in both 2011 and 2012. In each year, trifloxystrobin was significantly (P<0.0001) less effective in managing apple scab infections on mature fruit compared to the difenoconazole standard. The incidence of apple scab symptoms on mature fruit consistently exceeded 40% compared to

difenoconazole, which had less than 15% incidence of symptoms on mature fruit in both years. Although trifloxystrobin failed to provide a level of control comparable to that of difenoconazole, both fungicides still had significantly (P<0.0001) less apple scab than the untreated check, which exceeded 84% incidence in both years (Table 4.2). Not surprisingly, given the failure of trifloxystrobin to provide a level of control similar to that of difenoconazole, more than 35% of the *V. inaequalis* isolates collected from the research orchard were found to have qualitative resistance as determined by conidial germination on TR+S in 2011 and 2012 (Table 4.3). In addition, the relative abundance of the A143 allele in the composite samples collected from this research orchard exceeded 20% on average in 2011 and 2012 (Table 4.3).

Table 4.2. Incidence of apple scab symptoms on mature 'Empire' fruit in 2011 and 2012 from a research orchard at Geneva, New York, where treatments were designed to assess practical resistance to QoI fungicides.

Treatment programs (amt./Ha)	Timing[a]	Mature fruit scab (%)[b], 2011	Mature fruit scab (%)[b], 2012
Untreated	N/A	93.2 ± 1.4 a	84.6 ± 4.8 a
Penncozeb 75DF (3.3 kg) + Captan 80WDG (2.6 kg) Flint 50WG (140 g)	1-3, 6 4, 5, 7, 8	46.2 ± 5.6 b	37.3 ± 5.8 b
Penncozeb 75DF (3.3 kg) + Captan 80WDG (2.6 kg) Inspire (293 ml)	1-3, 6 4, 5, 7, 8	10.0 ± 1.6 c	10.0 ± 4.6 c

[a] Applications for each year were made at roughly 7 to 10 day intervals that corresponded to the following phenological stages: 1 = green tip; 2 = half-inch green; 3= tight cluster; 4 = pink; 5 = bloom; 6 = petal fall; 7 = 1st cover; 8 = 2nd cover.
[b] All values are the mean percent incidence and standard errors of apple scab lesions on mature 'Empire' fruit, expressed as the number of fruit with apple scab lesions out of five collected fruit with ten such collections assessed for each of four replicates. Values within columns followed by the same letter are not significantly different ($P \leq 0.05$) according to LSMEANS procedure of SAS (version 9.3; SAS Institute, Inc., Cary, NC)

Quantification of the QoI qualitative resistance responses in orchard populations of *V. inaequalis*. Across the selected baseline and commercial orchards, the percentage of *V. inaequalis* isolates with qualitative resistance to trifloxystrobin as determined by the conidial germination assay ranged from 0 to 100%, and the relative abundance of the A143 allele in composite samples ranged 0.3 to 50.6% (Table 4.3). While the ranges were different, there was concordance between the frequency of isolates with qualitative resistance and relative abundance of the A143 allele in composite samples as evidenced by a significant and strong linear relationship (R^2=0.989, P<0.0001). For orchards that were not reported to have practical resistance, less than 25% of the *V. inaequalis* population had qualitative resistance determined by conidial germination on TR+S, and the relative abundance of the A143 allele was less than 2%. In orchards that were reported to have practical resistance to trifloxystrobin, more than 35% of the isolate population had qualitative resistance determined by conidial germination on TR+S and the relative abundance of the A143 allele in composite samples always exceeded 20%. While we were unable to evaluate QoI resistance in vitro for orchard 12B due to the poor viability of conidia, the relative abundance of the A143 allele in orchard 12B (22.8%) exceeded the 20% relative abundance threshold associated with the practical resistance standards RS11 and RS12.

Table 4.3. Detection and quantification of the A143 allele associated with trifloxystrobin resistance for apple scab lesions on leaves for *V. inaequalis* populations with and without practical resistance to QoI fungicides.

Orchard Designation	County (state)	Practical Resistance to QoI fungicides (Yes/NO)	Frequency of isolates with qualitative resistance to trifloxystrobin[a]	Relative abundance of the resistant allele (%) (A143)[b]
5B	Thompson (NY)	Yes	100.0	50.6 ± 0.5
10B	Ontario (NY)	No	0.0	0.3 ± 0.1

11B	Franklin (MA)	Yes	50.0	35.8 ± 0.4
12B	Albany (NY)	Yes	-[d]	22.8 ± 7.0
22B	Penobscot (ME)	No	0.0	0.3 ± 0.1
25B	Penobscot (ME)	No	4.5	0.7 ± 0.0
26B	Penobscot (ME)	No	0.0	0.4 ± 0.2
32/34B	Seneca (NY)	No	0.0	1.9 ± 0.1
RS11[c]	Ontario (NY)	Yes	41.7	21.0 ± 2.0
RS12[c]	Ontario (NY)	Yes	36.8	20.5 ± 4.2

[a] The frequency of *V. inaequalis* isolates in an orchard (n = 50) determined to have the G143A mutation by conidial germination on media amended with 0.2 μg ml^{-1} trifloxystrobin and 100 μg ml^{-1} salicylhydroxamic acid.
[b] Relative abundance of the resistant allele for orchard populations determined by AS-qPCR. Values are means and standard errors of three technical replications and two biological replicates, with 25 *V. inaequalis* lesions per replicate.
[c] RS11 & RS12 are the research orchard populations from which practical resistance was evaluated.
[d] Qualitative QoI resistance on the basis of conidial germination could not be determined in vitro due to low viability of conidia.

DISCUSSION

Monitoring the development of qualitative resistance to QoI fungicides in *V. inaequalis* populations throughout NY and the New England region has been done via conidial germination assays, direct PCR and sequencing of the G143A region of the *cyt b* gene, and recently allele-specific PCR (3,5,9,27,29,31). Because these methods are designed to detect a single genotype, the contribution of heteroplasmy of the mitochondrial *cyt b* gene (50) is often disregarded, thereby limiting our understanding of isolate and orchard population genotypes and phenotypes. Indeed, heteroplasmy of the *cyt b* gene may contribute to the apparent quantitative resistance response to QoI fungicides in *V. inaequalis* (23). We developed an allele-specific real-time PCR (AS-qPCR) assay to determine how the relative abundance of the *cyt b* A143 allele resulting from *cyt b* gene heteroplasmy contributes to the quantitative resistance response in single

conidial isolates and how it relates to the phenomenon of practical resistance in orchard populations of *V. inaequalis*.

In this study, all of the isolates confirmed to have qualitative resistance according to conidial germination on TR+S medium (23) were found via DNA sequencing to contain the amino acid substitution from glycine to alanine at codon 143 of the *cyt b* gene (G143A mutation). In fungi, this point mutation has long been associated with qualitative resistance to QoI fungicides (4,7,14,45,50). Indeed, 60 to 100% of *V. inaequalis* isolates in Michigan apple orchards with documented QoI fungicide control failures were recently found to have qualitative resistance (27). We observed practical resistance in a research orchard when > 35% of the isolates in the *V. inaequalis* population were found to have the G143A mutation on the basis of conidial germination. Because losses in QoI fungicide efficacy have long been associated with the development of qualitative resistance in isolates on the basis of conidial germination and PCR-RFLP or DNA sequencing (3,9,23,27,28), we anticipated that the majority of individual isolates screened would be homoplastic for wild-type or mutant *cyt b* genes. However, only a third of the isolates screened possessed absolute homoplasmy and these isolates had either qualitative resistance with high resistance responses (> 81 %RG on TR medium), or were completely sensitive and had no growth (with the exception of isolate #11-48-10) on TR medium. The majority of isolates screened demonstrated some degree of *cyt b* heteroplasmy and demonstrated a range of quantitative trifloxystrobin resistance responses in vitro. Approximately half of the heteroplasmic isolates were found to possess the G143A mutation as determined by DNA sequencing, and for some isolates, the presence of both alleles could be observed on the electropherograms.

While heteroplasmy of the *V. inaequalis cyt b* gene was present in many of the isolates evaluated, isolates with quantitative resistance responses exceeding 50% RG on TR medium were consistently observed only when the relative abundance of the A143 allele exceeded 62%, suggesting that A143 abundance is an important contributor to high resistance responses. In regard to isolates with moderate resistance responses to QoI fungicides (29 to 49 %RG), heteroplasmy of the *cyt b* gene was not the primary factor involved in the quantitative resistance response. In such isolates, the relative abundance of the A143 allele never exceeded 8%. The low relative abundance of the Al43 allele in several isolates with %RG values approaching 50% suggests that other unidentified genetic factors may better explain the quantitative resistance response in some isolates. Interestingly, isolate #11-25-11 had an A143 relative abundance of 40.3% and approximately 40%RG on TR medium. For this isolate, it is possible that high A143 abundance explains the quantitative resistance response in vitro. However, this conclusion cannot be determined for certainty as the contributions of other unidentified genetic factors (7,23) potentially involved in the trifloxystrobin resistance response have not been quantified. Similar to the present study, Michalecka et. al. (37) was able to quantify the relative abundance of the A143 allele at the individual level, but only mixtures of DNA from homoplastic isolates were quantified rather than using field isolates of *V. inaequalis* with differing quantitative resistance responses. Furthermore, it is still not known if mitochondrial heteroplasmy plays a role in the quantitative resistance responses to QoI fungicides in other systems. In studies investigating mitochondrial *cyt b* heteroplasmy in other pathosystems (11,26,32), there is either no apparent quantitative response to QoI fungicides or the relationship between heteroplasmy and the quantitative resistance response was not examined or discussed.

While we found that a high relative abundance of the A143 allele (>60%) was associated with high resistance responses (>50 %RG) to trifloxystrobin, additional resistance mechanisms are likely involved in the resistance responses of both highly resistant isolates and of isolates with of low to moderate resistance responses accompanied by low A143 allele abundance. The involvement of other genes and resistance mechanisms in QoI insensitivity in vitro has been well documented in other pathogens (7). For example, in *Aspergillus nidulans,* drug efflux proteins from the ATP-binding cassette (ABC) transporter family (1) have been found to actively transport fungicides, including strobilurins, out of the fungal cell membrane, whereas in *M. graminicola* the major facilitator superfamily (MFS transporters) serves the same function (42). A mechanism such as a multi-drug efflux pump is certainly plausible in *V. inaequalis*, as multiple drug resistance between QoI and DMI fungicides has previously been documented in the pathogen (3,38). An alternative explanation for the moderate quantitative resistance responses in the absence of the A143 allele is the involvement of the alternative respiration pathway in vitro. In *V. inaequalis,* however, the activation of this pathway has been demonstrated to be highly dependent on strobilurin dose (39), with an increased likelihood of it becoming activated at higher doses. The discriminatory concentration of trifloxystrobin used in the absence of SHAM was specifically selected to monitor the quantitative resistance response (23) without being high enough to trigger the alternative respiration pathway. Hence, it is unlikely that the alternative respiration pathway is the reason for a quantitative resistance response in the absence of a high abundance of the resistant allele. Lastly, the presence of other *cyt b* gene mutations known to be associated with loss of QoI efficacy could account for quantitative resistance in the absence of the A143 allele. In this study, neither the F129L nor the G137R mutations, which have been associated with moderate resistance responses in other

pathosystems (2,13,18,41,44), were detected in any of the *V. inaequalis* isolates surveyed (data not shown).

In addition to being able to determine the relative abundance of the A143 allele for individual isolates of *V. inaequalis*, the application of the AS-qPCR technique for orchard composite samples (37,44) allows one to assess the abundance of the A143 allele for an entire orchard population of *V. inaequalis*. In regards to practical resistance, the relative abundance of the A143 allele was a low as 20% in the research orchard where trifloxystrobin failed to provide acceptable levels of apple scab control in 2011 and 2012. This suggested threshold was further verified with composite samples of sensitive and practically resistant orchards in the current study, as no commercial orchards with practical resistance to QoI fungicides had a relative abundance of the A143 allele below 22%. Similarly, Michalecka et al. (37) was able to quantify the abundance of the Al43 allele using AS-qPCR on composite samples from field populations *V. inaequalis*. Unfortunately, the authors only looked at the abundance of A143 allele in organic and conventionally managed orchard populations outside of the context of QoI resistance. The authors did report that the abundance of the A143 allele ranged from 50 to 100% in conventionally managed orchard populations, but the QoI resistance status for these orchards was not reported. While we are unable to validate or explain our A143 frequency threshold using the work of Michalecka et al. (37), the results from our study would suggest that these orchard populations in Poland could have practical resistance.

In conclusion, the results of this study demonstrate that heteroplasmy of the *cyt b* gene does play a role in the quantitative resistance response in highly resistant isolates. However, the low abundance of the A143 allele in isolates with moderate resistance responses (29 to 49% RG) suggests that the QoI resistance response is truly multigenic, although the importance of other

genes for resistance becomes masked as qualitative resistance when the abundance of the A143 allele is sufficiently high (>62%). Beyond understanding the mechanism of *cyt b* heteroplasmy in regards to QoI insensitivity, there are implications for evaluating and monitoring the development of practical resistance using A143 allele abundance. Since it is possible to detect and quantify low abundances of the A143 allele using AS-qPCR in orchard composite samples, it would be possible to identify orchards at risk for practical resistance to QoI fungicides, and prevent the development of practical resistance by removing the selective agent prior to the point where the population reaches the 20% A143 threshold.

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CHAPTER 5

MOLECULAR CHARACTERIZATION OF THE *sdhB* GENE AND BASELINE SENSITIVITIES TO PENTHIOPYRAD, FLUOPYRAM, AND BENZOVINDIFLUPYR IN *VENTURIA INAEQUALIS*

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ABSTRACT

The succinate dehydrogenase inhibiting (SDHI) fungicides are a class of single-site fungicides that are increasingly important in the management of *Venturia inaequalis*. In this study, the baseline sensitivity of *V. inaequalis* to penthiopyrad, fluopyram, and benzovindiflupyr was investigated. Thirty-five to 70 isolates with no prior exposure to single-site fungicides were used to determine the effective concentration at which conidial germ tube growth was inhibited by 50% (EC_{50}). Mean EC_{50} values for the conidial germ tube growth stage for penthiopyrad, fluopyram, and benzovindiflupyr were 0.086, 0.176 and 0.0016 μg ml^{-1}, respectively. Linear correlation analysis revealed a significant and positive correlation between fluopyram and penthiopyrad ($P \leq 0.0001$, r = 0.66) and fluopyram and benzovindiflupyr ($P = 0.0014$, r = 0.52). Baseline sensitivities of *V. inaequalis* during the mycelial growth stage was also determined for fluopyram and benzovindiflupyr. EC_{50} values were higher for fluopyram and benzovindiflupyr during this stage compared to the conidial germ tube growth stage with means of 0.043 and 2.02 μg ml^{-1}, respectively. In addition, the *sdhB* gene was characterized for three isolates of *V. inaequalis* collected from a research, baseline, and commercial orchard population. No common

mutation sites associated with SDHI resistance in other phytopathogenic fungi were discovered in these isolates, or isolates that were recovered following field applications of SDHI fungicides. The results of this study suggest that SDHI fungicides have a high level of activity during the conidial germ tube elongation stage in *Venturia inaequalis* and provides a basis for phenotypic and genotypic monitoring of shifts towards resistance of *V. inaequalis* populations to the SDHI fungicide class.

INTRODUCTION

Apple scab, caused by *Venturia inaequalis* (Cooke) G. Winter is one of the most devastating diseases of apple in the northeastern and mid-Atlantic regions of the United States. Due to the absence of desirable horticultural traits in cultivars with resistance to apple scab, commercially acceptable levels of apple scab control must be achieved by numerous fungicide applications throughout the growing season (Köller and Wilcox, 2001; Lesniak et al., 2011; MacHardy 1996). One class of fungicides available for the management of apple scab is the succinate dehydrogenase inhibitor (SDHI) fungicides. The SDHIs are a class of broad spectrum, single-site fungicides that interfere with mitochondrial respiration in fungi (Kuhn, 1984), including the apple scab pathogen, *Venturia inaequalis.* Although the carboximide SDHI fungicide, boscalid, has been registered for apple scab control as part of the formulated QoI/SDHI premixed product Pristine (BASF, Research Triangle Park, NC) for more than a decade, SDHI fungicides with greater levels of intrinsic activity against fungi have been released in recent years (Sierotzki and Scalliet, 2013; Thomas et al., 2012; Veloukas and Karaoglandis, 2012). As resistance to sterile demethylation inhibitor (DMI) and quinone outside inhibitor (QoI) fungicides in populations of *V. inaequalis* (Köller et al., 1997; Köller et al., 2004) becomes more prevalent throughout major apple production regions in the northeastern and the mid-Atlantic United States (Frederick et al, 2014; Villani et al., 2015), integration of next-generation SDHI fungicides (Sierotzki and Scalliet, 2013) into apple scab management programs will be increasingly important.

The SDHI fungicides comprise a diverse group of compounds that belong to eight distinct chemical groups. While structurally diverse (Sierotzki and Scalliet, 2013), all target the succinate dehydrogenase reductase enzyme, a component of complex II in the mitochondrial

electron transport chain. The target enzyme contains four nuclear-encoded subunits: SDHA, SDHB, SDHC, and SDHD, however all SDHIs currently labeled for crop protection target the ubiquinone-binding pocket, defined by the interface between the SDHB, SDHC, and SDHD subunits (Cecchini, 2003).

Similar to other single-site fungicides like the benzimidazoles (Jones, 1981) and QoI fungicides (Fernández-Ortuño et al., 2008; Köller et al., 2004), the highly specific mode of action of SDHI fungicides makes them particularly prone to resistance development. Indeed, resistance to one or more SDHI fungicides has been documented in individual field isolates or field populations of several plant pathogenic fungi including *Alternaria alternata* (Avenot et al., 2014), *Botrytis cinerea* (Amiri et al., 2014; Veloukas et al., 2013), *Podosphaera xanthii* (Ishii et al., 2011) and *Didymella bryoniae* (Avenot et al., 2012). Several mutations within the *sdhB, sdhC,* and *sdhD* genes have been associated with losses in SDHI fungicide sensitivity in all of these instances. Most frequently, a mutation of the *sdhB* gene within the region that codes for the fungicide-binding pocket results in the substitution of a highly conserved histidine (H) residue with tyrosine (Y), leucine (L), or arginine (R). Due to the variation among *sdhB* genes and differences in the substituting residue, this mutation may be denoted differently in fungal species such as H272Y/R/L in *B. cinerea* (Amiri et al., 2014), or H277Y/R/L in *A. alternata* (Avenot and Michailides, 2007; Stammler et al., 2011). Other mutations within the *sdhB* gene that have been associated with loss of SDHI fungicide efficacy include the substitution of a conserved proline residue (P225F/L/T in *B. cinerea*) (Leroux et al., 2010; Veloukas et al., 2011) and the substitution of a conserved asparagine residue (N230I in *B. cinerea*) (Veloukas, 2011).

Interestingly, substitution of one or more of the aforementioned conserved amino acids may impart resistance or partial resistance to only some SDHI fungicides depending on the

pathogen, the substituting residue, and the chemical group of the SDHI fungicide (Avenot et al., 2014; Sierotzki and Scalliet, 2013). The specific nature of reduced sensitivity or resistance to the SDHI fungicides and the growing importance of SDHIs in apple scab management programs prompted us to characterize the *sdhB* gene in *V. inaequalis* and determine baseline sensitivities and relative efficacies of SDHI fungicides used to control apple scab. Our specific objectives were to i) establish baseline sensitivities of isolates of *V. inaequalis* to penthiopyrad, fluopyram, and benzovindiflupyr collected from a population that had never been exposed to any single-site fungicides, including SDHI fungicides; ii) identify the *sdhB* gene in *V. inaequalis*, and characterize common mutation sites that confer SDHI resistance in other fungal plant pathogens; and iii) determine if mutations in the *V. inaequalis sdhB* gene are responsible for apple scab lesion development following applications of Fontelis SC (penthiopyrad; DuPont Crop Protection, Wilmington, DE), Luna Sensation (fluopyram + trifloxystrobin; Bayer CropScience, Research Triangle Park, NC), and Aprovia (benzovindiflupyr; Syngenta Crop Protection, Greensboro, NC).

MATERIALS AND METHODS

Collection of *V. inaequalis* isolates. From 2012 to 2014, a total of 105 isolates of *Venturia inaequalis* were collected from wild apple trees located in Geneva, NY. These trees had never been exposed to any single-site fungicide class including the SDHIs making the population appropriate for baseline sensitivity studies. To obtain isolates, young, fully expanded leaves with isolated sporulating primary apple scab lesions were arbitrarily selected throughout the baseline orchard. From each leaf, one individual lesion representing a single-spore infection event (Köller et al., 2004; MacHardy and Gadoury, 1989) was removed using a sterile cork borer (5 mm diameter), placed in 2.0 ml of sterile distilled water, and shaken for 60 s to dislodge conidia from

the lesion. Following agitation, leaf discs were removed and the resulting conidial suspensions (10^2 to 10^3 *V. inaequalis* conidia ml^{-1}) were stored at -20°C until the evaluation of SDHI fungicide sensitivity.

Assessment of minimal and complete media for the evaluation of SDHI sensitivity in isolates of *V. inaequalis*. Given concerns that SDHI fungicides lacked activity against both conidial germination and mycelial growth of fast growing fungal pathogens (e.g. *M. fructicola*) when those assays were conducted using complete media (Amiri et al., 2010; Hu et al., 2011), a subsample of 10 baseline isolates were evaluated on five different media: potato dextrose agar (PDA), Czapeck agar (CzA) (Markoglou et al., 2011), succinate medium (provided by Bayer) (Hu et al., 2011), yeast bacto acetate agar (YBA) (Stammler and Speakman, 2006) and glycerol yeast extract agar (GLYE) (10g Glycerol, 10g Yeast extract, 6g NaNo3, 1.5g KH2PO4, 0.5g KCl, 0.5g MgSO4, and 15g agar in 1 liter of water). All media were amended with 0 (control), 0.001, 0.01, 0.1, and 1.0 µg ml^{-1} technical grade benzovindiflupyr and fluopyram and EC_{50} values where determined using a conidial germ tube growth inhibition assay as described in detail below. After six days of incubation at 22°C, little to no growth was observed on CzA or YBA media at any of the fungicide concentrations tested (data not shown). Overall, growth rate of *V. inaequalis* on PDA was approximately 2x greater compared to GLYE when no fungicides were added to the medium, however inconsistent germination and conidial germ tube growth was observed at other fungicide concentrations and between the two fungicides (data not shown). Because of these observations, PDA, which was previously used to test for sensitivity to the new SDHIs in other fungi (Amiri et al., 2014; Avenot et al., 2012), was chosen for in vitro sensitivity tests for *V. inaequalis*.

Evaluation of sensitivity of *V. inaequalis* isolates to next generation SDHI fungicides. Because SDHI fungicides have been shown to be effective during several stages of fungal growth (Amiri et al., 2010; Veloukas and Karaoglanidis, 2012), two evaluation methods were used to determine baseline sensitivity of isolates of *V. inaequalis*. First, the sensitivity of baseline isolates of *V. inaequalis* to penthiopyrad, benzovindiflupyr, and fluopyram was evaluated using a conidial germ tube growth inhibition assay (Frederick et al., 2014; Köller et al., 2004; Villani and Cox, 2014, Villani et al., 2015). Conidial suspensions (100 µl aliquots) of the single lesion isolates were evenly distributed onto potato dextrose agar amended with streptomycin sulfate (50 µg ml $^{-1}$) and chloramphenicol (50 µg ml $^{-1}$) (PDA++). The PDA++ medium was also amended either with technical grade penthiopyrad (DuPont), technical grade fluopyram (Sigma-Aldrich, St. Louis, MO), or technical grade benzovindiflupyr (Syngenta) dissolved in acetone at concentrations of 0 (control), 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 µg ml^{-1}. Following six days of incubation at 22°C, the growth of germ tubes and secondary hyphae (for spores on low fungicide concentration with considerable growth) from five randomly selected conidia was determined using a SPOT Idea digital camera the with the SPOT Imaging Basic software package (Diagnostic Instruments Inc., Sterling Heights, MI) attached to an Olympus SZX12 stereoscope (Olympus America Inc., Center Valley, PA).

Given the number of concentrations needed to provide reliable EC_{50} values and the fact that single lesion isolates are composed of a finite number of conidia only two fungicides could be evaluated at all concentrations at a conidia density of 10^2 to 10^3 *V. inaequalis* conidia ml^{-1}. In this regard, the baseline data evaluating conidial germ tube growth inhibition for benzovindiflupyr and penthiopyrad was generated from 35 single lesion isolates, which is a number consistent with previous baseline studies in *V. inaequalis* and other pathosystems (Chen

et al., 2012; Köller et al., 1991). Since fluopyram is in a different SDHI chemical group from penthiopyrad and benzovindiflupyr, we wanted to examine the potential for cross-sensitivity between fluopyram and penthiopyrad, and between fluopyram and benzovindiflupyr. In this case, we wanted to make sure the same single lesion isolates were used. Hence, fluopyram baseline data was generated from 70 single-lesion isolates with 35 isolates from the collection used to determine cross-sensitivity with benzovindiflupyr and 35 isolates from the collection used to determine cross-sensitivity with penthiopyrad.

To examine the differences in SDHI baseline sensitivity between conidial and mycelial growth stages of *V. inaequalis*, 35 single lesion isolates were grown to colonies and evaluated for sensitivity to benzovindiflupyr and fluopyram at the same fungicide concentrations using mycelial growth inhibition assays as previously described (Amiri et al., 2010; Pfeufer and Ngugi, 2012). Briefly, a single germinating conidium was selected from each isolate and incubated on PDA medium for six weeks at 22°C. Subsequently, two agar plugs (5 mm diameter) were removed from the growing margin of each isolate and placed on PDA medium amended with technical grade fluopyram or benzovindiflupyr at each of the 12 concentrations listed above. Plugs were placed such that the fungal mycelium was in direct contact with the medium. Cultures were incubated at 22°C and the diameter of two colonies for each isolate at each fungicide concentration was measured biweekly for six weeks beginning at 14 days of incubation as described by Pfeufer and Ngugi (2012).

For both methods and for both years, relative percent inhibition of growth was calculated at each fungicide concentration as previously described (Yoshimura et al., 2004). Dose response curves were constructed for each isolate using relative percent inhibition of colony growth at each log-transformed (log_{10}) concentration for the respective fungicide to determine the value of

the effective concentration that inhibited isolate growth by 50% (EC_{50}). In addition, variation factors (V_f) for each fungicide were determined by dividing the isolate with the highest EC_{50} value by the isolate with the lowest EC_{50} value. Simple linear regression analysis was conducted and simple linear correlation coefficients (r) were calculated to determine the level of cross-sensitivity among the SDHI fungicides (SigmaPlot Version 11.0, Systat Software Inc., San Jose, CA).

Discovery of the *V. inaequalis sdhB* gene using Illumina Sequencing. Illumina sequencing was performed using the Illumina MiSeq v3 600bp kit (Illumina, San Diego, CA), to assist in the discovery of the *sdhB* gene in *V. inaequalis*. Following eight weeks of incubation at 22°C on PDA, approximately 50 mg of mycelium was harvested from a culture of the *V. inaequalis* baseline isolate 3a-27-10 and ground in liquid nitrogen using a sterile mortar and pestle. Extraction of genomic DNA was accomplished using the Omega Bio-Tek E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek, Norcross, GA) in accordance with the manufacturer's instructions. All sequencing and sample preparation, including library construction was completed at the Cornell University Genomic Facility (Ithaca, NY) using the TruSeq DNA Library Prep Kit (Illumina).

Genomic assembly of the baseline isolate was accomplished using the CLC Genomics Workbench (Version 8.0.1, CLCBio, Boston, MA). Using the BLAST function in CLC Genomics Workbench, the assembled contigs were searched for homologs to the *sdhB* gene from both *Sclerotinia sclerotiorum* and *Alternaria alternata* (accession no. XM001594527 and EU178856, respectively). Based on a single region of homology between the *sdhB* genes of the two reference fungi and *V. inaequalis* baseline isolate 3a-27-10 (see 'Results'), three primer sets were designed to amplify the *V. inaequalis sdhB* gene.

Using the primer sets ViSDHB-230F/ViSDHB415R, ViSDHb205F/ViSDHb860R, and ViSDHb504F/dsViDSHb1137R (Table 5.1), the *sdhB* gene from *V. inaequalis* was amplified, including a 178 bp region downstream and a 230 bp region upstream of the gene for three isolates collected in 2011 or 2014 from a baseline orchard, a research orchard, and a commercial orchard with no previous exposure to any SDHI fungicides. For each primer set, PCR reactions were performed in 25 µl reaction volumes and contained 1X EmeraldAmp GT PCR Master Mix (Takara Bio/Clontech Laboratories, Inc., Mountain View, CA), 0.4 µM each primer, and 5 to 10 ng of genomic DNA. All amplifications were performed in a T100 Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA) with the following program: 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at either 52°C (ViSDHB-230F/ViSDHB415R), 48°C (ViSDHb205F/ViSDHb860R) or 45°C (ViSDHb504F/dsViDSHb1137R), and 45 s at 72°C; followed by a final extension of 7 min at 72°C.

PCR products were separated on a 2% agarose gel (Bio-Rad Laboratories Inc.) stained with ethidium bromide in 1X Tris-acetate-EDTA buffer at 100 V for 1 h. Photographs of the gel were taken on a KODAK Gel Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY). Using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI), PCR products were purified and sequenced with internal primers (Table 5.1) in both directions for each primer set using an Applied BioSystems Automated 3730xl DNA Analyzer at the Cornell University DNA Sequencing facility in Ithaca, NY. For each isolate, sequences of the putative *V. inaequalis sdhB* gene were assembled using CLC Main Workbench (Version 6.8.2, CLCBio, Boston, MA).

RNA extraction, cDNA synthesis, and PCR. To elucidate the coding regions of the *V. inaequalis sdhB* gene, RNA was extracted from the same three isolates used for gDNA

amplification. Fifty to 100 mg of mycelium was harvested from isolates 41-45-11, 40-45-11, and 12-2b-14 following eight weeks of incubation on PDA at 22°C. The mycelium was subsequently ground in liquid nitrogen using a sterile mortar and pestle and total RNA was extracted using the Omega Bio-Tek E.Z.N.A.® Plant RNA Kit (Omega Bio-Tek). DNA was removed with the TURBO DNA-*free* Kit (Ambion® by Life Technologies, Carlsbad, CA). First strand complementary DNA (cDNA) was synthesized from the extracted RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories Inc.) in accordance with the manufacturer's instructions.

Table 5.1. Oligonucleotide primers used for amplification and sequencing of the *V. inaequalis sdhB* gene.

Primer	Sequence (5' to 3')	Amplification Target
ViSDHb-230F	AGA GGC TTC TCG CAA AGA AAG	Amplification of the region -230 to +415 relative to the first 5' adenosine of *sdhB*
ViSDHb415R	AGT GTT GAC ACC GTC AAT GTT	
ViSDHb205F	GAC CTT CCA CAT TTA CAG GT	Amplification of the region +205 to +860 relative to the first 5' adenosine of *sdhB*
ViSDHb860R	GTA CAA GGA CAT GGA GTT GTT	
ViSDHb504F	TGC CGG ATA TGA CAC AGT TCT	Amplification of the region +504 to +1137 relative to the first 5' adenosine of *sdhB*
DSViSDHb1137R	ATA TGT TCA TTT GTA CCC CT	
cdnaViSDHb1F	ATG GCT GCC CTC CGT ACC AC	Amplification of the *sdhB* coding region
cdnaViSDHb957R	ATA CGA AAG CCA TGC CCT TT	
seqViSDHb-200F	ACA TGG CAA TTC TTT TTT GC	Sequencing of the PCR product generated by primer pair ViSDHb-230F/ ViSDHb415R
seqViSDHb253F	CCG CAT GCA GTC ATA CAC CC	Sequencing of the PCR product generated by primer pair ViSDHb205F/ ViSDHb860R
seqViSDHb332R	CCT CGT TCT TAA TCC TGA TC	Sequencing of the PCR product generated by primer pair ViSDHb-230F/ ViSDHb415R
seqViSDHb517F	ACA GTT CTA CAA GCA ATA C	Sequencing of the PCR product generated by primer pair ViSDHb504F/ ViSDHb1137R
seqViSDHb795R	CGA TCC AGC GGT AGG ATT GAA	Sequencing of the PCR product generated by primer pair ViSDHb205F/ ViSDHb860R
sseqViSDHb1079R	ACA ATA AAA CAG ATG ACA G	Sequencing of the PCR product generated by primer pair ViSDHb504F/ ViSDHb1137R

Amplification of the coding region of the *V. inaequalis sdhB* gene was accomplished using primer sets cDNAViSDHb1F/viSDHB 415 and ViSDHb205F/cdnaViSDHB957R (Table 5.1). For each primer set, PCR reactions were conducted in 25 µl volumes as described above for gDNA. All amplifications were performed in a T100 Thermal Cycler (Bio-Rad Laboratories Inc.) with the following program: 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 52°C (cDNAViSDHb1F/viSDHB 415) or 50°C (ViSDHb205F/cdnaViSDHB957R), and 45 s at 72°C; followed by a final extension of 7 min at 72°C. PCR products were separated and photographs of the gel were taken as described above. Products were then purified and sequenced in both the forward and reverse direction as described above using the same primers used for amplification.

***sdhB* polymorphisms following applications of SDHI fungicides.** To monitor for the development of mutations in the *V. inaequalis sdhB* gene following applications of SDHI fungicides, a field trial was conducted in a research orchard at the New York State Agricultural Experiment Station in Geneva, NY in 2015. The orchard is an 18- year old planting of 'Empire' apples on M.9/M.111 interstem rootstocks with a history of DMI and QoI fungicide resistance (Villani et al., 2012, Villani et al., 2015) and SDHI fungicide applications. SDHI treatment programs consisted of either 1) Three applications of Aprovia (benzovindiflupyr, Syngenta) (511 ml/Ha); 2) Three applications of Luna Sensation (fluopyram + trifloxystrobin, Bayer CropScience, Cary, NC) (365 ml/Ha); 3) Three applications of Fontelis SC (penthiopyrad, DuPont) (1.2 liters/Ha); or 4) no fungicide applications ('untreated'). All treatments were applied dilute to drip (approximately 2,800 liters/ha) with an AA2 GunJet handgun (TeeJet Technologies, Glendale Heights, IL) handgun at approximately 2,000 kPA. Treatments were applied prior to infection events as predicted by the RIMpro apple scab forecast model (v 1.07.17:8, Marc Trapman, Zoelmond, The Netherlands) at the phenological stages of pink (8

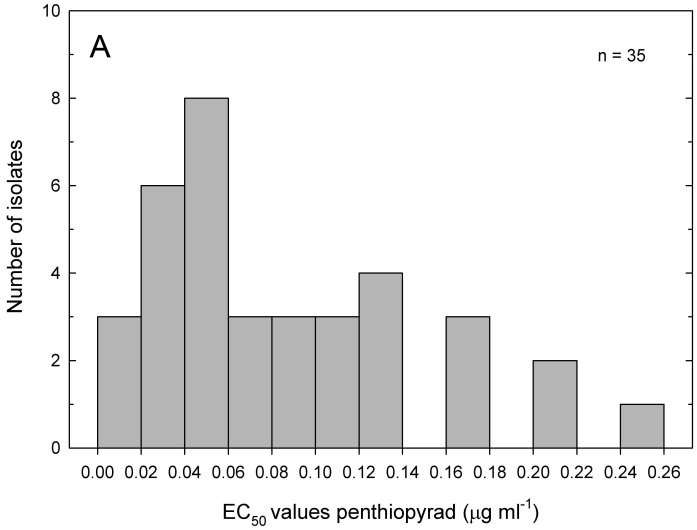
May) bloom (15 May), and 1st cover (29 May). With the exception of the untreated program, apple scab maintenance applications were made at green tip, half-inch green, tight cluster, and petal fall and consisted of captan (2.8 kg/Ha) (Captan 80 WDG, Arysta LifeScience Company, Cary, NC) mixed with mancozeb (3.3 kg/Ha) (Penncozeb 75DF, United Phosphorus Inc, King of Prussia, PA). All treatments were arranged in a randomized block design with four replications.

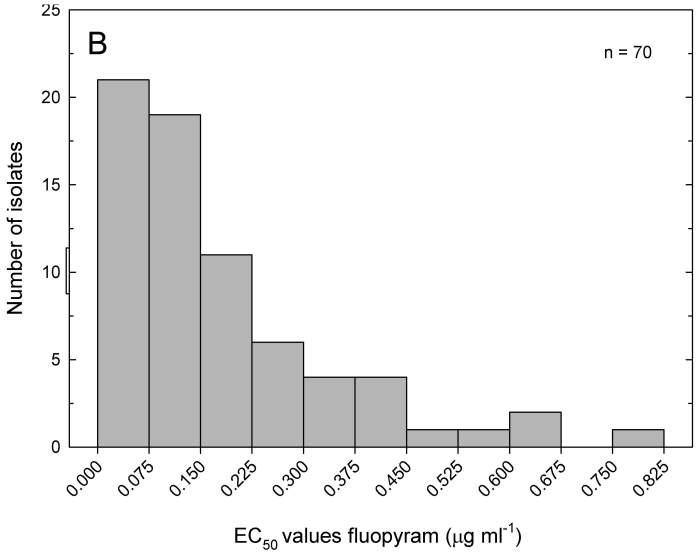
One hundred leaves with emerging apple scab lesions were collected from each of the four treatment programs (25 leaves per replication). Leaves were collected 10 days following a primary apple scab infection event that occurred on 30 May after the 1st cover application as predicted by RIMpro. From each leaf, a single lesion was excised as described above. A pooled sampling scheme of the lesions was utilized (Ma and Michailides, 2004; Villani and Cox, 2012) to determine if any mutations in the target *sdhB* gene were present in lesions that emerged following SDHI fungicide applications. Specifically, 100 excised lesions from each treatment (one lesion from each leaf) were pooled and then divided into ten replicate composite samples (ten lesions per sample). Each sample was ground in approximately 25 ml liquid nitrogen prior to DNA extraction. Extraction of genomic DNA was accomplished using the Omega Bio-Tek E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek) according to the manufacturer's instructions. PCR was performed on the extracted DNA from each of the ten treatment replicates using primer sets ViSDHB-230F/ViSDHb860R and ViSDHb504F/dsViDSHb1137R to amplify the *sdhB* gene. Cycling parameters were identical to those described above, however annealing temperatures of 51°C and 47°C were used for reactions containing primer pairs ViSDHB-230F/ViSDHb860R and ViSDHb504F/dsViDSHb1137R, respectively. PCR products were separated and photographs of the gel were taken as described above. Products were then purified and

sequenced in both the forward and reverse direction as described above using the same primers used for amplification.

RESULTS

Sensitivity of a baseline population of *V. inaequalis* to penthiopyrad, fluopyram, and benzovindiflupyr. In this study, a conidial germ tube growth inhibition assay was used to determine baseline sensitivities of isolates of *V. inaequalis* to the SDHI fungicides penthiopyrad, fluopyram, and benzovindiflupyr. For penthiopyrad, EC_{50} values ranged from 0.017 to 0.240 μg ml^{-1} (Figure 5.1a), with a mean EC_{50} value of 0.086 μg ml^{-1}. The majority of the baseline EC_{50} values for penthiopyrad occurred below 0.015 μg ml^{-1} with the minimum and maximum EC_{50} values separated by a V_f of 14. By comparison, the baseline EC_{50} values for fluopyram isolates ranged from 0.0002 to 0.815 μg ml^{-1} with a mean EC_{50} value of 0.176 μg ml^{-1} (Figure 5.1b). Fluopyram EC_{50} values occurred over a wide range with the minimum and maximum EC50 values separated by a V_f of 3,593. Benzovindiflupyr EC_{50} values for the baseline isolates were lowest ranging from 0.00013 to 0.0029 μg ml^{-1} with a mean of 0.0016 μg ml^{-1} (Figure 5.1c). Similar to penthiopyrad, the baseline EC_{50} values for benzovindiflupyr occurred over a relatively narrow range, with a V_f of 44. Linear regression analysis revealed significant and positive correlations of EC_{50} values between penthiopyrad and fluopyram ($P \leq 0.0001$, r = 0.66) (Figure 5.2a) and between fluopyram and benzovindiflupyr (P =0.0014, R = 0.52) (Fig 5.2b).


A
n = 35
Number of isolates
0
2
4
6
8
10
0.00 0.02 0.04 0.06 0.08 0.10 0.12 0.14 0.16 0.18 0.20 0.22 0.24 0.26
EC50 values penthiopyrad (µg ml-1)


B
n = 70
Number of isolates
0
5
10
15
20
25
0.000 0.075 0.150 0.225 0.300 0.375 0.450 0.525 0.600 0.675 0.750 0.825
EC50 values fluopyram (µg ml-1)

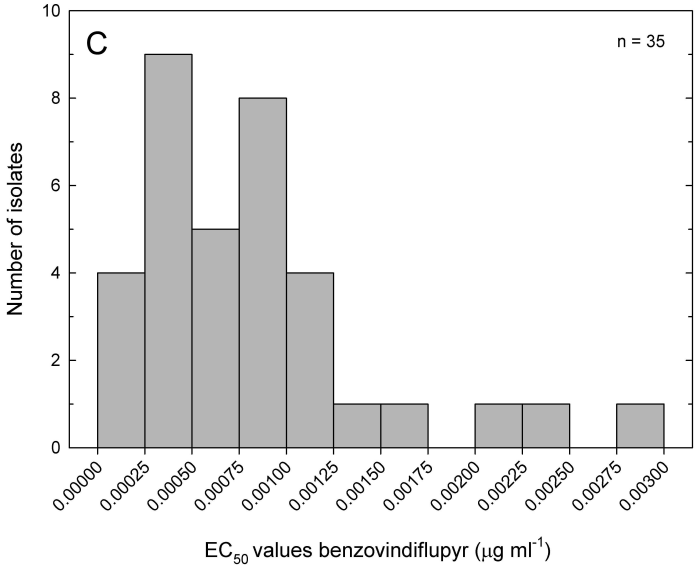

C
n = 35
Number of isolates
0
2
4
6
8
10
0.00000 0.00025 0.00050 0.00075 0.00100 0.00125 0.00150 0.00175 0.00200 0.00225 0.00250 0.00275 0.00300
EC50 values benzovindiflupyr (µg ml-1)

Figure 5.1. Distribution of EC_{50} values for baseline isolates of *V. inaequalis* for penthiopyrad (n=35) **(A)**, fluopyram (n=70) **(B)**, or benzovindiflupyr (n=35) **(C)**. Sensitivities were determined using a conidial germ growth inhibition assay. Baseline isolates were collected from apple orchards in New York that have never been exposed to single-site fungicides.

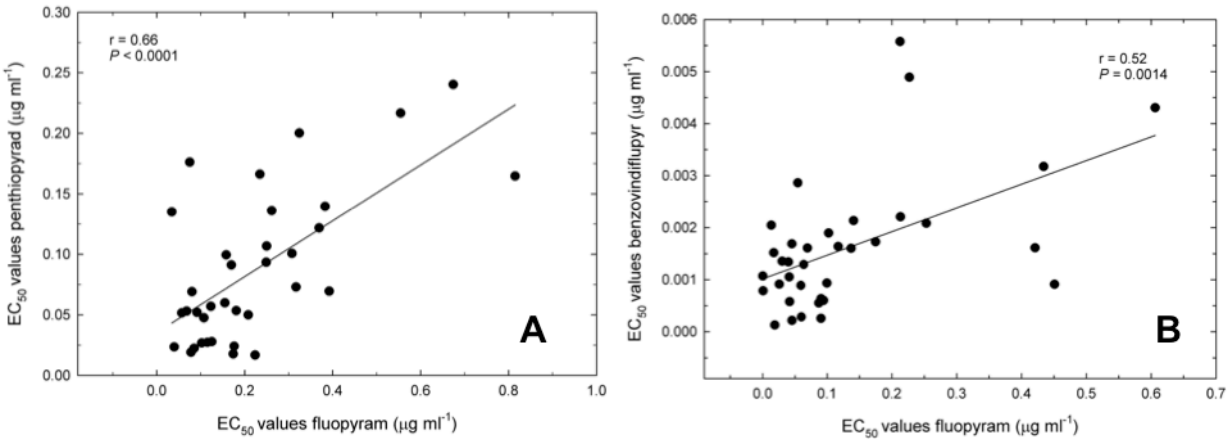


Figure 5.2. Linear correlations between fluopyram and penthiopyrad sensitivities **(A),** and between fluopyram and benzovindiflupyr sensitivities **(B)** for 35 baseline isolates of *Venturia inaequalis*. Sensitivity is expressed as the effective concentration that inhibited conidial germ tube growth by 50% (EC_{50}). Each curve was fitted with the linear distribution function [y = yo + ax].

The effective concentrations of benzovindiflupyr and fluopyram needed to inhibit mycelial growth by 50% were also evaluated using mycelial inhibition assays (Pfeufer and Ngugi, 2012). Mean EC_{50} values were determined for benzovindiflupyr and fluopyram every two weeks. After six weeks, benzovindiflupyr and fluopyram EC_{50} values ranged from 0.001 to 0.146 µg ml^{-1} (mean = 0.043 µg ml^{-1}) and from 0.127 to 4.00 µg ml^{-1} (mean = 2.02 µg ml^{-1}), respectively. These mean EC_{50} values were greater than those obtained for conidial germ tube growth inhibition. Interestingly, mean EC_{50} values increased by a factor of 1.5 for both fungicides from the initial two-week measurement to the final six-week measurement (six-week growth) (data not shown). Cross-sensitivity between benzovindiflupyr and fluopyram became

more apparent from 2 to 6 weeks. While there was a significant and positive correlation of EC_{50} values between benzovindiflupyr and fluopyram for the two-week ($P = 0.0038$) and six-week ($P \leq 0.0001$) evaluations, correlations became stronger over time. Indeed, the correlation coefficients (r) for benzovindiflupyr and fluopyram increased from 0.48 at 2 weeks to 0.93 at 6 weeks (Figure 5.3).

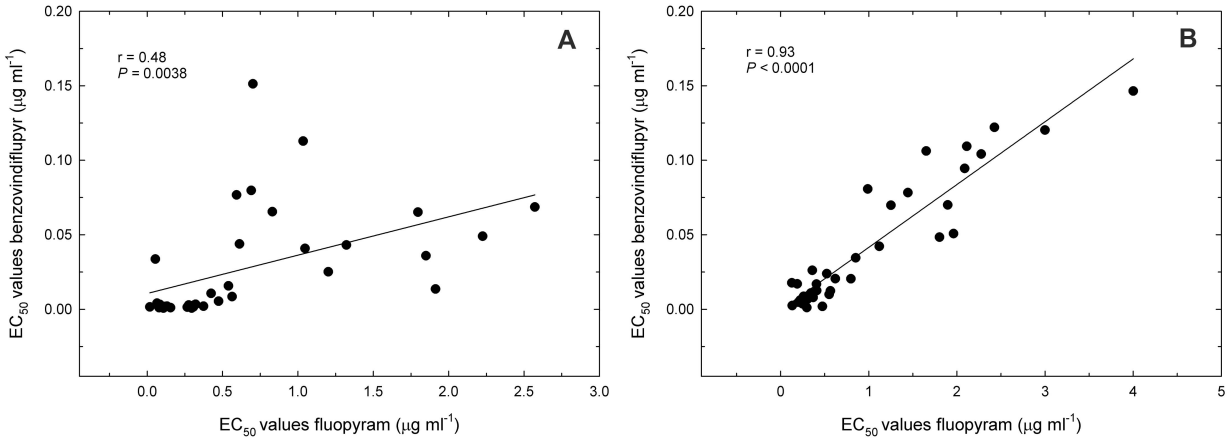


Figure 5.3. Linear correlations between fluopyram and benzovindiflupyr sensitivities for 35 baseline isolates of *Venturia inaequalis*. Sensitivities are expressed as the effective concentration that inhibited mycelial growth by 50% (EC_{50}) using a mycelial growth inhibition assay. Correlations between fluopyram and benzovindiflupyr EC_{50} values after two weeks **(A)** and six weeks of incubation **(B)**. Each curve was fitted with the linear distribution function [y = yo + ax]

Identification and characterization of the *V. inaequalis sdhB* gene. MiSeq reads for the *V. inaequalis* baseline isolate 3a-27-10 yielded a total of 8 million paired-end reads of Illumina sequence data and 7,300 contigs of between 200 to 300 bp in length were assembled. A BLAST search of the genome of the baseline isolate (CLC Main Workbench) with the *sdhB* sequences of *S. sclerotiorum* and *A. alternata* revealed a high level of homology within a single contig of the assembled genome. A search for open reading frames 200 bp upstream from the region of homology revealed a single putative start codon. The stop codon for the *sdhB* gene of

V. inaequalis was identified using a highly conserved region of 33 amino acids located four to five amino acids prior to the termination codon, 'TAA'.

Genomic DNA of the *sdhB* gene was amplified for three isolates of *V. inaequalis* collected from either a baseline (isolate 12-2b-14), a commercial (isolate 40-45-11), or a research (isolate 41-45-11) orchard population of *V. inaequalis*. Amplification was accomplished using primer pairs ViSDHB-230F/ViSDHB415R, ViSDHb205F/ViSDHb860R, and ViSDHb504F/dsViDSHb1137R, which produced fragments of 646 bp, 656 bp, and 634 bp, respectively. Single nucleotide polymorphisms (SNPs) were discovered at positions 169, 217, 229, 508, 653, 659, 680, 683, 689, 695, 698, and 722, however none of these mutations resulted in a change in the amino acid coding sequence.

Amplification of cDNA of the three isolates of *V. inaequalis* with primer sets cDNAViSDHb1F/viSDHB 415 and ViSDHb205F/cdnaViSDHB957R yielded fragments of 415 bp and 753 bp, respectively. Alignment of the *V. inaequalis sdhB* gene amplified with cDNA and gDNA from each of the three representative isolates, revealed a 58 bp intron located after nucleotide 45 and a 55 bp intron located after nucleotide 580. Including both introns, the *V. inaequalis sdhB* gene was 959 bp in length and the nucleotide sequence of the coding region was 84, 83, and 82% identical to the *sdhB* genes of *A. alternata* (accession no. KJ426260), *Leptosphaeria maculans* (accession no. FO90601), and *Pyrenophora tritici* (accession no. XM001933224), respectively. Nucleotide sequence data and corresponding amino acid sequence data for the *sdhB* gene of *V. inaequalis* isolates 12-2b-14 and 40-45-11 were deposited in GenBank under accession numbers KR139837 (12b-2b-14, gDNA), KR139835 (40-45-11, gDNA) and KR139836 (40-45-11, cDNA).

Screening for mutations in the *sdhB* gene following applications of penthiopyrad, fluopyram, and benzovindiflupyr. Following applications of Fontelis SC (penthiopyrad), Luna Sensation (fluopyram + trifloxystrobin), or Aprovia (benzovindiflupyr) single-lesion isolates of *V. inaequalis* were collected from each SDHI treatment program to determine if the presence of mutations in *sdhB* gene were associated with the emergence of apple scab lesions. Across all four treatment programs, there were no novel missense mutations identified at any location within the *SdhB* gene of *V. inaequalis* for any of the 10 replicate lesion samples. Also no missense mutations traditionally conferring SDHI fungicide resistance in other phytopathogens, including P205F/L/T (site for mutation P225F/L/T in *B. cinerea*), N210I (site for mutation N230I in *B. cinerea*), or H252Y/R/L (site for mutation H272Y/R in *B. cinerea* and H277Y/R/L in *A. alternata* were found).

DISCUSSION

Recent and impending releases of next generation SDHI fungicides provide additional alternatives to managing apple scab in the northeastern and mid-Atlantic United States. Knowledge on the sensitivity of *V. inaequalis* baseline populations to the SDHI fungicides is not only important for understanding the risk of SDHI resistance development in *V. inaequalis,* but can also provide information on relative SDHI fungicide efficacy and serve as the basis for monitoring population shifts towards resistance. In this study, the baseline sensitivity to three SDHI fungicides was determined for a population of *V. inaequalis* that had never been exposed to single-site fungicides. Because SDHI fungicides have been shown to be effective during several stages of fungal growth (Amiri et al., 2010; Veloukas and Karaoglanidis, 2012), two evaluation methods were used to determine baseline sensitivities of isolates of *V. inaequalis.*

First, sensitivities of penthiopyrad, fluopyram, and benzovindiflupyr were evaluated on conidia using a conidial germ tube growth inhibition assay. Secondly, the sensitivities of fluopyram, and benzovindiflupyr were evaluated on mycelial colonies using a mycelial growth inhibition assay.

Of the three SDHI fungicides evaluated, benzovindiflupyr demonstrated the highest level of activity against *V. inaequalis* during both the conidial germination and the mycelial growth stages of development. One possible explanation for the greater intrinsic activity observed in vitro might be a different interaction of benzovindiflupyr within the fungicide-binding pocket (Scalliet et al., 2012) compared with the other two fungicides. Indeed, a greater binding affinity of benzovindiflupyr could also account for the higher level of efficacy demonstrated by the fungicide (Schnabel and Dai, 2004). Alternatively, benzovindiflupyr could be affecting other additional metabolic functions in *V. inaequalis* that are essential to in the initial stages of fungal growth.

SDHI fungicides have been previously shown to inhibit conidial germination, germ-tube elongation, and mycelial growth, but the level of inhibition is often inconsistent throughout the different growth stages (Amiri et al., 2010; Amiri et al., 2014; Thomas et al., 2012; Veloukas and Karaoglanidis, 2012). Lower levels of sensitivity to the SDHI fungicide, boscalid, were observed for mycelium of *Monilinia fructicola* (Amiri et al., 2010) and *Botrytis cinerea* (Myresiotis et al., 2008; Veloukas and Karaoglanidis, 2012) compared to the sensitivity of germinating conidia of these isolates. Additionally, sensitivity to fluopyram was greater for germinating conidia in *B. cinerea* compared to mycelium (Veloukas and Karaoglanidis, 2012). Similarly, we found that germinating conidia of *V. inaequalis* were more sensitive compared to mycelium for both benzovindiflupyr and fluopyram with benzovindiflupyr demonstrating the highest level of activity against both stages. Considering SDHI fungicides inhibit complex II of fungal

respiration, the relatively high levels of efficacy demonstrated by both fluopyram and benzovindiflupyr during the conidial germination phase are not surprising. Indeed, conidial germination and germ tube elongation demand high levels of energy, which are primarily provided by aerobic respiration (Allen, 1965). Similar to the QoI fungicides, another class of respiration inhibitors, our results suggest that the SDHI fungicides should provide better control and should primarily be used as a protective agent to prevent conidial germination, germ-tube elongation, and the initiation of infection, rather than being used in the curative mode in the field.

Relatively narrow ranges in baseline EC_{50} values determined for the conidial germ tube growth phase were observed for penthiopyrad (0.017 to 0.240 μg ml^{-1}) and benzovindiflupyr (0.00013 to 0.0029 μg ml^{-1}), but not for fluopyram (0.0002 to 0.815 μg ml^{-1}). Despite the wide-range in baseline sensitivity to fluopyram, 75% of the isolates of *V. inaequalis* evaluated for fluopyram had EC_{50} values between 0.01 and 0.3 μg ml^{-1}. The range in penthiopyrad and benzovindiflupyr baseline sensitivities in *V. inaequalis* was narrower than that for baseline isolates of *A. solani* in which there was a 64-fold difference between the most and least sensitive members of the baseline population to penthiopyrad (Gudmestad et al., 2013). While the narrow range of EC_{50} values in this study could possibly be due to the low geographical diversity of baseline populations (Thomas et al., 2012) the number of isolates examined was similar to that of other reported studies in which baseline sensitivity to single-site fungicides was reported (Chen et al., 2012; Köller et al., 1991). Furthermore, similar ranges in baseline sensitivity have been observed in studies in which over 10 times as many isolates were evaluated (Olaya and Köller, 1999). Given the short range in sensitivity across the entire baseline population, selection for penthiopyrad and benzovindiflupyr resistance in *V. inaequalis* might be relatively slow compared

to other phytopathogens with a greater range in baseline SDHI sensitivity (Villani et al., 2015). However, other factors influencing rates of fungicide resistance development including resistance-management strategies, resistance-type (qualitative vs. quantitative), and disease pressure should not be overlooked.

Defined by their common biochemical mode of action, cross-sensitivity is commonly observed among fungicides belonging to the same FRAC group. In the current study, a moderate level of positive cross-sensitivity in baseline isolates was observed between penthiopyrad and fluopyram, and between benzovindiflupyr and fluopyram when the conidial germ tube growth inhibition assay was used to determine EC_{50} values. Since benzovindiflupyr and penthiopyrad are in a different SDHI chemical group than fluopyram, the observed level of cross sensitivity is a little surprising, but not unexpected since all three are in the same fungicide class and target the same enzyme complex. Interestingly, a higher level of positive cross-sensitivity between benzovindiflupyr and fluopyram was observed for mycelial growth suggesting that both fungicides may disrupt identical processes during the mycelial growth stage, or that non-primary target genes may be affected by SDHIs during the conidial germ tube growth phase compared to the mycelial growth phase (Sierotzki and Scalliet, 2013).

Despite the cross-sensitivity observed between penthiopyrad and fluopyram and benzovindiflupyr and fluopyram in baseline isolates of *V. inaequalis*, it cannot be concluded that cross-resistance will occur between the two fungicides. For example, cross-sensitivity between the DMI fungicides myclobutanil and difenoconazole was found in baseline populations of *V. inaequalis,* however, in populations resistant to myclobutanil, cross-resistance was not observed (Villani et al., 2015). In regards to the SDHI fungicides, little cross-sensitivity was observed between fluopyram and penthiopyrad in field isolates of *Didymella* bryoniae, *Corynespora*

cassicola and *Posdosphaera xanthii* (Avenot et al., 2012; Ishii et al., 2011). However, the majority of isolates used to evaluate cross-sensitivity between penthiopyrad and fluopyram in these studies had prior resistance to boscalid. Additionally, molecular mechanisms found to cause resistance to penthiopyrad may have no bearing on resistance to fluopyram. For example, the *sdhB* H277Y mutation imparted resistance to boscalid and penthiopyrad but not fluopyram in isolates of *A. alternata, D. bryoniae, C. cassicola,* and *P. xanthii* (Avenot et al., 2012; Avenot et al., 2014; Ishii et al., 2011). Thus, as isolates of *V. inaequalis* develop resistance to penthiopyrad, a corresponding decrease in sensitivity to fluopyram may not occur.

The *sdhB* gene in *V. inaequalis* was identified and characterized in isolates from a baseline, research, and commercial orchard so that presence of missense mutations could be identified in subsequent years following control failures of SDHIs in the field. Additionally, multi-lesion composite samples collected from trees exposed to treatment programs of different SDHI fungicides in the field were also evaluated for the presence of *sdhB* missense mutations. Following applications of Fontelis SC (penthiopyrad), Luna Sensation (fluopyram + trifloxystrobin), and Aprovia (benzovindiflupyr), apple scab lesions developed in each treatment, however no mutations resulting in changes to the amino acid coding sequence in the *sdhB* gene were identified within any of the multi-lesion composite samples. While we were unable to identify anomalies within the *V. inaequalis sdhB* gene, a number of factors including unidentified molecular mechanisms of resistance may have led to the development of lesions after fungicide application in the field. Mutations within the *sdhC* (H134R) *and sdhD* (H133R) genes have been associated with reduced sensitivity to boscalid and/or penthiopyrad in *A. alternata* (Avenot et al., 2009) and *A. solani* (Mallik et al., 2014). In *V. inaequalis,* the *sdhC* and *sdhD* genes and any corresponding mutations within those genes have yet to be identified. More

likely, other factors such as high inoculum pressure, inherent efficacy of the fungicide, cultivar susceptibility, and poor application coverage may have contributed to the presence of lesions following applications of SDHIs in the field.

In conclusion, the SDHI fungicides penthiopyrad, fluopyram, and benzovindiflupyr are able to inhibit conidial germ tube growth and mycelial growth in baseline isolates of *V. inaequalis*. Of these three fungicides, benzovindiflupyr provided the highest level of activity against conidial germination and mycelial growth with EC_{50} values below 1.0 μg ml^{-1} irrespective of the isolates and method used. In this regard, fungicide products formulated with benzovindiflupyr will likely provide a high level of control against apple scab when used during both protective and curative application modes. Aside from a sensitivity baseline, the *Venturia inaequalis sdhB* gene was identified and characterized. None of the missense mutations that have been associated with SDHI fungicide resistance in other fungal pathogens were found in the isolates used for gene discovery or those recovered following SDHI fungicide applications in the field. Despite the absence of missense mutations in the baseline and the one field population, the site-specific nature of this fungicide class and its widespread resistance in other fungal pathogens, suggests that rapid onset resistance in *V. inaequalis* is possible. Diligent monitoring for shifts towards resistance, screening of *sdh* genes for mutations, and practicing resistant management strategies will be imperative for sustained use of SDHI fungicides in the management of apple scab.

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CHAPTER 6

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Despite a relatively long history of research on the biology and management of *Venturia inaequalis* (Becker et al., 1992; Curtis, 1924; Köller and Wilcox, 1999; MacHardy, 1996; MacHardy et al., 2001) apple scab continues to be a primary disease concern in temperate apple growing regions throughout the world, including the northeastern United States. The planting of high acreages of susceptible cultivars, the difficulty of reducing primary inoculum, and the cool, humid spring climate throughout the northeastern United States all contribute to ideal conditions for apple scab epidemics. Currently, applications of single-site fungicides in tank mixture with protectant fungicides are the primary means for commercial growers to control the disease while meeting fruit quality expectations of consumers. As history has demonstrated, however, frequent use of single-site fungicides can and most likely will lead to the onset of resistance (Beckerman et al., 2014; Jones, 1981; Stanis and Jones, 1985). The work presented in this dissertation addresses the prevalence of DMI fungicide resistance throughout the northeastern and mid-Atlantic regions of the United States and examines molecular mechanisms responsible for practical resistance to DMI and QoI fungicides. In addition, the baseline sensitivity and genetic information that was established for SDHI fungicides a in *V. inaequalis* will enable rapid screening for resistance in future studies.

DMI fungicides have been used for more than 30 years to provide protective and curative activity against economically important fungal pathogens, including *V. inaequalis*. Although resistance to the DMIs was documented in individual isolates and isolated populations of *V. inaequalis* (Hildebrand et al,, 1988; Köller et al., 1997), the efficacy of DMI fungicides as a class has been fairly well-preserved since their introduction. There are many factors that have

contributed to the long-term efficacy of the DMI fungicides. However, the quantitative nature of DMI resistance in *V. inaequalis* and the diversity of DMI chemistries with varying levels of efficacy against *V. inaequalis* have certainly promoted the continued success of this fungicide class. In Chapter 2, the prevalence of practical resistance to the DMI fungicide, myclobutanil, was investigated for populations of *V. inaequalis* collected from major apple production regions throughout New England, the Midwest, and the Mid-Atlantic. Based on statistical comparisons with resistant and sensitive population standards, the majority of populations evaluated had reduced sensitivity or practical resistance to the fungicide. Previously marketed in Europe, the Federal and NY registrations of a newer DMI fungicide, difenoconazole, prompted the determination of baseline sensitivity to the fungicide in NY populations of *V. inaequalis,* and an investigation into its efficacy in populations of *V. inaequalis* found to have practical resistance to myclobutanil. It was found that difenoconazole had approximately 50x greater activity than myclobutanil against *V. inaequalis*, and despite widespread practical resistance and reduced sensitivity to myclobutanil, only one orchard population had reduced sensitivity to difenoconazole. These results suggested that newer DMI fungicides with high levels of intrinsic activity against *V. inaequalis* are still able to control apple scab in the field. Given the curative capability of DMI fungicides, growers might still have a potent fungicide for apple scab management following an infection event.

An unanticipated discovery from the DMI sensitivity investigation in Chapter 2 was the strong cross-sensitivity between myclobutanil and difenoconazole sensitivity present in a baseline population of *V. inaequalis*, but the absence of cross-resistance in populations of *V. inaequalis* with practical resistance to myclobutanil. Considering the higher level of efficacy demonstrated by difenoconazole, it was surprising that some isolates were less sensitive to

difenoconazole than myclobutanil *in vitro*. An explanation for the low level of cross-resistance between the two fungicides could be the presence of different mechanisms for resistance to myclobutanil and difenoconazole. To investigate this hypothesis in Chapter 3, reverse transcriptase-quantitative PCR was conducted to evaluate expression of the target *CYP51A1* gene in isolates of *V. inaequalis* with varying levels of sensitivity to myclobutanil and difenoconazole. Overexpression of the *CYP51A1* gene was observed for isolates resistant to difenoconazole regardless of sensitivity to myclobutanil. Alternatively, overexpression of *CYP51A1* was only observed for isolates resistant to myclobutanil when difenoconazole resistance was also present. The greater contribution of *CYP51A1* in difenoconazole sensitivity compared to myclobutanil sensitivity was further evaluated through linear correlation in which it was found that *CYP51A1* expression explained some of the variability in isolate sensitivity to difenoconazole ($R^2 = 0.630$) but little to none of the variability in isolate sensitivity to myclobutanil ($R^2 = 0.078$). Using Illumina sequencing to elucidate the upstream region of *CYP51A1* in *V. inaequalis*, a 169 bp element, termed El-3,1,2, was found only in isolates resistant to difenoconazole exhibiting overexpression of *CYP51A1*. While no promoters were predicted within this element, sites for the binding of transcription factors associated with activation of the *ERG11* (*CYP51A1*) were identified.

Chapter 4 transitioned to an investigation of the quantitative resistance response to QoI fungicides in *V. inaequalis.* While the substitution of glycine to alanine at codon 143 of the *cyt b* (G143A) has been extensively documented as qualitative resistance determinant to QoI fungicides in several phytopathogenic fungi including *V. inaequalis* (Fernández-Ortuño et al., 2008; Fontaine et al., 2009; Sierotzki et al., 2000), there has been little research on quantitative resistance to QoI fungicides or the mechanisms governing this type of resistance. Because each

single *cyt b* gene is encoded by a single mitochondrion there are several *cyt b* genes within just a single cell of *V. inaequalis.* Each copy of *cyt b* might either be resistant to QoI fungicides and thus have the A143 allele, or may be sensitive to QoIs and thus have the G143 allele. The nature of *cyt b* heteroplasmy has been well documented (Lesemann et al., 2006; Ma and Michailides, 2004). However its role in quantitative practical resistance to QoI fungicides had not been investigated. To better understand the role of heteroplasmy of the *cyt b* gene in the QoI resistance response for isolates and populations of *V. inaequalis,* I developed an allele-specific quantitative PCR assay to quantify the relative abundance of the A143 (resistant) allele in isolates of *V. inaequalis* with different levels of sensitivity to trifloxystrobin. Although a high relative abundance of the A143 allele was associated with isolates having high resistance responses, heteroplasmy of the *cyt b* gene was not the primary factor involved in isolates with moderate resistance responses suggesting that other resistance mechanisms are involved in reduced sensitivity to QoIs. To demonstrate the link between practical resistance and the abundance of the A143 allele, the AS-qPCR assay was also conducted on field populations of *V. inaequalis*. Results of this study showed that approximately 20% of the collective *cyt b* genes within a field population must be resistant (i.e. have the A143 allele) before QoI fungicide failures in the field are observed.

The final research chapter of this dissertation (Chapter 5) investigated the relative intrinsic activity of three new-generation SDHI fungicides in a baseline population of *V. inaequalis.* A moderate level of cross-sensitivity was found between fluopyram and benzovindiflupyr, and fluopyram and penthiopyrad in baseline isolates of *V. inaequalis*. However, benzovindiflupyr demonstrated the highest level of activity against conidial germination and mycelial growth. This indicates that benzovindiflupyr should perform well as

both a protective and post-infection fungicide in the field. The *sdhB* gene in *V. inaequalis* was also identified and characterized in this study. No missense mutations were identified in the coding regions of isolates of *V. inaequalis* recovered following SDHI fungicide applications.

In summary, understanding the prevalence of single-site fungicide resistance and the molecular mechanisms governing resistance will continue to be important for the sustainability of effective chemical management for apple scab throughout the northeastern United States. The identification of non-target gene involvement in fungicide resistance and the implications of multiple fungicide resistance imparted by these genes will also be crucial for selection of fungicides. For example, the mechanism behind myclobutanil resistance in *V. inaequalis* is still unknown and may be the result of overexpression of energy dependent drug efflux pumps (Hulvey et al., 2012). Additionally, overexpression of such non-specific efflux genes could also be associated with resistance to other single-site fungicides. Fortunately, the decreasing cost of 2^{nd} generation transcriptome sequencing should enable more rapid identification of non-target gene involvement in fungicide resistance and assist in the identification of minor candidate genes involved in multiple drug resistance.

There is still much research to also be conducted in regards to SDHI fungicides and resistance to these fungicides. While no missense mutations within the *sdhB* gene were identified in isolates of *V. inaequalis* recovered from the field following SDHI fungicide applications, the possibility of missense mutations within the *sdhC* and *sdhD* genes has yet to be determined in *V. inaequalis*. Furthermore, whether missense mutations will impart complete or partial resistance to SDHI fungicides, and the relationship between mutation location and resistance to a particular group of SDHI fungicides is also still unknown in the apple scab pathogen.

Ultimately, identifying and understanding the molecular mechanisms of single-site fungicide in *V. inaequalis* are important for the rapid detection of fungicide resistance, the development of fungicides with novel target sites and modes of action, the deployment of fungicide resistance management strategies, and the development of efficient and effective chemical management programs for apple scab control.

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