IMPLICATIONS OF PRODUCTION PRACTICES ON PREVALENCE AND STABILITY OF FUNGICIDE RESISTANT ORCHARD POPULATIONS OF *VENTURIA INAEQUALIS*

A Thesis

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ABSTRACT

Apple scab, caused by *Venturia inaequalis,* is one of the most economically important fungal diseases affecting apple production in temperate climates. Growers in these regions manage apple scab through the application of fungicide chemistries. The repeated usage of fungicide chemistries with a specific target site, such as Quinone outside Inhibitors (QoI) and demethylation inhibitors (DMIs), can have an impact on the orchard population structure in the number of resistant isolates that survive to reproduce. Many aspects regarding the development, prevalence, and stability of resistance to these fungicides is not completely understood for *V. inaequalis*. Two studies were carried out to determine how two different disease management practices affect the DMI sensitivity of *V. inaequalis* orchard populations. An additional study was performed to determine the prevalence and *in vitro* stability of QoI fungicide resistance in *V. inaequalis*. The first two studies provided data on the occurrence of DMI resistant isolates in *V. inaequalis* orchard populations either after a dormant chemical treatment or when difenoconazole (DMI fungicide) is applied throughout the production season as opposed to during the primary infection season only. The third study provided an understanding of the prevalence of QoI resistance in the northeastern United States, as well as the degree of orchard populations with the G143A mutation in the *cytochrome b* gene that confers a high degree of QoI resistance. With this understanding, attention is needed to understand the persistence of the G143A mutation in the absence of selective pressure. These studies contribute to the collective knowledge of how disease management options affect *V. inaequalis* DMI and QoI fungicide resistance.

BIOGRAPHICAL SKETCH

Zachary Andrew Frederick was born on June 10th, 1990 to Dennis and Susan Frederick, and has two younger brothers, Aaron and Jonathan. He is glad to have the support of his family and the welcome addition of his stepparents, Steven Frost and Carol Frederick.

Zack's first experience in a laboratory was in 2007 as a high school intern in Plant Genome Research Project under the supervision of Dr. Maria Harrison. At the conclusion of this internship, he asked Dr. Harrison what schooling would be required for a faculty position in this field. With Dr. Harrison's advice in mind, Zack participated in the Summer Scholars program at the New York State Agricultural Experiment Station in the lab of Dr. Kerik Cox in 2010. In supplement to his objective of examining sampling methods for assaying fungicide resistance in apple scab orchard populations, Zack also took further interest in plant pathology and more specifically in studying factors affecting the development and persistence of fungicide resistant phenotypes of *V. inaequalis* in regards to tree fruit production.

As the final step of Zack's bachelors in agricultural biotechnology at the State University of New York at Cobleskill, he returned to Dr. Cox's program in 2011 with additional aims to evaluate qualitative QoI testing methods for *V. inaequalis* and determine the prevalence of qualitative QoI resistance in northeastern orchard populations of *V. inaequalis*. In addition to his objectives, Zack participated in as many of the Dr. Cox's program activities as he could, which included attending regional extension meetings. In observing the interactions of stakeholders and faculty, as well as research driven by stakeholders on emergent issues, Zack became interested in Plant Pathology graduate programs with emphasis on extension.

DEDICATION

To family for unwavering support

The excellent company of friends

And astute mentors for guidance in all things

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PREFACE

Apple scab, caused by *Venturia inaequalis*, is one of the more destructive and economically important fungal diseases affecting apple production. Chemical management in form of fungicide applications are required to maintain crop profitability in apple orchards because the climate in the northeastern United States is favorable to apple scab disease development, and consumers prefer susceptible cultivars. Growers must use fungicide chemistries throughout the production season in such a manner to avoid selection for fungicide resistant in *V. inaequalis* orchard populations. This situation is the driving force behind many studies into the development and persistence of fungicide resistance, including the studies presented in this master's thesis concerning the persistence of QoI and DMI resistance. This particular problem is also of importance to regional growers who may be managing or may eventually manage fungicide resistant *V. inaequalis* populations. The contributions of each of the present studies can help refine grower disease management strategies so as to prevent management failures and a reduction of the crop value due to fungicide resistant populations. The results from these studies also contribute the understanding of the persistence of DMI and QoI fungicide resistance in *Venturia inaequalis*, and may be applicable to other agriculturally important ascomycete fungal plant pathogens.

CHAPTER 1

INTRODUCTION

Brief Overview of the Apple Industry as it Pertains to New York, Overview of Apple Scab Symptoms and Underlying Factors for Epidemics

Over the past century, the apple industry in New York has taken great strides to increase yields and profits to remain competitive. There are an estimated 42,000 bearing-age acres of apples which produce an average of 30,200 pounds of total harvest per acre, which puts New York in second place for national apple production (USDA National Agricultural Statistic Service, 2012). These bearing acres are spread across the state, but most are localized to particular regions such as the Niagara frontier, the Champlain Valley, the Hudson valley and the Finger Lakes. These 42,000 bearing-age acres are owned by approximately 694 commercial apple growers that provide over ten thousand jobs directly related to agricultural production. These farms also provide thousands of indirectly related jobs in chemical production and storage, shipping, equipment sales and repairs, research, packing and storage, marketing, financial and other services, and sales. In addition to the direct value to the apples themselves, their production is deeply intertwined with many other aspects of the local economy (New York Apple Association, 2011).

Apple scab, caused by *Venturia inaequalis* (Cooke) Winter, is one of the more economically important fungal diseases affecting apple production because of the pathogen's propensity to infect and damage foliar and fruit tissue, hence reducing the value of the crop. This pathogen can cause considerable, and potentially complete, crop loss if improper management

strategies are employed in areas with prevailing weather conditions that are conducive for severe infection (Holb et al, 2003, MacHardy, 1996, Roberts 1935). This pathogen has been observed worldwide (Holb et al, 2003, MacHardy et al, 2001, MacHardy, 1996, Sutton et al, 1996), including the northeastern United States (Merwin et al, 1994). Epidemics often occur during the spring and summer when conditions are conducive to infection, or when weather remains cool (< 30 ºC) and substantial wetting events (> 12 h of leaf wetness) occur (MacHardy et al, 1996). This necessitates chemical management in the form fungicide applications from bud break to early summer (MacHardy, Gadoury, and Gessler 2001, MacHardy, 1996, Gadoury, 1986). Early summer marks the point when primary inoculum has been discharged and heat renders conidia non-viable, however infection events can still occur during cool, wet periods in the summer (MacHardy, 1996).

Visible symptoms of apple scab infection begin as olive colored lesions on the upper surface of newly expanding leaves. The first lesions seen on newly expanding leaves and green apple tissue are often attributed to ascospore infections from inoculum that overwinter in leaf litters on the orchard floor (Agrios, 2005, Roberts, 1935). Lesions can increase in number and coalesce over leaf surface with continued secondary infections as the season progresses (Gessler et al, 2006, Agrios, 2005). Leaves that are severely infected will shrivel and abscise, which can defoliate a susceptible tree (Agrios, 2005, Jones et al, 1990). This extreme scenario can also cause the tree to not bud properly in the following season (Agrios, 2005). *V. inaequalis* is also a direct pathogen of apple fruit during all stages of development (Agrios, 2005, Jones et al, 1990, Roberts, 1935). *V. inaequalis* fruit infections reduce fruit size, quality, and can cause premature drop (Jones et al, 1990). Fruit infections that occur early in development crack the fruit as it expands, exposing inner tissues to secondary rots (Agrios, 2005, Roberts, 1935). Fruit infections

necrotize tissues near the fruit surface, which is the proximate cause of defined, corky lesions fruit that are of little commercial value (Gessler et al, 2006, Agrios, 2005, Jones et al, 1990). Fruit nearing maturity can still be infected, given favorable conditions, but may only develop small lesions that darken in storage and can infect surrounding fruit (Agrios, 2005). Despite management programs continuing throughout the production season, great emphasis has been placed on managing primary *V. inaequalis* overwintering inoculum and infections from this inoculum in the earliest parts of the primary apple scab season to stymy the start of an epidemic (Agnello et al, 2012, Holb et al, 2005, Holb et al, 2004a).

Both traditional management strategies and the body of literature highlight the significance of controlling *V. inaequalis* primary infections to prevent outbreaks that lead to epidemics (Agnello et al, 2012, Holb et al, 2005, Holb et al, 2004a). This hinges on preventing successful primary infections, which arise on apple leaves when hyphae penetrate the space between the cuticle and the upper epidermis of tissues (Agrios et al, 2005, Holb et al, 2005, Jones et al, 1990). *V. inaequalis* primary inoculum is partially derived from overwintering ascospores that are arranged in asci that are held within psuedothecia, which in turn are contained inside infected leaves that dropped in the fall. The loculostromata of *V. inaequalis* forms just after the leaves fall from the tree, and pseudothecia are formed from loculostromata in late spring. Pseudothecia release ascospores shortly after the buds of apple emerge, and susceptible tissue is exposed (Agrios, 2005, Gadoury et al, 2004, Jones et al, 1990). Ascospore discharge begins when the fallen leaves becomes moistened, and water moves into the pseudothecia through the ascocarp's walls or the ostiole at the vertex of the ascus (Aylor, 1996). Mature ascospores swell and protrude through the ostiole until they are ejected (Gadoury et al, 1992). This ejection period can range from 6 to 12 weeks, and is influenced greatly by prevailing

temperatures, relative humidity, light exposure (particularly 710 to 730 nm) , and the speed at which fallen leaves are degraded (Gadoury et al, 2004, Aylor, 1996, Brooke 1969). Research has indicated that aerial concentration of ascospores was highest at the source of inoculum and decreases as the distance increases. However, aerial concentrations are still high at 21 meters from the inoculum source. Further conjecture hypothesizes that ascospores have lower infection efficiencies on resistant cultivars, and is an area of ongoing research (Holb et al, 2004b, Gregory, 1968).

However, ascospores are not the sole source of overwintering inoculum. It has been previously determined that conidia can persist through winter, and that there are three main places where of *V. inaequalis* overwintering conidial inoculum resides in apple orchards: on exposed wood, on leaf litter, and both on and within diseased buds (Holb et al, 2004a, Becker et al, 1992). Inoculum on diseased wood and shoots has not proven to have high viability, with an average of 1.5% conidial viability (Becker et al, 1992). This has been attributed to unfavorable winter conditions. However, presuming 1.5% of millions of spores survive and are viable, that would theoretically still leave thousands of conidia that can potentially start a new epidemic, presuming no fungicide or biocide applications are made to reduce the surviving conidial population. Conidia have been shown to be more viable if they overwinter inside bud scales than on the surfaces of any apple tissue. Overwintering inside bud scales would place conidia in close proximity to susceptible green tissue as it emerges in the spring (Holb et al, 2004a, Becker et al, 1992).

Chemical Management: Copper Applications during Dormancy and Potential Selection for DMI sensitive *V. inaequalis* isolates.

Copper products are applied while the trees are dormant in either the late fall or early spring to avoid toxic effects on emerging green tissue (Clark, 1902). Applications of formulated copper biocides are often used on apple with the intent of reducing disease pressure from the overwintering fireblight inoculum (Norelli et al, 2003) in holdover cankers (Beer et al, 1977). These products are usually copper sulfate, copper hydroxide, copper oxide, copper sulfate and lime (Bordeaux mixture), or similar products and mixtures, which have been used agriculturally for decades or longer.

In 1949, it was agreed upon that copper based fungicides only displayed biocidal activity when wetted, even those of low solubility. The reason for this activity was still a point of debate, with some suggesting chemical activity by carbon dioxide and others holding fast to the notion that spore exudates combined with copper to make even more toxic precipitates (McCallan, 1949). In his review, McCallan concluded that it was most likely that copper fungicides inhibit successful spore germination and penetration because amino acids and malate spore exudates combine with dissolved copper and form copper complexes of increased toxicity. This localizes the most toxic copper compounds in close proximity to spores. However, research done my Martin et al, 1942 showed several potential amino acids that can have this effect with copper, but cuprimalate is the most directly toxic compound assayed from the interaction of model spore exudates and Bordeaux mixture. For example the biocidal activity of Bordeaux mixture, which protected wine grapes from downy mildew, was discovered in 1882 (MaCallan, 1949). Copper products have proven to be effective control measures for *V. inaequalis* (Montag et al, 2006, Holb et al, 2003). However, its use has been restricted parts of Europe due observed broad spectrum toxicological effects (Hunsche et al, 2011, Holb et al, 2003, Raw, 1962), and a

significant body of research focuses on reducing copper usage in apple production while still effectively managing *V. inaequalis* (Gobin et al, 2006, Montag et al, 2006).

Applications of copper products must be evenly distributed to ensure uniform coverage for greatest efficacy, while ensuring damage to plant tissue is limited (Clark, 1902). After Clark published these findings, prevailing logic suggested that the low solubility of copper fungicides was necessary to prevent tissue damage to treated plants. Even in 1949, efforts focusing on the effects on copper products on fungi aimed to figure out how these materials "exert their action to prevent the germination of spores" (McCallan, 1949). Further scientific inquiry indicated that three of the most prominent copper formulations (Copper hydroxide, copper oxide, and copper sulfate) were able to prevent fungal spore germination and inhibit the mitochondrial respiration pathway, and enter fungal hyphae when dissolved in solution (Montag et al, 2006). Copper hydroxide was the least effective of the three at preventing conidial germination, which has been attributed to a virtually negligible copper ion presence in solution (Montag et al, 2006). However, treating with this form of copper still prevents infections by damaging the appressorium once it is formed. Once damaged, penetration of the cuticle cannot occur. Copper sulfate is often formulated for smaller particle size and dissolves the most readily into solution out of the three common copper compounds listed above, implying that this product was the propensity to wash off. This would be an issue in the early apple season in the eastern United States when rains are prevalent. Growers want products stay on the tree, so copper hydroxide is commonly utilized because it is the least readily washed away. Research has identified the concentration and amount of copper products and the degree of active ingredient runoff into water supplies, albeit on a different crop system than tree fruit (Smith et al, 1981).

The outcome of this proposed work would offer insight into the effect of dormant copper and manganese biocidal applications on the DMI sensitivity of *V. inaequalis* orchard populations surviving applications. Little is known about the full range effects that copper applications have on surviving fungi. Additionally, the effects of other novel metal cations such as manganese have on factors such as fungicide sensitivity have not been determined for *V. inaequalis*. If the effect on increasing DMI sensitivity were to be consistent, manganese would be an alternative to copper products and lessen the potential negative impacts on fruit finish (Jama and Lateur, 2007, Montag et al, 2006). This study contributes the understanding of the full range of effects these metal cations have on orchards that are subject to treatments while the trees are dormant, and can potentially foster interest in evaluating the effect of cation products other than copper.

Although DMI resistance factors have been characterized, the factors involved in the persistence are not well understood. This study contributes to that knowledge in how applications of broad spectrum products already utilized in apple orchards in the northeast to manage fireblight inoculum will affect the persistence of fungicide sensitivity in *V. inaequalis* orchard populations. Understanding factors involved in the persistence of DMI resistance is a useful tool for regional growers, many of whom are already managing DMI resistant orchard populations. These findings can offer new insight into further management refinements that can be made to select for population members that are not DMI resistant on an orchard-wide scale through the usage of dormant copper applications. Any conclusions from this project are also potentially useful to the community of scientists that focus on agriculturally significant plant pathogens because once the trend has been observed; further inquiry can determine the molecular mechanisms responsible and elucidate if other metal cations have similar effects. This research can be incorporated into the large body of research already devoted to the subject of copper

resistance in fungi, and further experimentation can determine if these copper resistance factors alter the sensitivity of *V. inaequalis* to other fungicides later in the season. Changes in horticultural practices, such as the deployment of disease resistant cultivars, will continue to drive the tree fruit industry to disease management programs that rely on different fungicide usage and possibly fewer applications of pesticides (Jama and Lateur, 2007, Ishii et al, 2006). However, resistance problems with more site-specific fungicide applications (Ishii et al, 2006) means different management approaches are necessary to prolong the effective life of these products.

The Effect of Difenoconazole use on the Demethylation inhibitor Sensitivity in a DMI Resistant Population of *Venturia inaequalis*.

The application of demethylation inhibitor (DMI) fungicides has been one of the primary means for the management of apple scab caused by *Venturia inaequalis* in the northeastern United States (Agnello et al, 2012, Köller et al, 1997, Gilpatrick, 1982), and understanding how DMI usage affects the development of resistance is important to disease management. Applications of site specific fungicides, particularly sterol demethylation inhibitor (DMI) fungicides, have been favored in apple production in the management of many fungal diseases because of their effectiveness. First introduced in the 1970's, DMI fungicide resistance began to be noted in the early 1980's since each application selects for fungal population members with a stable, inherent genetic adjustment that limits the efficacy and ultimately the lifetime of effectiveness of DMI fungicide chemistries, granted most of the initial reports were on other agriculturally significant pathogens than *V. inaequalis* (Ma et al, 2005, Stanis et al, 1985, Delp,

1980). DMI fungicides offer both prophylactic protection against fungi and curative activity for infections that are already established (Kunz et al, 1997).

One DMI, Myclobutanil, has been commonly used to manage apple scab for decades due to its effectiveness (Marine et al, 2007, Köller et al, 1997). It is also effective against other pathogenic fungi, such as apple powdery mildew, making it a routine choice for management of apple diseases caused by fungi (Cox et al, 2012, Agnello et al, 2012, Cox et al, 2010). The mode of action of DMI fungicides hinges on the nitrogen aromatic ring portion of their chemical structure. The nitrogen aromatic ring inhibits the demethylation on C14 of 24-methyenedihydrolanosterol, preventing the biosynthesis of ergosterol; ergosterol being a key fungal cell membrane and cell wall component. The lone pair of electrons on the nitrogen ring of DMI fungicides have high affinity for the hydrophobic portion of 24-methyenedihydrolanosterol, and the DMI binding to the substrate noncompetitively inhibits the fungal C4 demethylase enzyme (Barrett-Bee et al, 1995). It has been suggested that the ergosterol precursors accumulate in the cell wall and prohibit normal functionality and further chitin synthesis, creating a fungistatic effect in sensitive fungi that prevents continued growth and sporulation (Barrett-Bee et al, 1995). Due to routine usage, there have been steady decreases in *V. inaequalis* sensitivity to myclobutanil (Marine et al, 2007), which has been contributed to multiple genetic factors. Observations of a continuous spectrum of DMI quantitative resistance suggests that many genetic elements work synergistically to create varying degrees of DMI resistance (Kunz et al, 1997). The various combinations of these resistance factors manifests as a range of fungicide sensitivities, as opposed to two discrete groups of sensitive and resistant fungi. This has conferred practical resistance to myclobutanil in some orchards, and a range of moderate resistance in others (Cox et al, 2009, Marine et al, 2007, Köller et al, 1997). Practical resistance

being defined as diminished disease control caused by the selection of resistant isolates (Köller, 1991). Practical resistance to myclobutanil was not widely documented under commercial orchard conditions as of the early 1990's (Hollomon et al, 1993). These had been exposed to selective pressures created by DMI fungicide usage for a decade, and gradually the surviving population was predominated by *V. inaequalis* members possessing more of the multiple genetic factors implicated in reduced DMI sensitivity (Köller et al, 1997).

Further research on the specific effects of difenoconazole on the fungicide sensitivity of *V. inaequalis* has yet to be experimentally evaluated because of the relatively recent introduction of this fungicide chemistry to the market for summer diseases of apple. The trials in the present study have demonstrated that difenoconazole applications have the greatest impact on DMI sensitivity during primary infection, and that difenoconazole application throughout the production season does not shift the *V. inaequalis* population towards increased DMI resistance. This is an important knowledge gap that should be addressed due to an incomplete understanding of how DMI resistance emerges in *V. inaequalis* orchard populations. Additionally, these conclusions are notable for the scientific community because resistance to one DMI has not always correlated to another, indicating that DMI cross resistance is an area of potential study with implications for management of fungal disease. Finally, any conclusions that determine the best methods of deployment and the precise timings of difenoconazole applications and how that effects the persistence of DMI resistance is of paramount importance to stakeholders because these factors have not been wholly elucidated. When a novel DMI fungicide is labeled for orchard use, further inquiry will be required to determine if that particular product will be effective with DMI resistant *V. inaequalis* populations, and understanding the development and

persistence of DMI fungicide resistance would offer insight into the probable lifetime of this product based on use.

The Prevalence of Quinone outside Inhibitor Resistance and the Stability of Qualitative QoI Resistance in *Venturia inaequalis.*

Another quintessential group of fungicides in the management of apple scab in many apple production areas are QoI fungicide chemistries. QoI fungicides were first isolated from wood rotting basidiomycete fungi like *Strobiluris tenacellus*, and have been used agriculturally since 1996 (Bartlett et al, 2002). These fungicides act on the Q_O-site of the cytochrome bc1 complex and prevent the continuation of mitochondrial respiration. The Q_O-site of the complex, which is the target site of trifloxystrobin and many QoI's, is involved in the reduction of O_2 while generating superoxide. Superoxide is the precursor to later reactive steps that generate reactive oxygen species (ROS). Interestingly, ROS generation can lead to excessive cellular free radicals which can cause DNA and protein degradation if unchecked due to a considerable conformational change to the cytochrome bc1 complex. It is at these redox sites where quinone mimics/inhibitors can enter into the system and prevent complete respiration (Croft, 2004).

Qualitative resistance has been attributed to a high degree of QoI resistance conferred by a single genetic locus, and is usually a glycine to alanine point mutation at site 143 in the *cyt b* gene in *V. inaequalis* (Vallières et al, 2011). In *Saccharomyces cerevisiae*, the G143A mutation has been observed to prevent QoI fungicides from binding to the cytochrome bc1 complex through simple steric hindrance (Fisher et al, 2005). The presence of G143A also dramatically increased resistance to azoxystrobin (QoI fungicide) in *S. cerevisiae* in laboratory studies (Fisher et al, 2005).

An overall goal of this research (Chapter 4) was to determine the prevalence of qualitative and quantitative QoI resistance in the northeastern United States. QoI resistant status may be based on the presence of one or more genetic elements in a *V. inaequalis* orchard population, and it is important to determine if qualitative or quantitative resistance is the predominant factor(s) in QoI resistance in the region. Another objective of this study was to assay the stability of qualitative QoI resistance in the absence of QoI-induced selective pressure. As previously mentioned, the G143A mutation imparts a high degree of resistance to QoI fungicides (Fisher et al, 2005). Growers who have the G143A mutation in a considerable proportion of their *V. inaequalis* orchard population are likely to see control failures if they rely on QoI fungicides for *V. inaequalis* disease management. In the case where orchard populations are resistant to QoI fungicides, the question arises as to the stability of G143A in the absence of selective pressure fostered by QoI applications. Stability of this mutation has yet to fully determined for *V. inaequalis*, and is another goal of the study presented in chapter 4. Overall, this study will contribute to the current understanding of QoI resistance in *V. inaequalis* and aspects of its stability, which provides relevant information that can be incorporated into apple scab disease management paradigms and could potentially be pertinent to the persistence of qualitative QoI resistance in other agriculturally significant ascomycete plant pathogens.

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CHAPTER 2

THE EFFECTS OF DORMANT CHEMICAL TERATMENT ON DEMETHYLATION INHIBITOR SENSITIVE OF *VENTURIA INAEQUALIS*

ABSTRACT

The application of fungicides is the primary means for the management of apple scab caused by *Venturia inaequalis*. Unfortunately, resistance to several fungicide chemistries including the demethylation inhibitor (DMI) fungicides is prevalent in regional populations of *V. inaequalis*. Orchard trials were conducted in a New York apple orchard in 2011 and 2012 to determine the influence of delayed dormant chemical treatment on the fungicide sensitivity of a *V. inaequalis* population with stable resistance to DMIs, but not resistant to dodine. Four delayed dormant chemical treatments consisted of either an application of copper, manganese, myclobutanil (DMI fungicide), or no fungicide application. Fungicide sensitivity to myclobutanil and dodine was evaluated for a minimum of 25 *V. inaequalis* clonal conidial isolates from each treatment program. In both years, mean percent relative growth of *V. inaequalis* isolates from the copper treatment were significantly ($P < 0.05$) lower than isolates that did not receive a delayed dormant treatment. The effect of the manganese treatment was inconsistent between years. There were no significant differences ($P > 0.05$) in dodine sensitivity among delayed dormant treatments in either year. These results suggest delayed dormant treatments can have an effect on increasing the *V. inaequalis* population's sensitivity to DMI fungicides.

INTRODUCTION

Apple scab, caused by the ascomycete pathogen *Venturia inaequalis* (Cooke) Winter, is the most economically important fungal disease affecting apple production worldwide (Agrios, 2005, Holb et al, 2003, MacHardy et al, 2001, MacHardy, 1996, Sutton, 2006). Apple scab can cause considerable loss if improper management strategies are employed in climates where weather conditions are conducive to severe infections (Agrios et al, 2005, Holb et al, 2003, MacHardy, 1996) such as the northeastern United States (Merwin et al, 1994). Conditions favorable to disease, which is when temperatures are between 12 and 26 °C with approximately 4 to 10 hours of leaf wetness (MacHardy and Gadoury, 1982), necessitate chemical management in the form fungicide applications from bud break to early summer (Gadoury and MacHardy, 1986, MacHardy et al, 2001, MacHardy, 1996). Early summer marks the point when primary inoculum has been discharged and heat renders conidia non-viable (MacHardy, 1996).

Growers in the northeastern United States should begin management of apple scab prior to bud break by targeting overwintering inoculum (MacHardy, 1996). Delayed dormant management of apple scab begins with shredding the leaf litter or an application of urea as a cultural control (Sutton et al, 2000). In addition to these cultural practices, some growers use copper at or prior to bud break to reduce overwintering fire blight inoculum (Norelli et al, 2003). Copper products have proven to be effective fungicides (McCallan and Wilcoxon, 1949) and control measures for apple scab (Holb et al, 2003, Montag et al, 2006). Because of negative effects on fruit finish with fruit russet as well as phytotoxic effects on foliage (Jama et al, 2007, Montag et al, 2006); most copper applications are not typically used directly on leaves and developing fruit and are applied to delayed dormant tissues instead. Another opportunity for

these applications to be utilized is in the period between when the buds swell and bud break, known as a delayed dormant application (Singer et al, 2010). It is unknown if a delayed dormant chemical treatment of copper would affect overwintering ascospores in leaf litter or conidial inoculum in bud scales (Becker and Burr, 1992). Moreover, it is not known if other novel metal cation products, such as manganese, would have similar effects on apple scab.

Demethylation inhibitor (DMI) fungicides have been utilized in apple production for more than 30 years (Gilpatrick, 1982, Köller et al, 1997). However, due to the highly specific mode of action, DMI fungicides have the propensity for the development of practical resistance (Gilpatrick, 1982, Ma et al, 2005), with practical resistant being defined as greatly diminished levels of disease control caused by the selection of fungicide resistant isolates (Köller et al, 1991). Reduced sensitivity to DMIs in *V. inaequalis* was observed in as early as 1985 (Stanis et al, 1985), and continues to be a problem in apple production in the Great Lakes region (Lesniak et al, 2011). Given the prevalence of DMI resistance in regional *V. inaequalis* orchard populations, investigations are needed to identify management practices that reduce the persistence of practical DMI resistance in orchard populations of *V. inaequalis*. To that end, Pfeufer and Ngugi (2012) conducted a survey to examine the correlation between different management practices and the presence of DMI resistant *V. inaequalis*. These authors noted that growers who applied copper products as delayed dormant chemical treatment were nearly twice as likely to have DMI sensitive *V. inaequalis* populations in their apple orchards. However, the direct impact of delayed dormant copper applications on the fungicide sensitivity of surviving *V. inaequalis* isolates later in the season is largely unknown. Aside from copper, metal cation solutions composed of manganese have been shown to affect the maturation of *V. inaequalis* ascospores *in vitro* (F. Trail, personal communication). As with copper, the potential effect of

delayed dormant applications of manganese on the DMI sensitivity of *V. inaequalis* has not been elucidated. Because of the central role of fungicide resistance in recent apple scab management failures in the northeastern United States (Lesniak et al, 2011, Köller et al, 1997), detailed investigations are warranted to determine if delayed dormant chemical treatments have any effect on the fungicide sensitivity profile of orchard populations of *V. inaequalis.* Specifically, we wish to (i) examine the effect of delayed dormant copper treatments on the DMI fungicide sensitivity of an orchard population of *V. inaequalis* with practical resistance to DMI fungicides; (ii) determine the effect of delayed dormant treatments on dodine fungicide sensitivity for an orchard population of *V. inaequalis* that does not have practical resistance to dodine; and (iii) determine if delayed dormant applications of another metal cation solution comprised of manganese would have a similar effect to copper on population sensitivity to DMI fungicides. The potential importance of delayed dormant applications on *V. inaequalis* fungicide resistance management and the persistence of DMI resistance will also be discussed.

MATERIALS AND METHODS

Orchard trial to evaluate the effect of delayed dormant chemical treatments on *V. inaequalis* DMI fungicide sensitivity. Trials were conducted in an orchard at the New York State Agricultural Experiment Station in Geneva, NY during the 2011 and 2012 apple production seasons. The orchard consisted of 35 year old paired 'McIntosh' and 'Cortland' onto the M.M. 111 rootstocks, and the trees alternated cultivar so that no cultivar was adjacent to the same cultivar. Both cultivars are also very susceptible to infection from apple scab. The *V. inaequalis* population in this orchard has been confirmed to be resistant to myclobutanil, and fungicide

sensitivity phenotypes for *V. inaequalis* isolates sampled over successive seasons have previously been demonstrated to be uniformly distributed throughout the planting, which designed for randomized block experimentrs with four replicates (Cox et al, 2011, Cox et al, 2010, Cox et al, 2009, Köller et al, 2008, Köller et al, 2007). Delayed dormant treatment programs were applied to orchard blocks approx. 1.2 Ha in 2011 and 1.0 Ha in 2012 in size using a handgun sprayer at 600 PSI with rates adjusted for a tree row volume of approx. 333.33 L/ Ha. Both the trees and the leaf litter underneath were treated during spring and summer to the point where uniform saturation of both the tree canopy and the ground. In the case of the untreated application, no delayed dormant chemical was applied.

All orchard blocks received one delayed dormant application which occurs at the phenological stage of silver tip in apple (8 April 2011 and 18 March 2012) of one of the following: Badge X2 (copper hydroxide and copper oxychloride, 1066.66 g/Ha, Isagrow USA, Morrisville, NC), F2 permanganate (manganese cation, 3,055 L/Ha, F2 Industries, Smyrna, TN), myclobutanil (Rally, 333.33 g/Ha, Dow Agrosciences, Indianapolis, IN), or nothing. Following the delayed dormant application, all treatment programs included a standard protectant program 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) during both primary apple scab seasons (green tip to 2nd cover). Applications were made to all treatment programs during the primary apple scab season on 7-10 day intervals from tight cluster (2 May 2011, 18 March 2012) through second cover (22 June 2011, 24 May 2012) in each trial year. During summer covers, all programs included applications of captan (2.8 kg/Ha) (Captan 80 WDG, Arysta LifeScience Company). Summer cover applications were made to the entire orchard on 14-21 day intervals from third (8 July 2011, 14 June 2012) through seventh cover (9

September 2011, 30 August 2012) in each year. To determine the potential effects of weather as a factor in the effectiveness of delayed dormant chemical treatments, data on rainfall, hours of leaf wetness, and predicted ascospore maturity were collected from the Network for Environment and Weather Applications (http://newa.cornell.edu/index.php?page=apple-diseases) for the delayed dormant application date in each year that orchard trials were conducted.

***In vitro* evaluation of DMI and dodine fungicide sensitivity of *V. inaequalis* clonal conidial isolates.** Clonal conidial isolates were evaluated for their mean percent relative growth (mean %RG) in order to evaluate the effect of delayed dormant chemical treatment on the DMI sensitivity of the *V. inaequalis* orchard population. The sensitivity to dodine was evaluated for the same collection of isolates in order to determine the effect of delayed dormant chemical treatment on a fungicide with an unrelated mode of action. In May of 2011 and 2012, 100 leaves with young, primary *V. inaequalis* lesions were collected uniformly from the 'McIntosh' trees in each delayed dormant chemical treatment to generate single leaf lesion isolates.

Single clonal conidial leaf lesions (Olaya and Köller 1999, Köller et al. 2004) were excised with a cork borer (7 mm diameter), and then placed in 1.2 ml of distilled water and shaken vigorously for 60s to dislodge conidia from the lesion. From each delayed dormant treatment program, the resulting clonal conidial isolates ($n > 25$) represented by the suspensions were then evaluated for fungicide sensitivity using microscopy aided mycelial relative growth assays, as described previously (Köller et al, 2004) with the following modifications. 100 µl of suspension was applied to the surface of un-amended medium, consisting of potato dextrose agar (Difco Laboratories, Detroit, MI) amended with two antibiotics: streptomycin sulfate (50 µg/ml, Sigma-Aldrich, St. Louis, MO) and chloramphenicol (50 µg/ml, Sigma-Aldrich, St. Louis, MO). A

conidial suspension of 100 µl was also applied to the surface of the PDA medium amended with myclobutanil (0.1µg/ml analytical standard; Sigma-Aldrich) (Köller et al, 1997). Additionally, 100 µl aliquots from each clonal conidial isolate suspension were applied to the surface of PDA media amended with dodine (0.2µg/ml, analytical standard; Sigma-Aldrich) (Köller et al, 1999).

Isolates were incubated for 7 days in the light at 23-25°C, and mean %RG for each *V. inaequalis* isolate was determined by measuring the mean growth of 5 micro-colonies originating from a single conidium in the presence of one of the evaluated fungicides relative to the mean growth of 10 micro-scale colonies on unamended medium. The mycelium from micro-scale mycelial colonies was measured (70-120X) 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).

Statistical Analyses. Logarithmic and arcsine data transformations were performed on observed sensitivity distributions for myclobutanil and dodine to determine which transformation best stabilized the variances and approximated normality. The distribution of sensitivity responses for each fungicide and transformation was analyzed for normality by the Shiparo-Wilks test using the continuous fit test of JMP v.9.0 (SAS Institute, Cary, NC) to assess the statistical appropriateness of procedures to determine significant differences in of the mean % RG of isolates for each delayed dormant treatment. The mean %RG of *V. inaequali*s isolates for each delayed dormant treatment was generated using the PROC Means procedure of SAS (SAS Institute), and differences in the mean %RG of isolates between delayed dormant treatments were determined using Generalized Linear Mixed Models with the GLIMMIX procedure of SAS v9.3 (SAS Institute).

RESULTS

The prevailing weather conditions and ascospore maturity on the date of delayed dormant application. According to the Network for Environment and Weather Applications, the average temperature on the 2011 delayed dormant chemical treatment application date of April 8th ranged from 1°C to 9.5°C while temperatures ranged between 11.6°C and 23.4°C on the 2012 delayed dormant chemical treatment application date of March 18th. There was no rain in the three days preceding the 2011 delayed dormant treatment, but there was 3cm of rain over the course of the three days prior to the 2012 application. There was also 18 hours of leaf wetness from dew in the three days following the 2012 application, and an accumulated 24 hours of leaf wetness. The ascospore maturity model (Gadoury and MacHardy, 1982) indicated that 1% of the ascospores were mature on the date of the delayed dormant treatments for the 2011 season. In the 2012 trial, approximately 2% of the ascospores were mature at the time of the delayed dormant treatment.

The effect of delayed dormant chemical treatments on *in vitro* sensitivity of *V. inaequalis* clonal conidial isolates to myclobutanil. In the 2011 trial, the mean %RG of clonal conidial isolates on myclobutanil-amended medium ranged from 53.9 to 88.7% with the manganese treatment (F2 permanganate) program (53.9 ± 6.8) and the untreated treatment program (88.7 ± 4.2) having the lowest and highest means, respectively (Figure 1). There was a significant effect of delayed dormant chemical treatment on *in vitro* sensitivity of *V. inaequalis* isolates to myclobutanil (P=<0.0001). The mean %RG on myclobutanil-amended medium for clonal conidial isolates from the copper treatment program was significantly different from the

delayed dormant treatment that received no fungicide (*P*=0.0015, Figure 1). Interestingly, the mean %RG on myclobutanil-amended medium was the lowest for clonal conidial isolates from the manganese (F2 permanganate) delayed dormant treatment, which was also significantly different from the treatment that received no fungicide (*P*=<0.0001, Figure 1). There was no significant difference between the mean %RG of clonal conidial isolates on medium amended with myclobutanil between the delayed dormant treatment of myclobutanil (Rally 40 WSP) and the treatment that received no fungicide (*P*=0.6148).

In 2012, the mean %RG of clonal conidial isolates on myclobutanil-amended medium ranged from 66.7 to 84.7% with the copper (67 ± 3.3) and manganese (F2 permanganate) (84 ± 3.0) delayed dormant chemical treatments having the lowest and highest means, respectively. Clonal conidial isolates from the treatment that received no fungicide had a mean %RG of 74.6 ± 2.7 (Figure 2). Similar to 2011, there was a significant (*P*=0.0003) effect of delayed dormant chemical treatment on *V. inaequalis* myclobutanil sensitivity. The mean %RG of clonal conidial isolates in on myclobutanil-amended medium was lowest for the delayed dormant copper treatment, which was significantly different from the treatment that received no fungicide (*P*=0.0146, Figure 2). There was no significant difference (*P*=0.7910) between the mean %RG of clonal conidial isolates on media amended with myclobutanil between the myclobutanil (Rally 40WSP) delayed dormant treatment and the delayed dormant treatment that received no fungicide.

***In vitro* dodine sensitivity of *V. inaequalis* clonal conidial isolates collected from an apple scab infected orchard.** In both 2011 and 2012, the sensitivity responses for *V. inaequalis* clonal conidial isolates expressed as mean %RG on media amended with dodine were log normally distributed. In 2011, the %RG of clonal conidial isolates on dodine-amended medium

ranged from 17.2 to 26.1% with the copper (17.2 ± 3.3) and manganese (26.1 ± 3.1) delayed dormant treatments having the lowest and highest means, respectively. Overall, the mean sensitivity of clonal conidial isolates to dodine was not significantly different among delayed dormant chemical treatments (P=0.5938). Clonal conidial isolates from the delayed dormant treatment that received no fungicide had a mean %RG of 23.0 ± 7.4, and based on linear contrasts, were not significantly different from the manganese (F2 permanganate) delayed dormant treatment (P=0.5432), copper delayed dormant treatment (P=0.2693) or myclobutanil (Rally 40 WSP) delayed dormant treatment (P=0.1496). In the 2012 trial, mean %RG of clonal conidial isolates on dodine-amended medium ranged from 24.2 to 34.7% with the untreated (24.2 ± 2.7) and myclobutanil (Rally 40 WSP) (34.7 ± 4.7) treatment programs having the lowest and highest means, respectively. The same trend in dodine sensitivity was observed in the 2012 season with no significant differences among delayed dormant chemical treatments for *in vitro* sensitivity to dodine, regardless of delayed dormant treatment (P=0.2038). Dodine sensitivity of clonal conidial isolates from the delayed dormant treatment that received no fungicide was not significantly different, based on linear contrasts, from the manganese (F2 permanganate) (P=0.4498), copper (P=0.7860), or myclobutanil (Rally 40 WSP) (P=0.2025) delayed dormant treatments.

DISCUSSION

In the present study, the delayed dormant chemical treatment consisting of a copper application resulted in *V. inaequalis* isolates with higher myclobutanil sensitivity (lower mean %RG). This result is consistent with observations by Pfeufer and Ngugi (2012) who found that

orchard populations of *V. inaequalis* were nearly twice as likely to be shifted toward DMI sensitivity where surveyed growers had reported using copper compared to orchards where surveyed growers had not reported using copper. One possible explanation for the effect of copper could be attributed to the elimination of highly DMI resistant isolates with reduced fitness. It could be hypothesized that highly DMI resistant isolates may not be able to reproduce as efficiently when treated with copper as a delayed dormant application. Although no information is available on apple scab fitness costs associated with either copper or DMI resistance, it has been shown that *Cercospora beticola* isolates with DMI resistance have a fitness penalty (Karaoglanidis et al, 2001), which has an effect on the long term persistence of this phenotype in the population depending on DMI usage (Anderson, 2005). Given the effects of copper on fungal spore production and viability (Montag et al, 2006, Cervantes et al, 1994), further research is necessary to understand how delayed dormant copper applications impact *V. inaequalis* isolates with DMI resistance. Another possible explanation for the effect of copper relates to the fact that even treatments of low (1mmol/L) concentrations of copper sulfate can damage *V. inaequalis* conidial membranes and inhibit complete mitochondrial respiration (Montag et al, 2006) in isolates with reduced fitness. By comparison, concentrations of copper hydroxide at 25mmol/L are required to have a similar effect on conidial viability (Montag et al, 2006). Both copper compounds have a high degree of toxicity, but the increased effectiveness on the part of copper sulfate has been attributed to its high solubility (Montag et al, 2006). However, a copper product with high solubility would be ineffective as a delayed delayed dormant application because this early point in the season is often predominated by rain events and prolonged periods of leaf wetness, which is why fairly insoluble copper products such as those containing copper hydroxide are recommended (Agnello et al, 2012, Montag et al, 2006). It has

been shown that as little as 1 mm of simulated rain can wash 35% of copper hydroxide off a leaf (Hunsche et al, 2011). The copper product Badge X2, which is 24.6% copper oxychloride and 22.9% copper hydroxide, was chosen for this study as it is marketed to be a weathering resistant and insoluble copper fungicide. This could have contributed to the product providing a consistent effect despite wetting surrounding the application date in the 2012 orchard trial.

A delayed dormant application of myclobutanil (Rally 40WSP) did not reduce or increase the DMI sensitivity of collected clonal conidial isolates over two consecutive years of orchard trials. This was probably due to the inability of this delayed dormant chemical treatment to eliminate most *V. inaequalis* population members because of stable DMI practical resistance that has been observed for this particular *V. inaequalis* orchard population. It has been determined that each DMI application increases DMI resistance while populations are sensitive (Ishii et al, 2005). However, further DMI applications are not anticipated to greatly impact the DMI sensitivity of clonal conidial isolates when the orchard practical resistance.

During both years of the study, the sensitivity of *V. inaequalis* isolates to dodine was not significantly different between the four chemical treatment programs. Additionally, clonal conidial isolates collected from delayed dormant chemical treatments were sensitive to dodine (%RG < 50%), which is consistent with the previously reported sensitivity for this *V. inaequalis* orchard population (Cox et al, 2012). The lack of any effect on dodine sensitivity resulting from the copper treatment could be due to a lack of resistant clonal conidial isolates in the population. It may also be that impacts on population fungicide sensitivity from delayed dormant applications are only realized in the context of practical resistance. Additionally, the fact that there were no differences in dodine sensitivity between treatment programs suggests that the sensitivity distributions of single conidial isolates is homogeneous throughout the orchard over

two consecutive growing seasons. This ensures no positional effects of trees within treatment blocks that could potentially bias results, as observed in another study (Gao et al, 2009). In this particular example, a *V. inaequalis* sub population in the lower portion of an orchard was observed to be much more insensitive to myclobutanil (Gao et al, 2009). If variation in dodine sensitivity was observed in the present study, and the pattern of differences among treatments was identical to that for myclobutanil, one might be tempted to suggest that the differences were due to patches of multiple resistant population member aggregates resulting from the placement of treatment program blocks. Given that treatment blocks were redesigned in the second year to encompass all treatment programs from the previous year and there were no differences in dodine sensitivity, the influence of a patchy distribution of population members with differences in sensitivity is unlikely.

A delayed dormant application of manganese impacted the DMI sensitivity of a collection of *V. inaequalis* isolates in 2011, but the effect was not observed in 2012. It is not currently known if apple scab ascospore formation is inhibited by manganese treatment, and knowledge of ascospore maturation in any ascomycete due to manganese treatment is limited. For example, manganese sulfate inhibits *Saccharomyces cerevisiae* ascospore production at doses above 0.0020 M (McClary et al, 1959). Similarly, a decrease of ascospore growth and viability of the lichen, *Hypogymnia physodes*, has been observed with an increasing manganese concentration in tree bark (Hauck et al, 2002, Hauck et al, 2001). Given the lack of information on the effects of manganese, further investigation is needed to address the potential benefits of this metal cation. The inconsistency of the manganese treatment program in affecting the DMI sensitivity of clonal conidial *V. inaequalis* isolates between years could be attributed to differences in seasonal weather conditions. For example, there was 0 versus 3cm of rainfall in

the three days prior to the 2011 (8 April) and 2012 delayed dormant application date (18 March), respectively, and 18 versus 24 hours of leaf wetness from dew in the three days following applications in 2011 and 2012, respectively. Since F2 Industries markets their manganese product primarily as a water sanitizer (http://www.f2ind.com/potable-water.html), it is likely that the product was not formulated with an adjuvant to increase adherence and rainfastness. High solubility in water would diminish the persistence of this product on treated plant tissue and prevent uniform distribution across the orchard floor based on drainage patterns. Even with the minor rain event (3 cm rainfall), it is possible that the product would have washed off. This would be consistent with observations for other soluble metal cation products including high solubility copper products, which have reduced persistence on treated tissues (Montag et al, 2006). Differences in the isolates between the copper and manganese programs were likely not observed in 2011 because the manganese had not weathered away following the application. In 2012, the survival of DMI resistant *V. inaequalis* isolates due to a lack of microbial degradation would increase if the manganese product was not as effective as the delayed dormant copper application because the former had weathered in the wetting following application, and was not uniformly distributed as the orchard floor as originally intended so that all leaf litter was treated.

Aside from rainfall, temperature may also have an effect on the persistence of a metal cation product used in a delayed dormant application, and on the rate of microbial decomposition of fallen foliage on which apple scab is overwintering. Moisture and temperature were suggested to influence the rate of degradation of fallen leaves (Whitkamp, 1966). In 2011, cooler temperatures (< 10 ºC) at the time of application may not have facilitated the rapid breakdown of leaves harboring ascospores. A lack of leaf litter breakdown could potentially lead to higher levels of initial inoculum, which could in turn, result in higher likelihood of resistant *V.*

inaequalis inoculum establishing new infections. Regardless, the adverse effects of temperature on effectiveness of manganese or other novel metal cation products would need to be more fully understood before such a product could potentially be used as a fungicide in a commercial setting.

In the present study, we have demonstrated that delayed delayed dormant copper treatments prior to bud break could be used to influence DMI sensitivity of *V. inaequalis* isolates in a DMI-resistant orchard population. This result suggests that the delayed dormant application of copper could be used as a means of resistance management. Copper applications have additional benefits of managing overwintering *E. amylovora* inoculum (Norelli et al, 2003). The specific mode of action and molecular basis for the effect observed in the orchard has yet to be elucidated. Aside from copper, the manganese product also appeared to have potential for resistance management, although the effect was not consistent between years possibly due to a lack of proper formulation to withstand environmental weathering. Understanding the relationship between chemical treatment and the sensitivity of *V. inaequalis* populations to various fungicide chemistries throughout the production season will be crucial for the identification and future implementation of effective resistance management strategies.

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FIGURES

Figure 1

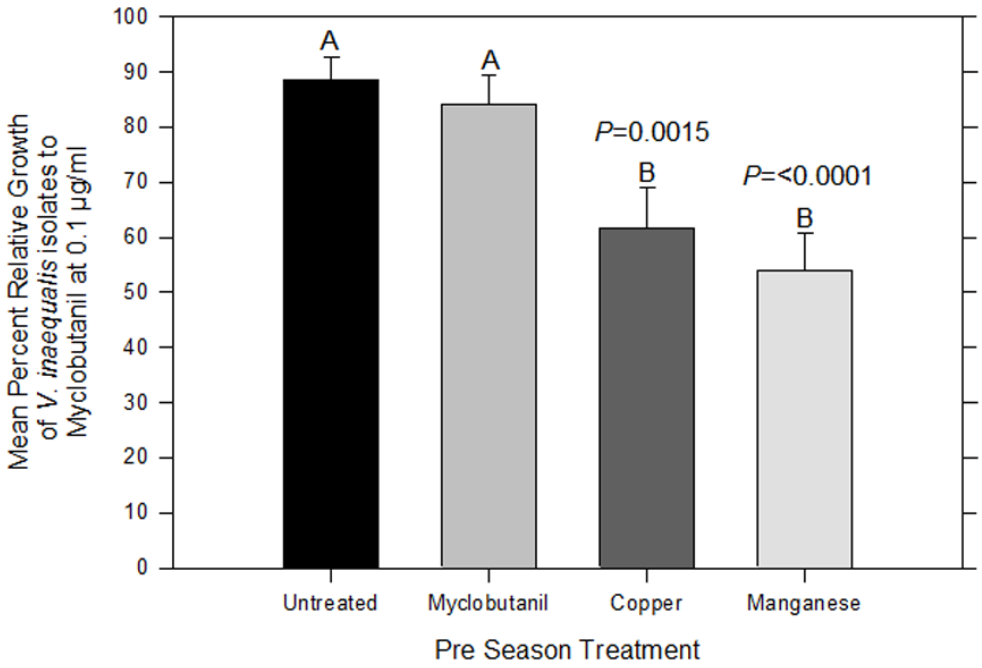

Figure 1: Mean relative growth representing the *in vitro* fungicide sensitivity of *V. inaequalis* isolates to myclobutanil for each treatment program for the 2011 trial. The *P*-values indicated in the graph are for the linear contrasts comparing the mean for the particular bar under the *P*-values to the untreated program. Treatments denoted by the same letter lack statistical significance ($\alpha = 0.05$) among adjusted estimated mean incidences.

Figure 2

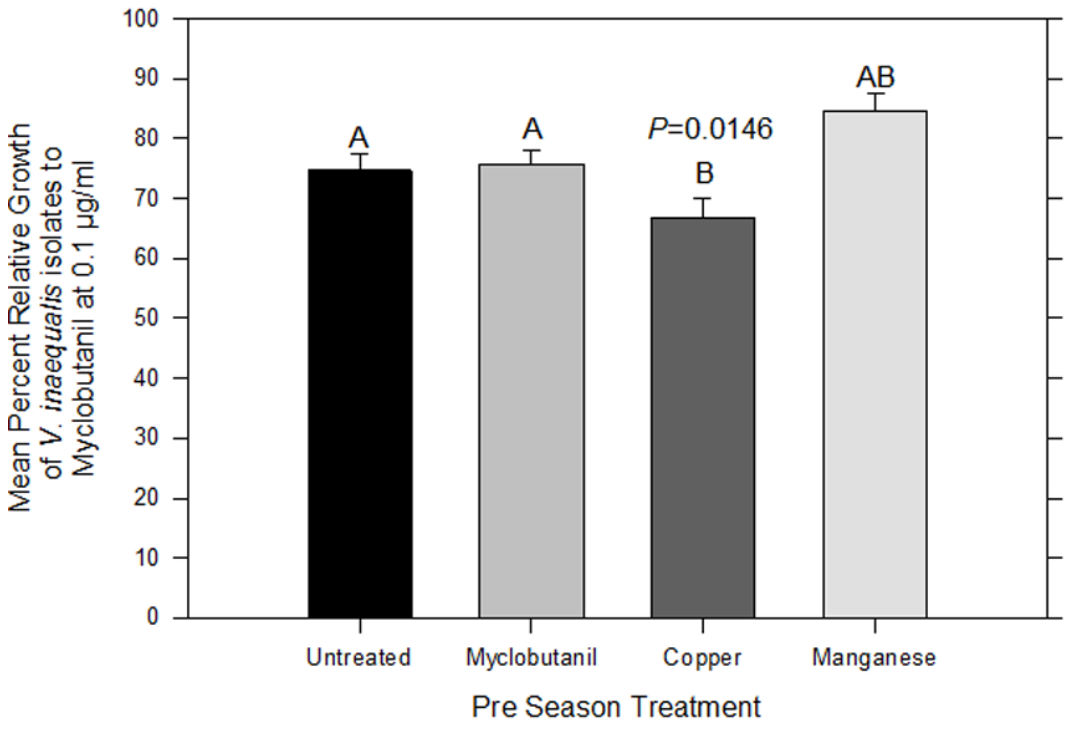

Figure 2: Mean relative growth representing the *in vitro* fungicide sensitivity of *V. inaequalis* isolates to myclobutanil for each treatment program for the 2012 trial. The *P*-values indicated in the graph are for the linear contrasts comparing the mean for the particular bar under the *P*-values to the untreated program. Treatments denoted by the same letter lack statistical significance ($\alpha = 0.05$) among adjusted estimated mean incidences.

CHAPTER 3

THE EFFECT OF DIFENOCONAZOLE USE ON THE DEMETHYLATION INHIBITOR SENSITIVITY IN A DMI RESISTANT POPULATION OF VENTURIA INAEQUALIS

ABSTRACT

Demethylation inhibitor (DMI) fungicides are used for the management of apple scab caused by *Venturia inaequalis*. To determine the effect of difenoconazole use on the DMI sensitivity of a *V. inaequalis* population with quantitative DMI resistance, trials were conducted in a New York apple orchard in 2011 and 2012. The three fungicide treatment programs consisted of difenoconazole applications in the spring to target primary apple scab infections, and throughout the growing season to reduce both primary and secondary infections, or no applications of fungicides. Sensitivity to two DMIs, e.g. difenoconazole and myclobutanil, and an alternative chemistry, e.g. dodine, was evaluated for a minimum of 25 *V. inaequalis* isolates from each fungicide program. In both years, mean percent relative growth of *V. inaequalis* isolates on difenoconazole-amended medium was highest in the fungicide program that received difenoconazole in the spring only. The effect of the season long program on sensitivity to myclobutanil was inconsistent between years, and there were no significant differences in dodine sensitivity among fungicide programs in either year. These results suggest that difenoconazole applications have the greatest impact on DMI sensitivity during primary infection, and that difenoconazole application may not necessary impact population sensitivity to other DMI fungicides.

INTRODUCTION

Apple scab, caused by the ascomycete pathogen *Venturia inaequalis* (Cooke) Winter, is the most economically important fungal disease of apples worldwide (Holb et al, 2003, MacHardy, Gadoury, and Gessler 2001, MacHardy, 1996, Sutton et al, 1996). Apple scab is most severe in temperate climates where weather conditions in the spring are conducive to infection (Holb et al, 2003, MacHardy, 1996). Such conditions necessitate chemical management in the form of fungicide applications from bud break to early summer, which coincides with the presence of primary inoculum in the form of ascospores and conidia (MacHardy, Gadoury, and Gessler 2001, MacHardy, 1996, Becker and Burr, 1992, Gadoury, 1986). Apple scab infections from surviving conidia are possible during the summer if the weather remains cool (< 30 ºC) and substantial wetting events (> 12 h of leaf wetness) occur (MacHardy et al, 1996). Chemical management for apple scab during the summer months prevents established, sporulating lesions from completing secondary infection cycles that result in the infection of developing fruit and leaves (Reardon et al, 2005, MacHardy, 1996).

Demethylation inhibitor (DMI) fungicides have been utilized in apple production for more than 30 years (Köller et al, 1997, Gilpatrick, 1982). While site specific fungicides including DMI fungicides have proven to be an effective means of managing fungal pathogen populations, repeated applications of these fungicides can create stable, adjustments that impart incremental levels of insensitivity in population members that limit efficacy over time (Ishii et al, 2006, Ma et al, 2005). Reduced sensitivity to DMIs in *V. inaequalis* populations in the Northeastern US was observed as early as 1985 (Stanis et al, 1985), and continue to be a problem in orchard populations in the Great Lakes region (Chapman et al, 2011). Repeated DMI usage in Virginia

and New York apple orchards for 10 to 15 years has provided strong selection for practical resistance to DMI fungicides, such as myclobutanil (Cox et al, 2009, Marine et al, 2007, Köller et al, 1997). In this case, practical resistance can be defined as greatly diminished levels of disease control caused by the selection for fungicide resistant isolates (Köller et al, 1991)

In addition to the 7 to 10 fungicide applications made during the spring to manage apple scab, growers in the eastern production regions of the US typically make four to eight additional fungicide applications during the summer to manage secondary infection cycles of apple scab and other diseases including Sooty blotch/fly speck (*Peltaster fructicola, Geastrumia polystigmatus, Leptodontium elatus, Zygophiala jamaicensis*), black rot (*Botryosphaeria obtusa*), bitter rot (*Glomerella cingulata*), and white rot (*Botryosphaeria dothidea*), which are collectively referred to as 'summer diseases' (Sutton, 1996). The DMI fungicide difenoconazole was recently registered as Inspire Super for the management of apple scab and the aforementioned summer diseases by the NY Department of Environmental Conservation in 2011 (http://128.253.223.36/ppds/525998.pdf). Despite the potential utility of difenoconazole to manage several diseases of apple, its use for both apple scab and summer diseases may have unforeseen effects on the DMI sensitivity of resident orchard populations of *V. inaequalis*.

Given the prevalence DMI resistance in regional orchard populations of *V. inaequalis* (Cox et al, 2009, Marine et al, 2007, Köller et al, 1997), and the recent introduction of difenoconazole for the management of apple diseases, investigations are needed to identify chemical management practices that impact the persistence and progression of DMI resistance in apple orchards. In order to provide insight on the effects of difenoconazole use on the persistence and progression of DMI resistance in an overwintering population of *V. inaequalis*, we determined the effect of difenoconazole treatment program during the spring for primary apple

scab infections and throughout the entire apple production season on the sensitivity of *V. inaequalis* isolates to (i) difenoconazole, and (ii) another DMI fungicide, myclobutanil. We also examined the effect of difenoconazole use on the sensitivity of overwintering *V. inaequalis* to a fungicide chemistry with an unrelated mode of action, dodine. This work sheds light on the effect of continued exposure to difenoconazole in a DMI resistant population of *V. inaequalis*.

MATERIALS AND METHODS

Field trial to evaluate the effect of difenoconazole application date on *V. inaequalis* DMI fungicide sensitivity. Trials were conducted during the 2010 and 2011 production seasons in a 16-year-old apple orchard at the New York State Agricultural Experiment Station in Geneva, NY planted with paired 'Empire' and 'Jonagold' apples on M.9/M.M.111 interstem rootstocks. Historically difenoconazole has been used in this orchard since 2006, but resistance has not been observed. However, the *V. inaequalis* population in this orchard has been confirmed to be practically resistant to myclobutanil, and sensitivity phenotypes for *V. inaequalis* isolates sampled over successive seasons have previously been demonstrated to be evenly distributed across the four replicate blocks with randomized treatment blocks (Cox et al, 2012, Cox et al, 2010). To evaluate the effect of difenoconazole use on the DMI sensitivity of overwintering population of *V. inaequalis*, an experiment was established with three fungicide programs (Table 1) which included a program where no fungicide applications were made (program 1), a program with four applications of difenoconazole (73 mL a.i./Ha) (research grade difenoconazole, Syngenta, Greensboro, NC) targeting primary apple scab infections in the spring (program 2), and a program with eight applications of difenoconazole throughout the entire apple growing

season targeting both apple scab and summer diseases (program 3). Fungicide programs (Table 1) were applied to four replicate 55.7 m^2 treatment blocks using a handgun sprayer with rates adjusted for a tree volume of approximately 2800 L/ Ha to the point of runoff from treated foliage. Fungicide applications targeting primary apple scab were made from April to June of each year on 7-10 day intervals. Summer cover fungicide applications targeting secondary apple scab infections and summer diseases were made on 14-21 day intervals from July to September (Table 1). Applications of captan (Captan 80 WDG, Arysta LifeScience Company, Cary, NC) alone (5.6 kg/Ha) or mixed (2.8 kg/Ha) with mancozeb (Penncozeb 75DF, United Phosphorus inc, King of Prussia, PA) (3.3 kg/Ha) were made at the phenological stages when difenoconazole was not applied in programs 2 (Table 1). During the seasons that overwintering isolates were evaluated for difenoconazole sensitivity, all treatment blocks received applications of captan (Captan 80 WDG, Arysta LifeScience Company) (2.8 kg/Ha) mixed with mancozeb (Penncozeb 75DF, United Phosphorus Inc) (3.3 kg/Ha) as a preventative measure from apple scab epidemics that would occur if otherwise unchecked until the time of collection.

***In vitro* evaluation of DMI and dodine fungicide sensitivity for collected *V. inaequalis* clonal conidial isolates.** In order to determine the effect of fungicide programs on the DMI sensitivity of overwintering *V. inaequalis,* clonal conidial isolates from each fungicide treatment program were collected and evaluated for sensitivity to two DMI fungicides, difenoconazole and myclobutanil, in the season following fungicide application. In order to determine the effect of difenoconazole use on a fungicide with an unrelated mode of action, dodine sensitivity was also evaluated using the same collection of isolates. In May of 2011 and 2012 (the calendar years following treatment), 100 leaves with primary apple scab lesions from

the overwintering population were collected equivalently from both ‘Jonagold’ and ‘Empire’ trees in each of the 55.7 m^2 fungicide treatment blocks established in the previous season. Single clonal conidial leaf lesions (Olaya and Köller 1999, Köller et al. 2004) were excised with a cork borer (7 mm diameter) from collected leaves. Excised single leaf discs were then placed in 1.2 ml of distilled water and shaken for 60s to dislodge conidia from the lesion. The clonal conidial isolates (n > 25) from each fungicide program, with the repetitions combined, were represented by these suspensions and were evaluated for fungicide sensitivity using microscopy aided mycelial relative growth assays that were previously described in Köller et al (2004) with the following modifications. Aliquots (100 µl) of each clonal conidial isolate suspension were added to the surface of a control medium, which was comprised of potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) amended with two antibiotics: streptomycin sulfate (50 µg/ml, Sigma-Aldrich, St. Louis, MO) and chloramphenicol (50 µg/ml, Sigma-Aldrich) to eliminate bacteria from the clonal conidial isolate suspensions. Aliquots (100 µl) of each clonal conidial isolate suspension were also applied to the surface of PDA media that was amended with either one of two DMI fungicides myclobutanil (0.1µg/ml as suggested by Köller et al, 1997, analytical standard; Sigma-Aldrich) or difenoconazole (0.1µg/ml analytical standard; Sigma-Aldrich). Additionally, 100 µl aliquots from each clonal conidial isolate suspension were applied to the surface of PDA media amended with dodine (0.2µg/ml, analytical standard; Sigma-Aldrich) (Köller et al, 1999).

Mean Percent Relative Growth (mean %RG) for each clonal conidial isolate was determined after 7 days at 21 to 25°C by measuring the mean growth of 5 micro colonies, each originating from a single conidium, in the presence of one of the evaluated fungicides relative to the mean growth of 10 micro colonies on the control medium. Measurements of mycelium (70-

120X) were determined using a SPOT Idea digital camera with the SPOT Imaging Basic software package (Diagnostic Instruments Inc., Sterling Heights, MI) attached to an Olympus SZX12 stereo scope (Olympus America Inc., Center Valley, PA).

Statistical Analyses. Logarithmic and arcsine data transformations were performed on observed sensitivity distributions for difenoconazole, myclobutanil and dodine to determine the transformation that best stabilized the variances and approximated normality. The distributions of sensitivity responses for each fungicide and transformation were analyzed for normality by the Shiparo-Wilks test using the continuous fit test of JMP v.9.0 (SAS Institute, Cary, NC) to assess the statistical appropriateness of procedures used to analyze the mean %RG of isolates for each treatment program. The mean %RG of *V. inaequalis* isolates for each treatment program was generated using the PROC Means procedure of SAS (SAS Institute), and differences in the mean %RG of isolates between fungicide programs were determined using Generalized Linear Mixed Models with the GLIMMIX procedure of SAS v9.3 (SAS Institute).

RESULTS

The effect of difenoconazole use on *in vitro* sensitivity of *V. inaequalis* clonal conidial isolates to difenoconazole. In 2011, the mean %RG of clonal conidial isolates evaluated on difenoconazole-amended medium ranged from 30.1% to 49.4% with program 1 (30.1% ± 5.8%, receiving no fungicide applications), and program 2 (49.4% ± 5.7%, 4 difenoconazole applications in the primary infection season only) having the lowest and highest means, respectively (Figure 3). There was a significant (P=0.0379) effect of fungicide program on *in*

vitro isolate sensitivity to difenoconazole. The mean %RG on difenoconazole-amended medium for clonal conidial isolates from program 2 was significantly higher than that of program 1 (P=0.0109, Figure 3). There was no significant difference between the mean %RG of clonal conidial isolates on medium amended with difenoconazole between programs 2 and 3 (program 3 being the treatment that received difenoconazole throughout the season) (P=0.1937). There also was no significant difference between the mean %RG of clonal conidial isolates on medium amended with difenoconazole between programs 1 and 3 (P=0.2136).

In 2012, mean %RG of clonal conidial isolates on difenoconazole-amended medium ranged from 29.9% to 42% with program 1 (29.9% ± 3.2%) and program 2 (41.7% ± 3.6%) having the lowest and highest means, respectively (Figure 4). There was a nearly significant (P=0.0549; a = 0.05) effect of fungicide program on *in vitro* isolate sensitivity to difenoconazole (Figure 4), which was consistent with the observed trend in 2011 (Figure 3). Based on linear contrast analysis, the mean %RG on difenoconazole-amended medium for clonal conidial isolates from program 2 was significantly higher than that of program 1 (P=0.0044) (Figure 4). There also was no significant difference between the mean %RG of clonal conidial isolates on medium amended with difenoconazole between programs 1 and 3 (P=0.3428).

The effect of difenoconazole use on *in vitro* sensitivity of *V. inaequalis* clonal conidial isolates to myclobutanil. In 2011, the mean %RG of *V. inaequalis* clonal conidial isolates on myclobutanil-amended medium ranged from 46.7% to 59.1% with the program 3 (46.7% ± 4.8%) and program 1 (59.1% ± 11.8%) having the lowest and highest means, respectively. Overall, there was no significant (P=0.4831) effect of fungicide program on isolate sensitivity to myclobutanil.

In 2012, the mean %RG of *V. inaequalis* clonal conidial isolates on myclobutanil-amended medium ranged from 74.5% to 85.8% with program 1 (74.5% ± 3.2%) and program 3 (85.8% ± 2.5%) and having the highest and lowest means, respectively. Interestingly, there was a nearly significant (P=0.0551 a = 0.05) effect of fungicide program on isolate sensitivity to myclobutanil in 2012 (Figure 5). Based on linear contrast analysis, the mean %RG on myclobutanil-amended medium for clonal conidial isolates from program 3 was significantly higher than that of program 1 (P=0.0066).

The effect of difenoconazole use on *in vitro* sensitivity of *V. inaequalis* clonal conidial isolates to dodine. In 2011, the mean %RG of clonal conidial isolates on dodine-amended medium ranged from 19.6% to 20.8% with the program 1 (19.6% ± 5.6%) and program 2 (20.8% ± 5.0%) having the lowest and highest means, respectively. The same clonal conidial isolates that were used for determining myclobutanil and difenoconazole sensitivity within each trial year were utilized for the dodine sensitivity assay. By comparison the mean %RG of clonal conidial isolates on dodine-amended medium in 2012 ranged from 19.0% to 23.1% with program 3 (19.0% ± 1.6%) and program 2 (23.1% ± 2.8%) having the lowest and highest means, respectively. Overall, there was no significant effect (2011, P=0.6693; 2012, P=0.2193) of fungicide program on isolate sensitivity to dodine in either year.

DISCUSSION

The results of these studies suggest that the use of difenoconazole throughout the apple production season (program 3) targeting both apple scab and other summer diseases (e.g. bitter

rot, fly speck, and sooty blotch) does not select for resistance to DMI fungicides any more than the application of difenoconazole only during the primary apple scab infection period (program 2). In particular, the level of difenoconazole resistance (represented by mean %RG) for isolates in program 2 was higher than isolates from program 1 (no fungicides) in both years. This observation may suggest that applications of difenoconazole targeting primary apple scab infections are likely to shift an orchard population of *V. inaequalis* towards difenoconazole resistance.

It was expected that applying difenoconazole eight times throughout the season (program 3) would select for isolates with lower sensitivity to difenoconazole than four applications in the spring (program 2). Our results did not confirm this hypothesis because there were no statistically significant differences in mean %RG of clonal conidial isolates on difenoconazole-amended medium between programs 2 and 3. One possible explanation is related to the level of overwintering inoculum at the seasons end. We have observed that trees receiving modern site-specific fungicides including fluxapyroxad, pyraclostrobin, fluopyram, and difenoconazole in summer cover applications have lower incidences of apple scab symptoms on fruit at harvest compared to trees that receive standard captan protectant applications (Cox et al, 2012). Similar observations regarding the incidence of apple scab at harvest following site-specific fungicide use have been previously reported (Taylor et al, 2009, Arauz et al, 1990). Such observations can be attributed to the curative action of site specific chemistries (Kuns et al, 1997). The use of protectant fungicides that lack curative activity may not prevent established infections from sporulating, which would increase the inoculum at the end of the season. A high level of inoculum at the end of the season might increase the probability that isolates with reduced sensitivity to difenoconazole would overwinter between trial years. Van den Bosch et al. (2011),

suggest the proportion of sensitive population members is affected by the basic reproduction rate (R_0) of sensitive lesions. This could be taken to support the concept that if these sensitive lesions survive in program 2 because captan has no curative activity during summer cover applications, then the proportion of sensitive overwintering inoculum would vary between programs 2 and 3. Further investigation in regard to end of season apple scab incidence would be warranted to verify this scenario.

Interestingly, the trends in isolate sensitivity to difenoconazole resulting from the fungicide programs did not result in corresponding trends for myclobutanil sensitivity. This was unexpected given that cross-sensitivity has been reported for DMI fungicides in several systems, including *V. inaequalis* (Ishii et al, 2006; Hildebrand et al, 1988, Fuchs and Drandarevski, 1976). The apparent deviation in DMI cross-sensitivity observed in the present study suggests that different mechanisms may be involved in the development of *V. inaequalis* resistance to myclobutanil and difenoconazole. Currently, little is has been documented about a lack of DMI cross-sensitivity for *V. inaequalis*. This observation could be attributed to sampling size, and a repeated trial and further experimental inquiry could determine if more samples are required to fully assay this deviation from previously reported DMI cross-sensitivity. However, differential selection for other DMI chemistries has been reported in other pathosystems (Fraiije 2007; Hsiang et al, 1997). For example, in the ascomycete, *Sclerotinia homoeocarpa*, EC_{50} values of isolates evaluated for sensitivity to propiconazole, myclobutanil, fenarimol and tebuconazole are not always highly correlated when pairwise comparisons are performed, which suggest that cross-resistance may not always operate between all DMI fungicides (Hsiang et al, 1997).

As anticipated, the sensitivity of *V. inaequalis* isolates to dodine was not significantly different between the three fungicide programs in our study. This demonstrates that differences

in isolate sensitivities to other fungicides evaluated in the present study are unlikely to be influenced by positional confounding of treatment blocks due to uneven distribution of fungicide sensitivity within the resident *V. inaequalis* population. An uneven distribution of fungicide sensitivity phenotypes has been reported for an orchard population of *V. inaequalis* in the United Kingdom (Gao et al, 2009). In this particular example, a *V. inaequalis* sub population in the lower portion of an orchard was much more resistant to myclobutanil (Gao et al, 2009). The authors suggested that this uneven distribution was likely due the combined effects of microclimate, uneven inoculum pressure, and fitness costs associated with fungicide resistance. If variation in dodine sensitivity among fungicide program had been observed in the present study, it is possible that such differences could be due to localized population members with multiple resistance mechanisms.

Overall, the results of the study suggest that the use of difenoconazole throughout the growing season to target both apple scab and summer disease problems has does not impact the DMI resistance of *V. inaequalis* populations any more than applications targeting apple scab during the spring and early summer. Despite the findings regarding the differences in isolate sensitivity regarding difenoconazole use, the repeated use of difenoconazole still places considerable selective pressure on the *V. inaequalis* population as a whole. This is evident from the observed differences in difenoconazole sensitivity between isolates from the difenoconazole fungicide programs (programs 2 and 3) and those from the program that received no fungicide applications (program 1). We have also demonstrated that the development of resistance to difenoconazole in a *V. inaequalis* population did not result in corresponding decreased sensitivity to another DMI fungicide myclobutanil, and similar observations have been reported for DMI chemistries in other pathosystems (Fraiije 2007; Hsiang et al, 1997). Our findings provide insight

on factors influencing DMI resistance in an ascomycete pathosystem, which could, in turn, drive the development of novel control strategies for apple diseases in the context of DMI resistance management.

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TABLE

Table 1. Fungicide application timings for each of the treatment programs used to evaluate effects on the DMI sensitivity of *Venturia inaequalis* isolates.

	Primary apple scab season [a]			
Program [c]	Pre-bloom	Pink and bloom	Petal fall	1st and 2nd cover
1	untreated	untreated	untreated	untreated
2	Captan 80 (2.8 kg/Ha) + Mancozeb (3.3 kg/Ha)	Difenoconazole (73 mL a.i./Ha)	Captan 80 (2.8 kg/Ha) + Mancozeb (3.3 kg/Ha)	Difenoconazole (73 mL a.i./Ha)
3	Captan 80 (2.8 kg/Ha) + Mancozeb (3.3 kg/Ha)	Difenoconazole (73 mL a.i./Ha)	Captan 80 (2.8 kg/Ha) + Mancozeb (3.3 kg/Ha)	Difenoconazole (73 mL a.i./Ha)

	Summer covers [b]	
Program [c]	3rd cover	4th, 5th, 6th, and 7th cover
1	untreated	untreated
2	Captan 80 (2.8 kg/Ha)	Captan 80 (2.8 kg/Ha)
3	Captan (2.8 kg/Ha)	Difenoconazole (73 mL a.i./Ha)

[a] Fungicide treatment programs targeting primary apple scab infections were applied at 7-10 day intervals from the phenological stages of green tip (2 May 2011 and 28 March 2012) to 2nd cover (22 June 2011, 24 May 2012)

[b] Fungicide treatment programs targeting secondary apple scab infections and summer diseases were applied at 14-21 day intervals designated 3rd, 4th, 5th, 6th, and 7th cover. Cover applications 3rd 4th, 5th, 6th, and 7th for the 2011 season were applied on 21 June, 8 July, 27 July, 10 August, and 25 August, respectively. Cover applications for the 2012 season were at these same phenologic stages on 14 June, 3 July, 25 July, 15 August, and 30 August, respectively.

[c] All applications were made using a handgun sprayer at 600 PSI with rates adjusted for a tree row volume of approx. 2800 L/ Ha

FIGURES

Figure 3

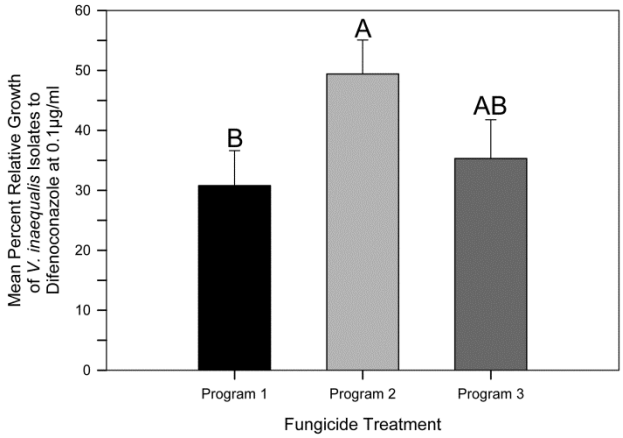

Figure 3: Mean relative growth values representing the *in vitro* fungicide sensitivity of *V. inaequalis* isolates to a discriminatory dose of difenoconazole for each treatment program for the 2011 trial. Treatments indicated with the same letter lack statistical significance ($\alpha = 0.05$) among adjusted estimated mean incidences.

Figure 4

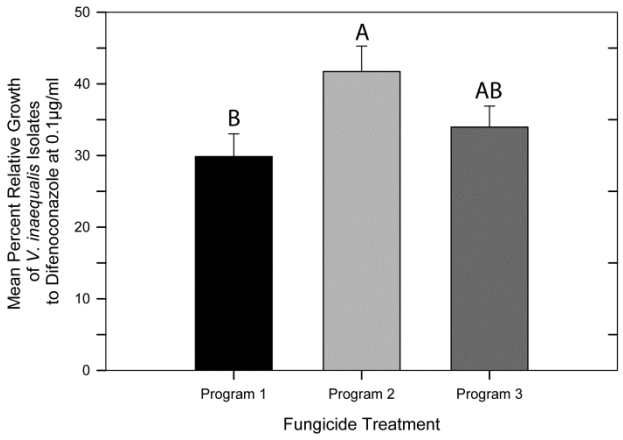

Figure 4: Mean relative growth values representing the *in vitro* fungicide sensitivity of *V. inaequalis* isolates to a discriminatory dose of difenoconazole for each treatment program for the 2012 trial. Treatments denoted with the same letter lack statistical significance ($\alpha = 0.05$) among adjusted estimated mean incidences.

Figure 5

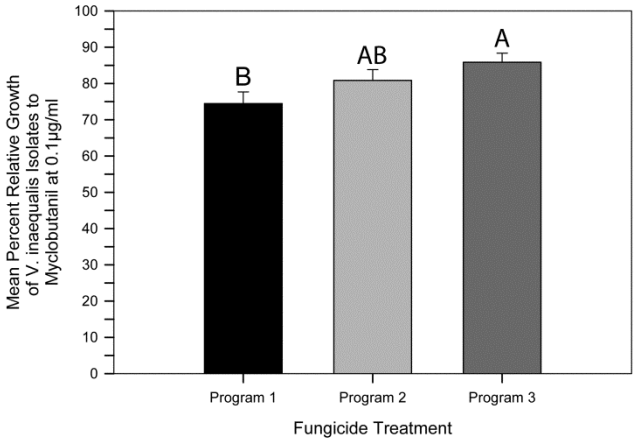

Figure 5: Mean relative growth values representing the *in vitro* fungicide sensitivity of *V. inaequalis* isolates to a discriminatory dose of myclobutanil for each treatment program for the 2012 trial. Treatments indicated with the same letter lack statistical significance ($\alpha = 0.05$) among adjusted estimated mean incidences.

CHAPTER 4

THE PREVALENCE OF QUINONE OUTSIDE INHIBITOR RESISTANCE IN *VENTURIA INAEQUALIS* IN THE NORTHEASTERN UNITED STATES

ABSTRACT

The use of fungicides, including the Quinone outside Inhibitors (QoIs), is one of the primary means for the management of apple scab disease caused by *Venturia inaequalis*. To determine the prevalence of both quantitative and qualitative QoI resistance in the northeastern United States, we sampled clonal conidial isolates (n = 4,481) from 120 orchard populations representing commercial and research orchards, as well as unexposed baseline orchards, from 2004 to 2011. The majority of sampled orchards (67%, 80 of 120) were sensitive to QoI fungicides, 34 of 120 (28%) orchards had *V. inaequalis* populations with QoI resistance, and six of the sampled orchards (5%) had *V. inaequalis* populations with reduced sensitivity to QoI fungicides. Isolates with qualitative QoI resistance, conferred by the G143A *cytochrome b* gene mutation, were found in 13 of the 34 QoI resistant orchard populations with 26.9 to 95.8% of the isolates possessing qualitative resistance. To evaluate the stability of the G143A mutation, a total of 27 isolates (19 resistant and 8 sensitive isolates) were subcultured continuously in the presence or absence of the QoI fungicide trifloxystrobin. All isolates that initially possessed qualitative resistance maintained the resistant genotype (G143A) for six subcultures in absence or presence of a QoI fungicide. Given the observed propensity for QoI fungicide resistance in many *V. inaequalis* populations and the stability of the G143A mutation in individual isolates,

regional apple scab management paradigms must encompass strategies to limit selection of QoI resistance.

INTRODUCTION

Apple scab, caused by the ascomycete fungal pathogen *Venturia inaequalis*, is one the most economically important diseases of apple (Agrios, 2005, MacHardy et al, 1996, Jones et al, 1990). Apple scab is most severe in temperate climates where weather conditions during spring are conducive to infection (Holb et al, 2003, MacHardy, 1996), which is when temperatures are between 12 and 26 °C with approximately 4 to 10 hours of leaf wetness (MacHardy and Gadoury, 1989). Little to no marketable fruit can be produced if proper chemical management paradigms are not in place (Agrios, 2005, Holb et al, 2003, Jones et al, 1990). Indeed, losses in orchards bereft of management may exceed 70 to 80% of the total harvest (Holb et al, 2003).

Successful management of apple scab is primarily achieved through the use of fungicides (Agnello et al, 2012, MacHardy et al, 1996). Of these fungicides, the Quinone outside Inhibitors (QoIs) have proven to be highly effective for the management of apple scab (Färber et al, 2002, Olaya and Köller, 1999). The antifungal activity of QoI's was first noted in the 1970's (Anke et al, 1977), and was first used in apples for the management of apple scab in the mid to late 1990s (Olaya et al, 1998, Köller et al, 2004). QoI fungicides prevent fungal respiration by inhibiting electron transfer at the ubiquinol-oxidation center (Qo site) of the bc1 enzyme III complex in the mitochondria (Ma et al, 2005, Färber et al, 2002, Bartlett et al, 2002). Because of this highly specific mode of action, repeated applications of QoI fungicides can create stable, incremental adjustments that limit efficacy in fungal populations over time (Ishii et al, 2006, Ma et al, 2005).

One mechanism of resistance described for QoI fungicides is a single point mutation that codes for alanine instead of glycine at position 143 (G143A) in the *cytochrome b* gene. This mutation is most commonly observed in field pathogen populations developing fungicide resistance (Bartlett et al, 2002). The G143A mutation has been observed to confer a high degree of qualitative QoI resistance in orchard populations of *V. inaequalis* in Michigan (Lesniak et al, 2011), and the presence of this mutation was previously reported in a *V. inaequalis* population in New York (Cox et al 2008). Other point mutations, both naturally and artificially induced, also confer qualitative resistance to QoI fungicide chemistries in other fungal pathogens (Kim et al, 2003; Karaoglanidis et al, 2011, Ma et al 2003), but none of these have been reported in *V. inaequalis*. Additionally, there is an alternative respiration pathway or cyanide insensitive pathway (Färbe et al, 2003), which influences fungal response to respiratory inhibitors (Zheng et al, 2000, Day et al, 1995). This pathway allows the fungal pathogen to bypass the inhibited respiration pathway and persist through selective pressures created by QoI application, as shown for *Magnaporthe grisea* (Mizutani et al, 1996), *Septoria triciti* (Ziogas et al, 1997), *Botrytis cinerea* (Tamura et al, 1999), and in *V. inaequalis* (Olaya et al, 1998).

There is an additional quantitative mechanism of QoI resistance, in which multiple genetic elements work in concert to impart a wide degree of resistance in *V. inaequalis* (Köller et al, 2004). Unlike the discrete categories of resistant and sensitive phenotypes observed with qualitative QoI resistance in *V. inaequalis* (Lesniak et al, 2011), isolates may display a range of sensitivity phenotypes in the absence of a *cyt b* mutation when exposed to a QoI fungicide (Köller et al, 2004). It has been suggested that quantitative resistance precedes QoI qualitative resistance in *V. inaequalis* isolates in orchard populations, making a two-step pathway to practical QoI resistance (Köller et al, 2004); whereby practical resistance is defined as greatly

diminished levels of disease control under field conditions resulting from the selection of fungicide resistant phenotypes within the population (Köller et al, 1991).

Given the potential for heteroplasmy in the *cyt b* gene in fungi (Gisi et al, 2002), information on the stability of the G143A mutation in the absence of selective pressure is of key importance for understanding qualitative resistance in *V. inaequalis*. While investigations into the stability of such *cyt b* gene mutations has been carried out for a number of plant pathogenic fungi (Mizutani et al, 1996, Ziogas et al, 1997, Tamura et al, 1999), the stability of the G143A mutation in *V. inaequalis* is largely unknown. This stability within isolates of *V. inaequalis* would be an important knowledge gap to address and the first step in understanding the persistence of qualitative resistance in an orchard population. In this regard, the objectives of this study were to first (i) determine the prevalence of quantitative and qualitative QoI resistance in *V. inaequalis* orchard populations in the northeastern United States, and to (ii) assess the stability of qualitative QoI resistance in *V. inaequalis* in the absence of QoI-induced selective pressure. The resulting information improves our understanding of qualitative QoI resistance in the region, and provides an initial assessment of the potential for an orchard population to revert toward QoI sensitivity in the absence of use.

MATERIALS AND METHODS

In vitro sensitivity of *V. inaequalis* populations to the QoI fungicide trifloxystrobin. From 2004 to 2011, *V. inaequalis* populations were examined from 120 apple orchards located in the northeastern United States. Specifically, populations from 94 commercial, 16 research, and 10 baseline orchards in multiple states (Table 2) were evaluated. Baseline orchards are defined as

orchard plantings never having exposure to site-specific fungicide chemistries (e.g. anilinopyrimidines, DMIs, or QoIs). Twenty three of the 120 total orchard populations were sampled in multiple years (Table 2, highlighted in bold). For each orchard, 50-100 leaves were randomly collected from trees from across the planting. From each single orchard collection, a minimum of 15 *V. inaequalis* clonal conidial leaf lesion isolates (Olaya and Köller 1999, Köller et al. 2004) were evaluated for sensitivity to the QoI fungicide trifloxystrobin.

Each clonal conidial leaf lesion was excised with a cork borer (7 mm diameter) and placed in 1.2 ml of distilled water and shaken for 60s to rinse conidia from the lesion. Resulting clonal conidial isolates were then evaluated for quantitative QoI sensitivity using microscopy aided mycelial relative growth assays as described in Köller et al. (2004). In short, 100 µl aliquots of each clonal conidial isolate suspension were applied to the surface of control medium, consisting of potato dextrose agar (PDA) (Difco Laboratories) amended with two antibiotics: streptomycin sulfate (50 µg/ml, Sigma-Aldrich, St. Louis, MO) and chloramphenicol (50 µg/ml, Sigma-Aldrich). A 100-µl aliquot of each clonal conidial suspension was also applied to the surface of media amended with trifloxystrobin (0.02 µg/ml analytical standard; Sigma-Aldrich) (Köller et al, 2004). Isolates were incubated for seven days at 23-25°C. Mean percent relative growth (mean %RG) for each clonal conidial isolate was determined by measuring the mean growth of five micro colonies, each originating from a single conidium, in the presence of trifloxystrobin (0.02 µg/ml) relative to the mean growth of 10 micro colonies on un-amended medium. The mycelium from micro-scale colonies was measured (70-120X) using a SPOT Idea digital camera with the SPOT Imaging Basic software package (Diagnostic Instruments Inc., Sterling Heights, MI) attached to an Olympus SZX12 stereo scope (Olympus America Inc., Center Valley, PA).

Distributions of quantitative QoI fungicide sensitivity responses in the form of isolate mean %RG values were generated for each orchard and classified by comparisons to standards as previously described (Köller et al, 1997). This was specifically accomplished by statistical comparison of distributions of observed mean %RG from the 120 test orchard populations verified as being practically resistant or sensitive to trifloxystrobin. The two latter isolate categories were validated during farm consultations or by performing the applications and disease assessment directly. Comparisons of distributions were made using the nonparametric Kolmogorov-Smirnov test with SAS (version 9.3, SAS Institute, Cary, NC). Nonparametric statistical procedures were employed because of the potential for bimodal distribution of trifloxystrobin sensitivity resulting from qualitative QoI resistance.

Determination of qualitative QoI resistance in *V. inaequalis* populations. In order to determine the presence QoI qualitative resistance in *V. inaequalis***,** both cultural and molecular assays were used. All clonal conidial isolates from each of the orchard populations sampled were subjected to the cultural assay by placing 100 µl of the conidial suspension (see above) on PDA (Difco Laboratories) amended with trifloxystrobin and salicyl hydroxamic acid (sham) to inhibit the alternative respiratory pathway (100 µg/ml, analytical standard; Sigma-Aldrich), as suggested by Köller et al. (2004). Indeed, when sham is combined with a QoI fungicide such as trifloxystrobin, greater mycelial growth inhibition is observed through the synergistic effects of the two compounds (Färber et al, 2002, Steinfield et al, 2001), which eliminates the quantitative QoI resistance response for this facet of the present study. To score qualitative resistance phenotypes, isolates were incubated for 14 days at 23-25°C and evaluated for the presence of *V. inaequalis* micro-colonies (Köller et al, 2004).

To evaluate the molecular basis of qualitative resistance for a subset of *V. inaequalis* isolates, polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) was performed as described by Fontaine et al. (2009) with several modifications. Prior to DNA extraction, colonies were grown from clonal conidial isolate suspensions on trifloxystrobin-amended media (0.02 µg/ml) for 5 weeks at 23-25°C. Isolation of *V. inaequalis* tissue for DNA extraction was accomplished by gently scraping mycelium from the surface of the medium using a scalpel and sterilizing between isolates. Mycelia of each clonal conidial isolate (100 mg) was ground using liquid nitrogen and total DNA was extracted using an Omega Bio-Tek Plant DNA extraction kit (Omega Bio-Tek, Norcross, GA) following the protocol provided. To determine the presence of the *cyt b* G143A mutation, a PCR was performed to amplify a 950-bp amplicon from the *cyt b* gene using *V. inaequalis* specific primers ViCytB-5F (5'-GGACCAAGTAATCACTGGTGTATGG-3') and ViCytB-6071R (5'-TTGAAAGCTAGGCTAGGGCGAACA-3') (Cox et al, 2008). Restriction Fragment Length Polymorphism (RFLP) analysis was performed on PCR products using 5 units of Fnu4HI restriction enzyme (New England Biolabs, Ipswich, MA). The reaction was incubated at 37°C for 12 hours. After incubating, a final inactivation step of 25 minutes at 65°C was performed with an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) to inactivate the Fnu4HI restriction, as required by the manufacturers protocol, and DNA fragments analyzed by electrophoresis on a 1.0% agarose gel (Figure 6).

Stability of QoI qualitative resistance in isolates of *V. inaequalis* in the absence of QoI-induced selective pressure. A subset of isolates from orchards surveyed in 2010 and 2011 with a range of quantitative resistant phenotypes was evaluated for the presence and stability of

the *cyt b* G143A mutation (Table 3). In order to establish the presence of qualitative QoI resistance and the *cyt b* G143A mutation, isolates were subjected to the cultural assay, PCR, and RFLP analysis (described above). To test for stability, colonies were grown on medium amended with trifloxystrobin (0.2 µg/ml) and sham (100 µg/ml) (Figure 7), and transferred to medium amended with trifloxystrobin and allowed to grow for five weeks at 23-25°C. Once *V. inaequalis* colonies were five weeks of age, two agar plugs were removed using a 5mm diameter cork borer and transferred to freshly made trifloxystrobin medium and un-amended PDA medium (Figure 7). At each successive 5-week time point, each isolate was transferred to either fresh PDA or fresh trifloxystrobin-amended (0.02µg/ml) medium to mimic the absence of selection pressure or constant selective pressure, respectively (Figure 7). By the end of the experiment, each isolate had undergone a total of six transfers over the course of six months. At the outset and at the time of each transfer, the presence of the *cyt b* G143A mutation was assessed for each isolate as described above.

Eight isolates that were not resistant to trifloxystrobin were included in the study as controls. Since these isolates would not grow on trifloxystrobin-amended media, they were grown on un-amended PDA medium for five weeks at 23-25°C, and transferred to both trifloxystrobin amended and un-amended PDA medium. These isolates were also evaluated for the presence of the *cyt b* G143A mutation at each evaluation point.

Results

Sensitivity of *V. inaequalis* clonal conidial isolates to trifloxystrobin. From 2004 to 2011, 120 individual *V. inaequalis* orchard populations were evaluated throughout the northeastern

United States (Table 2). While a few orchards were represented by a minimum of 15 clonal conidial isolates successfully cultured from collected leaves, most orchard populations were represented by 25 or more clonal conidial isolates (Table 2).

Across all seven years, the majority (67%, 80 of 120) of orchard populations were found to be sensitive to trifloxystrobin on the basis of statistical comparison (Kolmogorov-Smirnov test) of quantitative resistance phenotype distributions to known standards (Figure 8). The mean %RG for the sensitive populations ranged from 0.0% to 36.3% with a baseline orchard (0.0% ± 0.0%) and a sensitive commercial orchard (36.3% ± 4.3%), representing the endpoints of the range. Of the 80 apple scab orchard populations that were sensitive to trifloxystrobin, 56 were from commercial apple orchards, 14 were from research orchards, and 10 baseline orchards had isolates with mean %RGs ranging from 0.0% - 18.0% with an orchard from Geneva, NY (0.0% ± 0.0%) and Montezuma, NY (18.0% ± 3.9%), representing the endpoints of the baseline range.

Throughout the seven years of orchard surveying, only 6 of the 120 apple scab populations were found to have reduced sensitivity or shifted toward trifloxystrobin resistance. The mean %RG for these populations ranged from 33.9% to 39.2% with a research orchard (33.9% ± 6.6%) and a commercial orchard (39.2% ± 3.6%) representing the endpoints of the range. Of the orchard sites with *V. inaequalis* populations that had reduced sensitivity to trifloxystrobin, four were commercial orchards with a QoI use history of approximately 2-3 times per season according to pesticide application records. The remaining three were research orchards where pesticide application records indicated some QoI use (Table 2).

During the course of the seven years of testing, there were 34 *V. inaequalis* orchard populations with practical resistance to trifloxystrobin on the basis of statistical comparison of quantitative resistance phenotype distributions to known standards (Table 1; Figure 8). The mean

%RG ranged from 41.0% to 100.4% with commercial orchards from Pataskala, OH (41.0% ± 4.1%) and Newfield, NY (100.4% ± 3.2%) representing the endpoints of the range. Of the orchard populations of *V. inaequalis* with resistance to trifloxystrobin, 38 were commercial orchards with a QoI use history of approximately 2-3 times per season according to pesticide application records. Additionally, all of these commercial orchards had a history of 15 or more QoI applications. Only 2 of the 40 orchard sites with *V. inaequalis* populations that were resistant to trifloxystrobin were collected from research orchards (Table 1).

Percentage of *V. inaequalis* isolates with qualitative QoI resistance in orchard populations. As anticipated, many of the *V. inaequalis* orchard populations that had practical resistance to trifloxystrobin on the basis of quantitative resistance also had a large percentage (>25%) of isolates that tested positive for qualitative resistance conferred by the *cyt b* G143A mutation, as determined by growth on trifloxystrobin and sham within two weeks (Köller et al, 2004). Using this threshold of more than 25% of the population members possessing qualitative resistance, 14 orchard populations were classified as being practically qualitatively resistant to QoI fungicide chemistries. The remaining 20 resistant orchard populations had little to no isolates that tested positive for the *cyt b* G143A mutation (< 18%), indicating a quantitative resistance mechanism responsible for the orchard's resistant classification. None of the six orchard populations with a reduced sensitivity to trifloxystrobin had any isolates with the *cyt b* G143A mutation. Interestingly, 4 of the 80 trifloxystrobin sensitive orchard populations did have isolates with the *cyt b* G143A mutation (Table 2), but these members were not sufficiently present (2-14%) to have the population classified as qualitatively or quantitatively (by the Kolmogorov-Smirnov test) resistant to trifloxystrobin. Somewhat surprisingly, five of the *V.*

inaequalis orchard populations (orchards 69a, 81b, 86a, 107, and 118;Table 2) had more than 25% isolates with the G143A mutation, but were classified as sensitive to trifloxystrobin on the basis the distribution of quantitative resistance phenotypes (mean %RG).

Evaluation of the stability of the G143A mutation in isolates of *V. inaequalis*.

Trifloxystrobin resistance was maintained through six consecutive transfers for all 19 of the 27 isolates with qualitative resistance (Table 3). Similarly, the presence of the G143A mutation was consistently observed by PCR-RFLP analysis for all isolates after each transfer on both trifloxystrobin-amended and un-amended medium (Figure 6). As expected, all 8 sensitive or baseline isolates failed to grow on trifloxystrobin-amended medium and did not have the *cyt b* G143A mutation, as shown by PCR RFLP analysis.

DISCUSSION

Across all seven years, the majority of *V. inaequalis* orchard populations were found to be sensitive to trifloxystrobin on the basis of the distribution of quantitative sensitivity phenotypes for population members. Considering the number of commercial orchard sites evaluated, there appears to be widespread occurrence of sensitivity to QoI fungicides for populations in New York and other regions included in this study. At the specific locations with sensitive populations, QoI fungicides are and should still be an effective means of managing apple scab. However such broad sweeping generalizations should be made with caution, as it is clear from the study that population sensitivity is highly specific to the local operation and production practices. Although some orchards were tested several times over the course of seven

years, little can be said about the temporal change in QoI sensitivity because most sites were not analyzed every year.

Similar to previous studies, the present study focused on many *V. inaequalis* populations from commercial orchards undergoing disease management programs (Lesniak et al, 2011, Köller et al, 2004, Färber et al, 2002, Steinfeld et al, 2001). The current study was also able to evaluate a comparably large number of baseline orchards (15%; 18 of 120) which had never been exposed to a QoI fungicide or other site specific chemistry. None of the isolates in the baseline *V. inaequalis* populations had the *cyt b* G143A mutation, which suggests that it may not be found in *V. inaequalis* populations which are unexposed to QoI fungicides. While this doesn't suggest that QoI, or any other fungicide, is inherently mutagenic, it does provide indirect evidence supporting the hypothesis of Van den Bosch et al. (2011) who suggested that stresses induced by fungicide applications may predispose population members to more rapidly develop mutations that serve as the initiation point for selection of fungicide resistance.

In the seven years of orchard population evaluation, few *V. inaequalis* orchard populations were classified as reduced sensitive to trifloxystrobin. One explanation is that reduced sensitivity or incremental resistance is not conferred by qualitative resistance, which usually confers a high degree of fungicide resistance (Lesniak et al, 2011, Vallières et al, 2011). Instead, reduced sensitivity in the population is likely due to a majority of individuals with a myriad of unknown genetic factors working synergistically to confer a range of fungicide sensitivity (Köller et al, 2004). In six *V. inaequalis* orchard populations with reduced sensitivity, none of the isolates possessed the *cyt b* G143A mutation (Table 2). Hence, qualitative QoI resistance was not contributing to the observed reduced sensitivity. Interestingly, the majority of instances where the *V. inaequalis* populations were classified as reduced sensitive to

trifloxystrobin were research orchards, which are treated more unconventionally in regards to commercial fungicide application practices. In these somewhat artificial production systems, the transient quantitative resistance phase suggested by Köller et al. (2004) may be prolonged. Many of the research orchard populations are not yet practically resistant to QoIs, but have the potential for practical resistance depending on continued QoI fungicide usage, as suggested by Chapman et al. (2011).

The results of this study indicate that QoI resistant *V. inaequalis* populations are present in apple orchards in the northeastern United States, but not in the majority of orchards included in the present study. Of the 40 QoI resistant *V. inaequalis* orchard populations, 17 populations had more than 25% of the isolates with the G143A mutation. In those cases, qualitative QoI resistance was likely responsible for the observed QoI resistant phenotypes, which is in agreement with a previous study in Michigan (Lesniak et al, 2011). Köller et al (2004) suggested that QoI resistance occurs in two phases in *V. inaequalis* orchard populations, which begins with quantitative resistance that is succeeded by isolates with the *cyt b* G143A mutation that confers a high degree of qualitative resistance. It was observed that 23 of the 40 *V. inaequalis* orchard populations were resistant to QoI fungicides, but few to none (0% - 18%, Table 2) of the isolates possessed the *cyt b* G143A mutation. The 23 orchard populations comprised of quantitatively resistant isolates may be in the first phase of this process while the remaining 17 orchards with qualitatively resistant isolates may be in the second phase. Given that there is no temporal relevance to the findings, our results cannot provide compelling evidence for the two phase development of QoI resistance in orchard populations, as previously proposed (Köller et al, 2004). However, our results do support the idea that genetic factors other than the *cyt b* G143A mutation are involved in QoI resistance.

Interestingly, there were five *V. inaequalis* orchard populations (orchards 69a, 81b, 86a, 107, 118, Table 2) that had more than 25% isolates with the *cyt b* G143A mutation and were determined to be sensitive to trifloxystrobin based on the distribution of quantitative resistance phenotypes (mean %RG). Compared to the other orchard populations evaluated in the study, these five orchards had fairly skewed bimodal distributions (data not shown). The majority of isolates from these orchard populations were sensitive isolates with low %RGs, but these populations also had 25-30% of the isolates with qualitative resistance and high relative growth values. Such a distribution would cause the median to fall in the sensitive range despite the presence of isolates with qualitative resistance. In these scenarios, orchard classification by the Kolmogorov-Smirnov test maybe less appropriate. The five orchard populations are also counter-examples of when the development of QoI resistance does not follow the hypothesized two phase response in that qualitative resistance developed in the absence of a predominantly quantitative resistant phenotype for the majority of *V. inaequalis* orchard population.

In this study we found that nearly all orchards with practical resistance to QoI fungicides had received two or more QoI fungicide applications per season (data not shown), which suggests that frequent QoI usage creates strong selective pressure for resistance. For example, orchard 117 (Table 2) had a population mean %RG of 100.4 with 95.8% of the isolates having the *cyt b* G143A mutation (Table 2). In this extremely resistant population, QoI fungicides had been applied three times per season for eight seasons according to pesticide application records. Similar levels of QoI resistance following intensive application schedules have been reported in other systems. It has been shown that more than 10 QoI applications in a kiwifruit planting will strongly select for highly resistant isolates of *Botrytis cinerea* (Bardas et al, 2010) populations, and six QoI applications annually in strawberry resulted in sufficient selective pressure for the

development of resistant *B. cinerea* isolates (Samuel et al, 2011). Intensive QoI usage for nine years in Michigan was sufficient to detect *V. inaequalis* isolates with the *cyt b* G143A mutation in several orchard populations (Lesniak et al, 2011).

In the present study, it was demonstrated that the *cyt b* G143A mutation appears to be stable in isolates for six transfers in the absence of QoI selective pressure. Since a reversion or loss of mutation was never observed for any isolates during the course of the six-month experiment, a reliable estimate of the duration allowing for a potential loss of the G143A mutation cannot fully be determined without further experimentation. Indeed, it has been reported that more than 30 similar cultural transfers were needed before a loss of the G143A mutation or reversion to wild type were observed for the wheat pathogen *Blumeria graminis* f . sp . *tritici* (Fraaije et al, 2002).

The persistence of the G143A mutation in an isolate or population operates on the assumption that there is a fitness cost associated with the mutation. Specifically, the potential for a loss of the G143A mutation assumes that mitochondria without the mutation would make the isolate more fit, and will overtake resistant populations in the absence of QoI fungicide use. The results of other studies have shown little (Ma and Uddin, 2009) or no (Karaoglanidis et al, 2011) fitness effects of qualitative QoI resistance in *Magnaporthe oryzae* and *Alternaria alternata*, respectively. Further analysis of *V. inaequalis* isolate fitness in regards to *cyt b* genotype and monitoring of changes in the proportion isolates with qualitative resistance in an orchard setting where QoI applications have ceased would provide better insight into the stability of the G143A mutation.

Overall, the results of this study suggest that orchard populations with quantitative and qualitative resistance to QoI fungicides are present in the northeastern United States. Hence,

apple producers in the region can still use QoI fungicides, but should use them with caution given the propensity for resistance. In several orchards, the distribution of quantitative phenotypes alone suggested that these populations may have practical resistance to trifloxystrobin. Köller et al. (2004) suggested that QoI resistance management for *V. inaequalis* could be accomplished by limiting the number of QoI applications made seasonally. Such curtailment of QoI use, if practiced, could slow the selection of population members with qualitative resistance, which would be important since we have demonstrated that qualitative QoI resistance is stable in isolates beyond several transfers. However, further research is needed to understand the persistence of qualitative QoI resistance in the absence of QoI use over successive field seasons.

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TABLES

Table 2: List of orchards by designation, year, and classification from which *V. inaequalis* populations were evaluated for trifloxysrtobin resistance. Orchard populations that were reexamined additional times in later years are highlighted in bold.

Designation	Classification[b]	n	% RG[a]	Pr>Ksa Resist	Pr>Ksa Sensitive	Percent G143A	Year	Orchard Type	State
1	Sensitive	32	0.0 ± 0.0	<0.0001	<0.0001	0	2009	Commercial	NY
2	Sensitive	30	19.8 ± 4.7	0.0125	0.3876	0	2007	Commercial	NH
3	Sensitive	27	14.5 ± 8.0	<0.0001	0.006	0	2007	Commercial	MA
4	Sensitive	30	12.5 ± 6.4	<0.0001	0.0072	0	2007	Commercial	RI
5	**Sensitive**	**30**	**0.5 ± 0.5**	**<0.0001**	**<0.0001**	**0**	**2007**	**Baseline**	**NY**
6	**Sensitive**	**31**	**12.8 ± 4.1**	**0.0002**	**0.0566**	**0**	**2005**	**Commercial**	**MA**
6a	**Sensitive**	**29**	**14.6 ± 4.6**	**0.0002**	**0.1618**	**0**	**2006**	**Commercial**	**MA**
7	Sensitive	30	23.2 ± 5.2	0.0242	0.586	0	2006	Commercial	VT
8	Sensitive	16	7.6 ± 4.5	0.0002	0.018	0	2007	Commercial	ME
9	Sensitive	30	26.1 ± 3.5	0.0002	0.3876	0	2005	Commercial	MA
10	Sensitive	30	2.8 ± 2.8	<0.0001	0.0001	0	2007	Baseline	NY
11	Sensitive	30	17.9 ± 4.3	0.0015	0.586	0	2007	Commercial	ME
12	**Sensitive**	**30**	**29.2 ± 4.0**	**0.0033**	**0.799**	**0**	**2005**	**Research**	**NY**
12a	**Sensitive**	**30**	**18.6 ± 5.6**	**0.0005**	**0.3876**	**0**	**2006**	**Research**	**NY**
12b	**Sensitive**	**30**	**18.6 ± 3.6**	**0.0007**	**0.2365**	**0**	**2007**	**Research**	**NY**
13	**Sensitive**	**30**	**11.3 ± 2.7**	**<0.0001**	**0.0165**	**0**	**2004**	**Baseline**	**NY**
13a	**Sensitive**	**30**	**1.67 ± 1.62**	**0.0004**	**0.586**	**0**	**2005**	**Baseline**	**NY**
13b	**Sensitive**	**32**	**0.0 ± 0.0**	**<0.0001**	**<0.0001**	**0**	**2006**	**Baseline**	**NY**
13c	**Sensitive**	**30**	**3.0 ± 1.8**	**<0.0001**	**0.0004**	**0**	**2007**	**Baseline**	**NY**
13d	**Sensitive**	**34**	**0.9 ± 0.5**	**<0.0001**	**<0.0001**	**0**	**2008**	**Baseline**	**NY**
13e	**Sensitive**	**22**	**0.1 ± 0.1**	**<0.0001**	**0.0003**	**0**	**2010**	**Baseline**	**NY**
13f	**Sensitive**	**25**	**4.51 ± 2.7**	**<0.0001**	**0.0021**	**0**	**2011**	**Baseline**	**NY**
14	Sensitive	36	8.2 ±	<0.0001	0.0008	0	2008	Commercial	RI

			2.2						
15	Sensitive	24	33.6 ± 5.2	0.0222	0.1379	0	2007	Commercial	MA
16	Sensitive	30	2.2 ± 1.7	<0.0001	0.0001	0	2007	Commercial	NH
17	Sensitive	19	35.5 ± 3.4	0.003	0.1207	0	2007	Commercial	NY
18	**Sensitive**	**30**	**27.6 ± 5.3**	**0.0366**	**0.9525**	**0**	**2005**	**Research**	**NY**
18a	**Sensitive**	**31**	**4.1 ± 2.0**	**<0.0001**	**0.0023**	**0**	**2006**	**Research**	**NY**
18b	**Sensitive**	**30**	**15.5 ± 3.6**	**0.0002**	**0.0354**	**0**	**2007**	**Research**	**NY**
18c	**Sensitive**	**32**	**0.5 ± 0.5**	**<0.0001**	**<0.0001**	**0**	**2009**	**Research**	**NY**
19	**Sensitive**	**21**	**10.6 ± 3.2**	**0.0002**	**0.0114**	**0**	**2008**	**Commercial**	**RI**
20	Sensitive	30	34.8 ± 4.9	0.0366	0.2365	0	2007	Commercial	RI
21	Sensitive	30	20.6 ± 3.3	<0.0001	0.1344	0	2006	Commercial	MA
22	Sensitive	32	4.4 ± 3.1	<0.0001	0.0003	0	2009	Commercial	*ME*
23	**Sensitive**	**30**	**12.2 ± 3.3**	**0.0005**	**0.0354**	**0**	**2007**	**Commercial**	**RI**
23a	**Sensitive**	**15**	**0.0 ± 0.0**	**<0.0001**	**0.0015**	**0**	**2009**	**Commercial**	**RI**
24	Sensitive	30	12.1 ± 5.4	<0.0001	0.0165	0	2006	Commercial	NY
25	Sensitive	22	25.13 ± 6.3	0.0674	0.5995	0	2009	Commercial	MI
26	**Sensitive**	**47**	**20.7 ± 3.5**	**0.0012**	**0.4843**	**0**	**2008**	**Commercial**	**WV**
26a	**Sensitive**	**39**	**18.1 ± 6.4**	**<0.0001**	**0.0177**	**0**	**2010**	**Commercial**	**WV**
27	Sensitive	30	33.1 ± 7.0	0.0242	0.0713	0	2006	Commercial	NY
28	Sensitive	33	7.8 ± 3.6	<0.0001	0.0036	0	2009	Commercial	NY
29	**Sensitive**	**30**	**5.0 ± 3.7**	**0.0354**	**0.004**	**0**	**2005**	**Baseline**	**NY**
29a	**Sensitive**	**30**	**5.1 ± 3.7**	**<0.0001**	**0.0004**	**0**	**2006**	**Baseline**	**NY**
30	**Sensitive**	**30**	**2.3 ± 1.2**	**<0.0001**	**0.0001**	**0**	**2007**	**Research**	**WV**
31	**Sensitive**	**30**	**33.0 ± 5.1**	**0.0657**	**0.2365**	**0**	**2005**	**Commercial**	**NY**
32	**Sensitive**	**30**	**5.9 ± 2.8**	**<0.0001**	**0.0029**	**0**	**2005**	**Commercial**	**MA**
32a	**Sensitive**	**30**	**7.6 ± 2.9**	**<0.0001**	**0.0165**	**0**	**2006**	**Commercial**	**MA**
33	**Sensitive**	**30**	**25.9 ± 4.4**	**0.0025**	**1**	**0**	**2005**	**Commercial**	**NY**
34	**Sensitive**	**36**	**0.1 ± 0.1**	**<0.0001**	**<0.0001**	**0**	**2009**	**Research**	**NY**
34a	**Sensitive**	**16**	**5.5 ± 5.1**	**<0.0001**	**0.0068**	**0**	**2011**	**Research**	**NY**
35	**Sensitive**	**30**	**16.2 ± 4.2**	**0.0005**	**0.1344**	**0**	**2006**	**Research**	**NY**
36	Sensitive	22	32.7 ±	0.0263	0.7232	0	2007	Commercial	NY

			5.3						
37	Sensitive	30	36.3 ± 4.3	0.0056	0.1494	0	2007	Commercial	MA
38	Sensitive	19	3.8 ± 3.9	<0.0001	0.0019	0	2005	Baseline	NY
39	**Sensitive**	**30**	**34.0 ± 7.4**	**0.0543**	**0.0713**	**0**	**2005**	**Commercial**	**WV**
39a	**Sensitive**	**30**	**11.3± 4.1**	**<0.0001**	**0.0204**	**0**	**2006**	**Commercial**	**WV**
40	Sensitive	16	18.0 ± 3.9	0.003	0.616	0	2004	Baseline	NY
41	Sensitive	30	20.5 ± 3.8	0.011	0.0713	0	2006	Commercial	WV
42	Sensitive	30	17.0 ± 6.5	<0.0001	0.1344	0	2005	Research	NY
43	Sensitive	30	2.7 ± 1.7	<0.0001	0.0001	0	2006	Commercial	VT
44	Sensitive	31	8.3 ± 3.8	<0.0001	0.0023	0	2005	Commercial	WV
45	Sensitive	30	28.9 ± 6.9	0.0657	0.586	0	2005	Research	NY
46	Sensitive	30	12.7 ± 3.9	0.0004	0.0354	0	2005	Commercial	OH
47	Sensitive	30	25.4 ± 9.9	<0.0001	0.0713	0	2007	Commercial	NY
48	**Sensitive**	**30**	**16.4 ± 4.4**	**0.0037**	**0.1344**	**0**	**2007**	**Commercial**	**RI**
49	Sensitive	27	0.5 ± 0.4	<0.0001	<0.0001	0	2009	Commercial	RI
50	Sensitive	30	14.0 ± 3.2	<0.0001	0.0354	0	2005	Research	OH
51	Sensitive	30	0.00 ± 0.0	<0.0001	<0.0001	0	2009	Commercial	NY
52	Sensitive	30	11.2 ± 4.2	<0.0001	0.0165	0	2005	Commercial	NH
53	Sensitive	27	4.4 ± 4.4	<0.0001	0.0005	0	2008	Research	PA
54	Sensitive	34	0.1 ± 0.1	<0.0001	<0.0001	0	2009	Baseline	IN
55	Sensitive	20	0.0 ± 0.0	<0.0001	0.0004	0	2009	Commercial	*ME*
56	Sensitive	26	19.6 ± 8.1	0.0072	0.1849	0	2006	Commercial	MA
57	Sensitive	30	8.4 ± 3.0	<0.0001	0.0165	0	2005	Commercial	WV
58	Sensitive	30	6.0 ± 2.4	<0.0001	0.0029	0	2006	Commercial	NY
59	**Sensitive**	**30**	**5.3 ± 2.2**	**<0.0001**	**<0.0001**	**0**	**2007**	**Baseline**	**NY**
59a	**Sensitive**	**34**	**0.6 ± 0.3**	**<0.0001**	**<0.0001**	**0**	**2008**	**Baseline**	**NY**
59b	**Sensitive**	**33**	**0.3 ± 0.3**	**<0.0001**	**0.0003**	**0**	**2009**	**Baseline**	**NY**
60	**Sensitive**	**30**	**27.7 ± 4.7**	**0.0061**	**0.586**	**0**	**2007**	**Commercial**	**MA**
60a	**Sensitive**	**34**	**3.4 ± 1.6**	**<0.0001**	**0.0001**	**0**	**2008**	**Commercial**	**MA**
61	Sensitive	27	12.6 ± 0.3	<0.0001	0.0448	0	2008	Commercial	RI
62	**Sensitive**	**29**	**0.00 ±**	**<0.0001**	**<0.0001**	**0**	**2009**	**Commercial**	**ME**

			0.0						
63	Sensitive	32	28.1 ± 4.0	0.0035	0.4994	0	2005	Commercial	OH
64	**Sensitive**	**30**	**8.5 ± 3.7**	**<0.0001**	**0.0165**	**0**	**2005**	**Research**	**NY**
64a	**Sensitive**	**30**	**3.7 ± 2.3**	**<0.0001**	**0.0008**	**0**	**2006**	**Research**	**NY**
64b	**Sensitive**	**30**	**4.1 ± 2.4**	**<0.0001**	**0.0011**	**0**	**2007**	**Research**	**NY**
65	Sensitive	41	1.6 ± 1.6	<0.0001	<0.0001	0	2009	Research	MI
66	Sensitive	30	15.0 ± 4.4	<0.0001	0.0703	0	2007	Commercial	RI
67	Sensitive	30	5.0 ± 2.2	<0.0001	0.0029	0	2006	Commercial	NY
68	Sensitive	24	20.2 ± 4.6	0.0013	0.2348	0	2005	Commercial	MA
69	Sensitive	20	20.7 ± 6.5	0.024	0.2904	0	2006	Commercial	MA
70	Sensitive	33	8.5 ± 3.1	<0.0001	0.0096	0	2009	Research	NY
71	Sensitive	29	35.5 ± 7.3	0.0145	0.2775	0	2007	Commercial	NY
72	Sensitive	30	34.0 ± 3.7	0.0048	0.2365	0	2005	Commercial	WV
73	Sensitive	30	23.5 ± 4.6	0.0048	0.799	0	2005	Commercial	WV
74	Sensitive	29	23. 0 ± 3.9	0.0006	0.8397	0	2006	Commercial	RI
75	Sensitive	30	31.7 ± 4.9	0.0269	0.9525	0	2006	Commercial	MA
76	Sensitive	30	31.8 ± 9.1	0.0598	0.586	0	2005	Commercial	WI
77	Sensitive	33	19.9 ± 3.1	<0.0001	0.1737	0	2004	Commercial	NY
78	Sensitive	21	18.6 ± 5.3	0.0164	0.7232	0	2010	Research	ME
79	Sensitive	26	17.86 ± 4.9	0.0052	0.3752	2.5	2011	Commercial	ME
80	**Sensitive**	**25**	**0.316 ± 0.31**	**<0.0001**	**<0.0001**	**4**	**2011**	**Commercial**	**VT**
81	Sensitive	24	32.70 ±3.8	0.0133	0.2656	4.76	2011	Commercial	ME
12c	**Sensitive**	**32**	**6.3 ± 3.6**	**<0.0001**	**0.0009**	**9.68**	**2009**	**Research**	**NY**
82	Sensitive	38	11.1 ± 4.0	<0.0001	0.0013	10.81	2008	Commercial	RI
83	Sensitive	16	2.39 ± 2	<0.0001	0.0048	14.15	2011	Commercial	NC
12d	**Sensitive**	**50**	**12.7 ± 4.3**	**<0.0001**	**0.0007**	**14.29**	**2008**	**Research**	**NY**
5a	**Sensitive**	**26**	**10.4 ± 3.6**	**<0.0001**	**0.0101**	**18.18**	**2011**	**Baseline**	**NY**
84	Resistant	30	67.6 ± 4.9	0.0088	<0.0001	0	2005	Commercial	NY
85	Resistant	31	75.6 ± 3.3	0.0001	<0.0001	0	2005	Commercial	NY
86	Resistant	30	43.2 ± 7.1	0.8005	0.0165	0	2006	Commercial	NY
87	Resistant	30	44.2 ±	0.1225	0.0129	0	2007	Commercial	NY

			6.1						
88	Resistant	30	50.1 ± 5.2	0.1032	0.0165	0	2007	Commercial	NH
89	Resistant	30	51.8 ± 8.6	0.8005	0.0029	0	2006	Commercial	NY
90	Resistant	30	71.0 ± 5.1	0.0033	<0.0001	0	2007	Commercial	NY
91	Resistant	31	44.1 ± 5.5	0.6083	0.0855	0	2005	Commercial	OH
92	Resistant	30	51.6 ± 5.3	0.2145	0.0165	0	2007	Commercial	RI
93	Resistant	30	54.5 ± 3.9	0.0217	0.0072	0	2007	Commercial	NY
94	Resistant	30	45.0 ± 4.1	0.0543	0.0165	0	2007	Commercial	CT
33a	**Resistant**	**30**	**76.7 ± 3.9**	**0.0002**	**<0.0001**	**0**	**2007**	**Commercial**	**NY**
95	Resistant	30	57.1 ± 7.8	0.1988	0.0011	0	2006	Commercial	NY
96	Resistant	30	67.9 ± 5.4	0.0111	<0.0001	0	2005	Commercial	NY
97	Resistant	31	41.0 ± 4.1	0.0493	0.1344	0	2005	Commercial	OH
98	Resistant	30	49.7 ± 6.9	0.2145	0.0713	0	2007	Commercial	MA
99	Resistant	30	78.8 ± 5.4	0.017	<0.0001	0	2005	Commercial	NY
100	Resistant	30	45.2 ± 3.0	0.0147	0.0032	0	2007	Commercial	RI
81a	**Resistant**	**30**	**46.7 ± 4.0**	**0.1333**	**0.0354**	**0**	**2007**	**Commercial**	**VT**
101	Resistant	30	48.4 ± 5.5	0.1032	0.0072	0	2004	Commercial	NY
102	Resistant	30	47.4 ± 6.9	0.4249	0.0354	0	2007	Commercial	NY
103	Resistant	30	70.9 ± 4.4	0.0019	<0.0001	0	2005	Commercial	NY
104	Resistant	30	46.3 ± 5.3	0.0269	0.0165	14.29	2007	Commercial	NY
105	Resistant	19	41.8 ± 9.6	0.6632	0.0546	26.92	2010	Commercial	WV
106	Resistant	20	31.64 ± 7	0.2391	0.5121	26.92	2011	Commercial	ME
107	Resistant	32	21.3± 5.6	0.0001	0.086	28.57	2009	Commercial	MI
81b	**Resistant**	**35**	**25.6 ± 6.1**	**0.0012**	**0.3934**	**29.41**	**2008**	**Commercial**	**VT**
108	Resistant	20	50.95 ± 58.87	0.285	0.0107	30.77	2009	Commercial	MI
48a	**Resistant**	**31**	**28.8 ± 4.9**	**0.0125**	**0.9525**	**34.48**	**2008**	**Commercial**	**RI**
109	Resistant	15	10.73 ± 6.1	0.0007	0.0815	35.71	2011	Commercial	ME
62a	**Resistant**	**24**	**12.03 ± 5.8**	**<0.0001**	**0.0134**	**36.67**	**2011**	**Commercial**	**ME**
110	Resistant	15	94.19 ±4.9	0.0001	<0.0001	40	2011	Research	VT
111	Resistant	24	19.8 ± 27.7	<0.0001	0.0466	41.6	2011	Commercial	ME

31a	**Resistant**	**17**	**68.7 ± 13.0**	**0.2329**	**0.003**	**47.06**	**2008**	**Commercial**	**NY**
112	Resistant	25	58.4 ± 12.7	0.27	0.002	52.17	2008	Commercial	NY
113	**Resistant**	**34**	**54.7 ± 7.2**	**0.1799**	**0.0033**	**64.29**	**2010**	**Commercial**	**NY**
114	Resistant	35	67.7 ± 9.4	0.0002	<0.0001	64.52	2008	Commercial	NY
115	Resistant	18	3.04 ± 2.2	<0.0001	0.0035	75	2011	Research	VA
116	Resistant	21	69.92 ± 7.2	0.0449	0.0001	87.5	2011	Commercial	MA
117	**Resistant**	**21**	**100.35 ± 3.2**	**<0.0001**	**<0.0001**	**95.83**	**2011**	**Commercial**	**NY**
19a	**Reduced Sensitive**	**30**	**39.8 ± 3.0**	**0.0088**	**0.0165**	**0**	**2007**	**Commercial**	**RI**
30a	**Reduced Sensitive**	**30**	**33.9 ± 6.6**	**0.0016**	**0.0308**	**0**	**2006**	**Research**	**WV**
118	Reduced Sensitive	28	39.1 ± 3.6	0.0118	0.0428	0	2006	Commercial	MA
35a	**Reduced Sensitive**	**30**	**34.3 ± 4.1**	**0.0088**	**0.2365**	**0**	**2004**	**Research**	**NY**
119	Reduced Sensitive	30	39.2 ± 3.6	0.0366	0.0165	0	2007	Commercial	NY
120	Reduced Sensitive	30	35.3 ± 4.4	0.3074	0.1344	0	2005	Commercial	NY
18d	**Reduced Sensitive**	**25**	**36.3 ± 11.6**	**0.2404**	**0.5774**	**6.67**	**2010**	**Research**	**NY**

[a] The mean percent relative growth at 0.02 µg/ml trifloxstrobin for an orchard population.

[b] Orchard classification was determined by comparing the distribution of quantitative QoI resistant phenotypes for a given *V. inaequalis* orchard population to standards for QoI resistant and sensitive *V. inaequalis* orchard populations using a Kolmogorov-Smirnov test in SAS (Version 9.3, SAS Institute, Cary, NC)

[c] Baseline orchards are defined as orchard plantings never having exposure to site-specific fungicide chemistries (e.g. anilinopyrimidines, DMIs, or QoIs)

Table 3: List of *V. inaequalis* clonal conidial isolates used for the stability of the *cyt b* G143A mutation assay.

Isolate	Classification [a]	Mean %RG [b]	*cyt b* genotype [c]	Origin
11.1.11	Resistant	132.65	A143	Montague, MA
11.25.11	Resistant	132.65	A143	Montague, MA
5.44.11	Resistant	124.23	A143	Newfield, NY
5.40.11	Resistant	108.49	A143	Newfield, NY
14.4.10	Resistant	108.49	A143	Geneva, NY
11.13.11	Resistant	100.63	A143	Montague, MA
12.19.10	Resistant	100.63	A143	Geneva, NY
6.47.10	Resistant	92.81	A143	Newfield, NY
8.33.10	Resistant	90.47	A143	Geneva, NY
6.40.10	Resistant	90.18	A143	Newfield, NY
11.11.11	Resistant	83.88	A143	Montague, MA
11.45.10	Resistant	83.88	A143	Geneva, NY
11.17.11	Resistant	72.8	A143	Montague, MA
11.20.11	Resistant	66.5	A143	Montague, MA
41.3.10	Resistant	54.26	A143	Geneva, NY
2.7.10	Resistant	51.69	A143	Shepherdstown, WV
41.11.10	Resistant	48.51	A143	Geneva, NY
27.35.10	Resistant	45.54	A143	Geneva, NY
28.24.11	Resistant	26.49	A143	Buckfield, ME
37.22.10	Sensitive	52.47	G143	Sanford, ME

11.22.11	Sensitive	46.93	G143	Montague, MA
25.6.11	Sensitive	46.93	G143	Manchester, ME
11.8.11	Sensitive	45.54	G143	Montague, MA
28.17.11	Sensitive	41.46	G143	Buckfield, ME
3.35.10	Baseline	0	G143	Geneva, NY
32.11.11	Baseline	0	G143	Geneva, NY
32.42.11	Baseline	0	G143	Geneva, NY

[a] Classification was determined by whether or not the isolate had more than 48% RG and grew on trifloxystrobin (0.2 µg/ml) and sham (100 µg/ml)-amended medium.

[b] The mean percent relative growth at 0.02 µg/ml trifloxstrobin for an orchard population.

[c] At postion 143 in the cyt b gene of *Venturia inaequalis*, A indicates resistant (A143) allele and G indicates sensitive (G143) allele

FIGURES

Figure 6

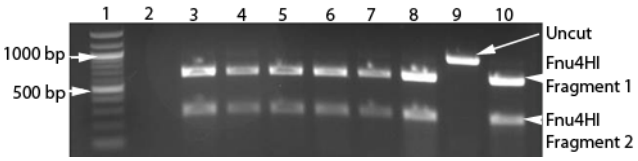

Figure 6: A Fnu4HI digestion of the 950 bp DNA fragment of the *V. inaequalis cytochrome b* generated by PCR using primer pair 6017R and 5F containing codon 143. The QoI resistant isolates (lanes 3-8 and 10) produce two fragments of approximately 600 and 350 bp in length. The QoI sensitive isolate (lane 9) is not affected by Fnu4HI digestion, and has a single uncut fragment (950 bp). Lane 1 has the 1 kb DNA ladder (New England Biolabs, Ipswich, MA).

Figure 7

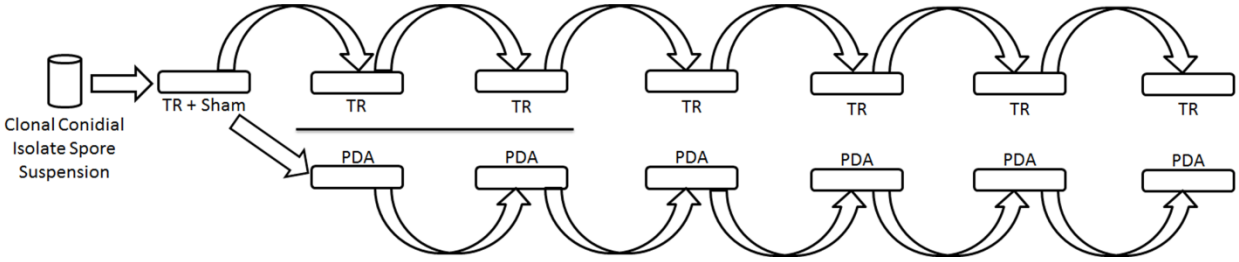

Figure 7: Flowchart showing the assay designed to evaluate the stability of the G143A mutation for a single isolate. A 100 µL aliquot of clonal conidial suspension was placed on trifloxystrobin (0.2 µg/ml) and salicyl hydroxamic acid (100 µg/ml) (TR+Sham) amended medium. Single colonies were transferred every 5 weeks (curved arrows) for 6 months to impose constant QoI selective pressure or no QoI selective pressure. Qualitative resistance for resulting colonies on both TR (trifloxystrobin, 0.02 µg/ml) and PDA (Un-amended potato dextrose agar) medium (rectangles) was determined by PCR and RFLP analysis.

Figure 8

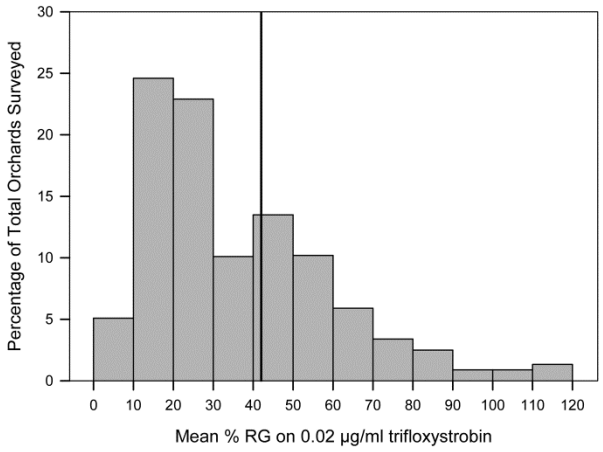

Figure 8: Distribution of mean relative growth values at 0.02 µg/ml trifloxystrobin in orchard populations of *V. inaequalis* sampled from 2004-2011. The bar denotes a suggested threshold for orchards with quantitative QoI resistance (41% RG), which was positioned so that no sensitive or reduced sensitive orchards as determined the Kolmogorov-Smirnov test would be positioned above that point.

CHAPTER 5

CONCLUSIONS

In the northeastern United States, apple scab caused by *Venturia inaequalis* continues to be a problem in apple production (Merwin et al, 1994). In addition to disease management, growers must be conscientious of the development of fungicide resistance in orchard populations if site specific fungicides such as DMI and QoI fungicides are employed.

Demethylation inhibitor (DMI) fungicides have been used in apple production for more than 30 years (Köller et al, 1997, Gilpatrick, 1982). However, repeated applications of these fungicides can create stable, incremental adjustments that lead to DMI resistance in population members (Ishii et al, 2006, Ma et al, 2005). Reduced sensitivity to DMIs in *V. inaequalis* populations in the Northeastern US was observed as early as 1985 (Stanis et al, 1985), and continue to be a problem in orchard populations in the Great Lakes region (Chapman et al, 2011). Two studies were performed to determine the implications of shifts in production practices regarding dormant chemical management or summer difenoconazole fungicide applications on the DMI sensitivity in *V. inaequalis* orchard populations in the northeastern United States. The study presented in chapter 2 focuses on the implications of delayed dormant metal cation applications on the DMI sensitivity of *V. inaequalis* in an orchard population that is already DMI resistant. In addition to formulated copper products, our study incorporates a novel cation product, manganese. Understanding the relationship between chemical treatment and the sensitivity of *V. inaequalis* populations to various fungicide chemistries throughout the production season will be crucial for the identification and future implementation of effective

resistance management strategies. This also indicates that these findings are of interest to the scientific community because of the potential for dormant chemical management to affect the persistence of DMI fungicides. Amongst *V. inaequalis* isolates that survived copper treatment, we observed an increase in DMI sensitivity in two consecutive years of trials. Evaluation of novel metal cations could lead to sparked interest in these products and perhaps the release of novel cation chemistries aimed at reducing overwintering inoculum, but may have less environmental impact that copper products are known to have. Our research on one example, manganese, showed an inconsistent effect in increasing DMI sensitivity of treated *V. inaequalis* isolates.

The second study (chapter 3) examining the effect or orchard practices on DMI resistance was performed to determine the implications of shifts in production practices regarding the use of difenoconazole fungicide applications to the summer season on the DMI sensitivity in *V. inaequalis* orchard populations. Recently, DMI fungicides such as difenoconazole have been labeled for use in summer fungal disease management of apple (http://128.253.223.36/ppds/525998.pdf). However, it is not currently known what the full effect of changing applications to different times in the season will have on DMI sensitivity of *V. inaequalis*. The conclusions from this study indicate that using difenoconazole throughout the production season dose not significantly increase DMI resistance in *V. inaequalis* populations compared to untreated populations, which suggests that difenoconazole use during the summer season will not predispose *V. inaequalis* populations to DMI resistance any more so than difenoconazole use during the primary apple scab season. We have also demonstrated that the development of resistance to difenoconazole in a *V. inaequalis* population did not result in corresponding insensitivity to another DMI fungicidem, myclobutanil. Our findings provide

insight on factors influencing DMI resistance in an ascomycete pathosystem, which could drive the development of novel control strategies for apple diseases in the context of DMI resistance management.

A final and third study (Chapter 4) was implemented to determine the prevalence of quantitative and qualitative QoI resistance in *V. inaequalis* orchard populations in the northeastern United States. This study also set out to address the stability of qualitative resistance in the absence of selective pressures created by QoI fungicide applications, although that was not conclusively determined. Knowledge gained from these studies will directly answer stakeholder questions on modifications that can be made to management paradigms to ensure proper *V. inaequalis* disease management through careful fungicide selection to avoid resistance selection. Overall, the results of this study suggest that orchard populations with quantitative and qualitative resistance to QoI fungicides are present in the northeastern United States. Many apple producers in the region can still use QoI fungicides to great effect on *V. inaequalis* orchard populations, but care should be given in using them because of the propensity for resistance (Lesniak et al, 2011). The distribution of quantitative phenotypes in several orchard populations alone suggested that these populations may have practical resistance to trifloxystrobin. Köller et al. (2004) suggested that QoI resistance management for *V. inaequalis* could be accomplished by limiting the number of QoI applications made seasonally. Such use could slow the selection of population members with qualitative resistance, which would be important since we have demonstrated that qualitative QoI resistance is stable in isolates beyond several transfers. This study contributes to the knowledge of the development and persistence of QoI resistance. Although the stability of qualitative QoI resistance was not fully elucidated beyond 6 transfers,

this study contributes to the understanding of that stability, which has been called for in the literature (Lesniak et al, 2011).

In short, conclusions from each chapter impact management paradigms on DMI sensitivity or the prevalence of QoI resistance, and are pertinent to those interested in fungicide resistance management by demonstrating important factors for DMI resistance in orchard trials and the stability of qualitative QoI resistance *in vitro*. Given the importance of apple scab to regional apple production, as well as the problem with fungicide resistance, investigations are warranted to determine how disease management practices affect fungicide resistance in *V. inaequalis*.

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