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STATUS OF RESISTANCE TO STEROL-DEMETHYLATION INHIBITING FUNGICIDES IN POPULATIONS OF *VENTURIA INAEQUALIS* FROM PENNSYLVANIA APPLE ORCHARDS

A Thesis in

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ABSTRACT

Apple scab, caused by *Venturia inaequalis*, is the most economically important fungal disease of apple in the eastern United States. Over the past 25 years, apple growers have relied on sterol demethylation inhibiting (DMI) fungicides for scab control, but reduced efficacy has recently been noted. The aim of this study was to evaluate the sensitivity of *V. inaequalis* isolates from Pennsylvania to DMI fungicides. In 2008 and 2009, leaves and immature fruit with scab symptoms were collected from 20 commercial orchards. Growers provided management history of the sampled plots. A total of 479 single-spore cultures were isolated from the tissues and maintained individually. Each isolate was tested for sensitivity to DMI fungicides on 1/4-strength PDA plates amended with a range of concentrations of myclobutanil, fenbuconazole, or difenoconazole. Mean effective concentration for 50% inhibition (EC_{50}) values of each fungicide were calculated for populations from 14 orchards. Relative growth (RG) values were calculated and isolates with RG > 75 on plates amended with 0.5 μg/mL, were scored as resistant to the particular fungicide. Widespread shifts toward resistance to the three DMI fungicides were noted with EC_{50} values of 2.139 ± 0.090 (mean ± standard error), 0.839 ± 0.053 and 0.158 ± 0.008, for myclobutanil, fenbuconazole, and difenoconazole, respectively. Based on a 0.5 μg/mL threshold, 64% of isolates were resistant to myclobutanil, and 24% were resistant to fenbuconazole. Less than 1% of the isolates were cross-resistant to all three fungicides, but 22% were cross-resistant to myclobutanil and fenbuconazole. Failure to use dormant copper sprays, older trees, larger orchards, orchards comprised of ten or more cultivars, mixed-cultivar orchards, and isolates from

the ‘York Imperial’ cultivar were positively correlated ($0.0001 < P < 0.05$) with the incidence of resistant isolates. Isolates from orchards that were not treated with copper were twice as likely to be resistant to myclobutanil (odds ratio = 2.015; $P = 0.0009$). Growers can focus on using management practices, particularly dormant copper sprays, to reduce their risk of developing resistance to DMI fungicides.

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Chapter 1

Introduction

Apple scab, caused by *Venturia inaequalis* (Cooke) G. Wint., is one of the most economically damaging diseases of apple, the most important fruit crop in the northeastern United States. While apple scab has been recognized for over 400 years, control is difficult and scab epidemics are a problem for many of today's growers (MacHardy et al., 2001; Carisse and Dewdney, 2002). Apple scab displays superficial lesions on fruit and leaves and scabbed apples are adequate for consumption, however, the aesthetic value of the fruit is reduced such that the crop can only be sold for juice, cider, or applesauce. A bushel of scabbed apples, sold for processing, garners as little as 10% of the value of the same bushel of intact fresh-market apples (J. Travis, personal communication).

Control of apple scab consists of two main strategies; use of cultural control methods and application of chemical fungicides. Fungicides may be classified either as protectants or eradicants, and are an essential part of commercial apple production. Protectants include copper and sulfur, captan and mancozeb, and benzimidizoles. Copper compounds, captan, and mancozeb have multiple modes of action; one of which is to react with sulfhydryl groups (-SH) in certain amino acids, disrupting protein conformations and inhibiting the efficacy of critical fungal enzymes (Leroux, 1996; Russell, 2005). Sulfur acts to disrupt electron transport chains in fungi, among other modes of action (Leroux, 1996). Benzimidazole fungicides are systemic protectants that

interfere with fungal microtubule movement, disrupting nuclear division (Lalancette et al., 1987; Russell, 2005). Strobilurins, which are considered to have protectant, systemic, and eradicant action, work by binding the quinone outer (Qo) site on cytochrome B, effectively inhibiting fungal cellular respiration (Russell, 2005). Sterol-demethylation inhibiting fungicides (DMIs) are systemic protectants with curative action against fungal pathogens (Ngugi, 2006; Russell, 2005).

DMI fungicides prevent the synthesis of ergosterol, the critical sterol of the fungal cell wall. DMI fungicides are arguably the most effective fungicides in use against apple scab because they are particularly safe, were extremely effective at their outset, and have broad spectrum uses (Russell, 2005). As a class, DMIs move translaminarly in plant tissue, which facilitates their effectiveness on diseases that occur on the underside of leaves. Translaminar movement is characterized by local movement, such as from the upper leaf surface, through leaf tissue, to the lower leaf surface. DMIs are also effective post-infection, which allows growers to apply the products up to 96 hours after an infection period (PA Tree Fruit Production Guide, 2009). This allows for longer intervals between sprays and more flexibility in relation to the control of other diseases (Ngugi, 2006). Growers have come to rely on DMI fungicides as part of their disease management programs.

The first publication detailing practical apple scab resistance to DMI fungicides was in Michigan in 1997 (Koller et al., 1997), yet scientists were reporting populations with reduced sensitivity to DMIs in Germany, the Netherlands, and Italy as early as 1985 (Fiaccadori et al., 1987; Stanis and Jones, 1985). Since then, varying levels of resistance to DMI fungicides have developed in *V. inaequalis* populations around the world, ranging

from mild shifts in sensitivity to near-complete resistance (Errampalli, 2004; Fiaccadori et al., 1987; Gao et al., 2009; Koller et al., 1997; Koller and Wilcox, 2001; Kunz et al., 1997; MacHardy, 1996; Marine et al., 2007; Schnabel and Jones, 2001; Stanis and Jones, 1985; Xu et al., 2010). DMI fungicides possess a single mode of action: the azole ring of the active ingredient interferes with the heme group of the fungal enzyme P450 14-α-demethylase. This enzyme performs three successive hydroxylation reactions on eburicol, a precursor for ergosterol. Without these hydroxylation reactions, ergosterol does not form the appropriate molecular conformations in the fungal cell membrane and fluid regulation in the cell is disabled (Joseph-Horne and Holloman, 1997). Because DMI fungicides have a single-site mode of action, there is high potential for *V. inaequalis* to develop resistance to the fungicides.

Although the sensitivity to DMI fungicides in populations of *V. inaequalis* has been characterized in other major apple production states in the eastern US (Koller et al., 1997; Kunz et al., 1997; Koller and Wilcox, 2001; Marine et al., 2007), the status in Pennsylvania has never been investigated. The overall goals of this study were to assess and document the prevalence of *V. inaequalis* resistance to commonly used DMI fungicides in Pennsylvania apple orchards, and to identify orchard management practices that predispose *V. inaequalis* populations to developing resistance. The specific objectives of the study were to:

1. determine the prevalence and incidence of resistance to the DMI fungicides myclobutanil, difenoconazole, and fenbuconazole in populations of *V. inaequalis* from Pennsylvania apple orchards;

2. determine the frequency of *V. inaequalis* isolates that are resistant to more than one DMI fungicide; and

3. identify apple production-related factors that contribute to the development of resistance to DMI fungicides in *V. inaequalis* populations.

Chapter 2

Literature Review

2.1. Disease cycle of apple scab

V. inaequalis is pathogenic on certain rosaceous species, including domesticated apple (*Malus x domestica* Borkh.), various crabapple species (*Malus* spp.), hawthorn (*Crataegus* spp.), firethorn (*Pyracantha* spp.), and mountain ash (*Sorbus* spp.) (MacHardy, 1996). The pathogen is a cosmopolitan hemibiotrophic ascomycete that undergoes a single primary and multiple secondary reproduction cycles each year, infecting leaves and fruit of susceptible hosts. With the exception of overwintering conidia on budscales (Carisse and Dewdney, 2002; Gao et al., 2009), *V. inaequalis* commonly overwinters in leaf litter, during which mating occurs between individuals of different mating types (MacHardy, 1996). In the spring, ascospores resulting from sexual recombination are forcibly discharged from pseudothecia, just as host foliar tissue is most susceptible. This primarily occurs during or following a wetting event, with most ascospores trapped during the day (MacHardy and Gadoury, 1989). For primary infections to occur, ascospores require temperatures roughly between 5°C and 26°C and at least 6 hours of continuous wetness to complete germination. Thus, the development of apple scab is favored by temperate climates with wet spring seasons. Infection prediction models, such as the Mills Curves, have been devised to aid growers in timing their control strategies (MacHardy and Gadoury, 1989), and infections may occur on both the

abaxial and adaxial leaf surfaces, in addition to the surface of young fruit (Carisse and Dewdney, 2002).

Fungal hyphae grow beneath the cuticle post-infection, forming a stroma, with conidiophores bursting through the surface to disseminate conidia, the asexual spores. Fungal lesions are brown to olive green, generally occurring in rough circles (Carisse and Dewdney, 2002). Conidial production and ensuing secondary infection occurs numerous times over the summer, requiring similar climatic conditions as primary infection; however, secondary infections are largely restricted to the tree that sustained the primary infection (MacHardy and Gadoury, 1989; MacHardy et al., 2001). In severe cases or when lesions develop on the petiole, apple scab can result in chlorosis and defoliation (Carisse and Dewdney, 2002). *V. inaequalis,* enters its saprophytic stage in the fall, only penetrating the host mesophyll cells after leaf abscision. In the litter, sexual structures are formed and mating takes place to produce ascospores, which constitute the primary inoculum for the following year (MacHardy et al., 2001).

2.2 Apple Scab Control

Control of apple scab around the world generally consists of two main strategies; use of cultural control methods and application of chemical fungicides. Cultural control of apple scab includes planting a mixture of cultivars, including resistant selections (Didelot et al., 2007), establishing and maintaining distance for appropriate air flow between trees, and using various tactics to promote breakdown of leaf debris (Carisse and Dewdney, 2002). Several research programs centered on developing scab-resistant apple cultivars began their work earlier than the 1970s (Carisse and Dewdney, 2002; Janick,

2006). Quantitative scab resistance genes are present in wild crabapple relatives and notably in the 'Golden Delicious' cultivar and some of its progeny (Bus et al., 2009; Parisi et al., 1993). However, *V. inaequalis* populations have already shown the ability to gradually overcome apple resistance genes, due to high diversity and a yearly sexual cycle (Parisi et al., 1993). Planting cultivars in mixtures that include resistant varieties has been shown to significantly reduce incidence and severity of apple scab, however, this was achieved while using a moderate fungicide schedule (Didelot et al., 2007).

Proper planting distance and canopy pruning allows for better fungicide applicator coverage and shortens periods of leaf wetness, critical steps in preventing *V. inaequalis* infections. Litter infestation with earthworms, compost tea amendments, urea applications, and leaf shredding help leaf litter break down more quickly, destroying the substrate on which *V. inaequalis* mates and produces primary inoculum (Carisse and Dewdney, 2002; Bus et al., 2009). However, most cultural control measures are minimally used by commercial farmers, because none confer complete disease control and the economic benefits are difficult to quantify (MacHardy, 1996).

In commercial apple production, fungicides are by far the most important part of scab control programs. Copper or sulfur compounds have been used in the treatment of apple scab since the early 1900s, and may be amended with small amounts of other fungitoxic ions, such as zinc. However, coppers are known to induce phytotoxicity, sometimes even at low levels. Copper has several modes of action, including the interaction of the Cu^{2+} ion with sulfhydryl groups (-SH) in certain fungal amino acids, disrupting protein conformations and inhibiting the efficacy of critical fungal enzymes (Leroux, 1996; Russell, 2005; PA Tree Fruit Production Guide, 2009). Coppers may be

"fixed" with sulfides, yet sulfur applications are also independently effective in fungal disease management. Sulfur also has several modes of action, including the interruption of electron transport chains in fungi (Leroux, 1996). A number of other formulations, marketed by chemical companies, exist to control apple scab as well.

Other chemical fungicides fall into one of two categories, protectants or eradicants. Like copper, contact protectants like mancozeb and captan inactivate sulfhydryl groups, in addition to possessing other modes of action (Leroux, 1996; Russell, 2005). Contact protectants can induce phytotoxicity, but this risk is much lower than the phytotoxicity risk from copper. Captan may be used throughout the apple production season, while mancozeb is subject to strict EPA restrictions and can only be used before bloom (PA Tree Fruit Production Guide, 2009). Phytotoxicity can be reduced by amending with ions, such as zinc (Russell, 2005). Benzimidazole fungicides are systemic protectants that interfere with nuclear division in fungi. *V. inaequalis* populations developed resistance to these fungicides about twenty years after benzimidazoles were put into use (Lalancette et al., 1987; Russell, 2005). Strobilurins, which are considered to have protectant, systemic, and eradicant action, work by binding the quinone outer (Qo) site on cytochrome B, and inhibiting fungal cellular respiration (Russell, 2005). *V. inaequalis* resistance to these fungicides has been found in Europe and the United States, though not to the extent of resistance to DMI fungicides (Koller et al., 2004; Russell, 2005; Steinfeld et al., 2002).

2.3 Information on DMI fungicides

Sterol-demethylation inhibiting fungicides (DMIs) are systemic protectants with curative action against fungal pathogens (Ngugi, 2006; Russell, 2005). DMI fungicides prevent the synthesis of ergosterol, the critical sterol of the fungal cell wall. DMIs are arguably the best fungicides in use against apple scab because they are touted as having few nontarget effects, were extremely effective at their outset, and have broad spectrum uses (Iris et al., 1993; Russell, 2005; PA Tree Fruit Production Guide, 2009). As a class, DMI fungicides move translaminarly through leaves, providing locally systemic protection on both sides of the leaf surface. This is particularly important in managing fungal diseases, such as apple scab and powdery mildew, which occur on either side of the leaf surface. DMI fungicides are also effective post-infection, which allows growers to apply the products up to 96 hours after an infection period. This allows longer intervals between sprays and more flexibility in relation to the control of other diseases (Ngugi, 2006).

Unfortunately, DMI fungicides are prone to the development of resistance in target pathogens. As few as six years after their introduction in apple farming in the 1980s, orchards that had been using DMI fungicides appeared to have reduced control of the disease (Stanis and Jones, 1985; Fiaccadori et al., 1987). The first publications detailing practical apple scab resistance to DMIs were in Michigan and Switzerland in 1997 (Koller et al., 1997; Kunz et al., 1997), yet scientists were reporting populations with reduced sensitivity to DMI fungicides in Germany as early as 1985 (Stanis and Jones, 1985). Since then, varying levels of resistance have developed to DMI fungicides in *V. inaequalis* populations around the world, ranging from mild shifts in sensitivity to

near-complete resistance (Errampalli, 2004; Fiaccadori et al., 1987; Gao et al., 2009; Jobin and Carisse, 2007; Koller et al., 1997; Kunz et al., 1997; MacHardy, 1996; Marine et al., 2007; Stanis and Jones, 1985; Xu et al., 2010). Several studies have calculated the EC_{50} values from orchard populations treated with DMI fungicides as eight times higher than the EC_{50} values from untreated populations (Stanis and Jones, 1985; Errampalli, 2004).

Sterol-demethylation inhibiting fungicides were labeled for agricultural use on United States beginning in the 1980s. Myclobutanil (Nova 40W / Rally 40W, Dow Agrosciences) was registered in 1989, and fenbuconazole (Indar 75 DSW; Dow Agrosciences) and difenoconazole (Inspire Super MP; Syngenta) were registered in 1995 and 2007, respectively (Koller et al., 2005; NPIRS Public, 2010). Myclobutanil has been used on apples since its registration in the 1980s, however, fenbuconazole and difenoconazole were only registered for apples in 2007 and 2008, respectively (PA Tree Fruit Production Guide, 2009). Since myclobutanil has been used for such an extensive amount of time, greater resistance to this active ingredient is expected. Some scientists have investigated the possibility of cross resistance within the DMI class of fungicides among *V. inaequalis* isolates with orchard-specific results (Xu et al., 2010), while other research found the incidence of *V. inaequalis* cross-resistance between DMIs and other fungicides (Koller and Wilcox, 2001). The former study also suggests the likelihood of resistance to DMI fungicides is increased if existing *V. inaequalis* populations are resistant to products with modes of action other than 14-α-demethylase inhibition (Koller and Wilcox, 2001). A number of other fungal plant pathogens have confirmed resistance to DMIs, including *Aspergillus nidulans* (Del Sorbo et al., 1997), *Erysiphe graminis*

(Delye et al., 1997a), *Uncinula necator* (Delye et al., 1997b), *Sclerotinia homeocarpa* (Hsiang et al., 1997), *Cercospora beticola* (Karaoglanidis and Thanassoulopoulos, 2003), and *Blumeriella jaapii* (Wyand and Brown, 2005).

DMI fungicides possess a single mode of action: the azole ring of the active ingredient interferes with the heme group of the fungal enzyme P450 14-α-demethylase. This enzyme performs three successive hydroxylation reactions on eburicol, a precursor for ergosterol. Without these hydroxylation reactions, ergosterol does not form the appropriate molecular conformations in the fungal cell membrane and fluid regulation in the cell is disabled (Joseph-Horne and Holloman, 1997). Most DMIs have different active ingredients, but each active ingredient performs a similar action on the demethylase. In addition, some fungicides with the same name have several different formulations and inactive ingredients, which could enhance efficacy in the field. A number of possibilities exist in terms of how *V. inaequalis* actually overcomes DMI fungicides' effects.

2.4 Possibilities for determinants of resistance to DMI fungicides

Overexpression of the *CYP51A1* gene, which is involved in the synthesis of P450 14-α-demethylase, has been shown to be related to resistance to myclobutanil in *V. inaequalis* (Schnabel and Jones, 2001). In that study, the *CYP51A1* genes of resistant and sensitive isolates were sequenced and the resulting nucleotide sequences were identical, although the authors did document a 553-bp insertion upstream of some isolates' demethylase gene. This insertion was present in a large proportion of the isolates resistant to myclobutanil, however, some tested isolates demonstrated resistance without carrying it (Schnabel and Jones, 2001).

Studies on related fungi could help elucidate how *V. inaequalis* achieves its resistance against DMI action. Three studies suggest that point mutations in *CYP51* may confer resistance to DMI fungicides in the powdery mildew pathogens, *Erysiphe necator* and *E. graminis,* as well as the cherry leaf spot pathogen, *Blumeriella jaapii* (Delye et al., 1997a; Delye et al., 1997b; Wyand and Brown, 2005). This point mutation changes a single amino acid in 14-α-demethylase from phenylalanine to tyrosine, though most authors suggest this single change is not the only mechanism for resistance to DMI fungicides (Delye et al., 1997a; Delye et al., 1997b; Gisi et al., 2000; Wyand and Brown, 2005).

ATP-binding cassettes have been detected in multi-drug resistant *Aspergillus nidulans* isolates (Del Sorbo et al., 1997), so efflux mechanisms present another possibility for the determinant of *V. inaequalis* resistance to DMI fungicides. ATP-binding cassettes are membrane transporters hypothesized to pump toxic substances outside the fungal cell (Del Sorbo et al., 1997; Gisi et al., 2000). Since no definitive study has proven a single genetic origin, most agree that populations resistant to DMI fungicides have multiple mechanisms and develop resistance quantitatively over an extended period of time (Delye et al., 1997a; Delye et al., 1997b; Gisi et al., 2000; Jobin and Carisse, 2007; Joseph-Horne and Holloman, 1997; Koller et al., 1997; Schnabel and Jones, 2001; Wyand and Brown, 2005).

2.5 Previous studies on *Venturia inaequalis* resistance to DMI fungicides

The severity of resistance to DMI fungicides may be measured in an EC_{50} value; that is, the average concentration of fungicide required to reduce colony growth by half.

EC_{50} values are usually computed as an average of a population (Errampalli, 2005; Gao et al., 2009; Jobin and Carisse, 2007; Koller et al., 1997; Xu et al., 2010), though they may also apply to individuals if experiments are performed in replication. In contrast, other studies chose a single discriminatory concentration after some preliminary trials, and growth above a certain level at that concentration indicates whether an individual is resistant or sensitive (Marine et al., 2007; Schnabel and Jones, 2001). EC_{50} values and discriminatory concentrations are informative ways to compare resistance in one area to resistance in another area. The table below shows EC_{50} averages and discriminatory concentrations compiled from a number of studies on *V. inaequalis* resistance to the DMI fungicides investigated in this study (Table 1). The higher frequency of research projects evaluating myclobutanil is indicative of the longer length of time that ingredient has been marketed as well as its widespread use.

Table 1: Summary of effective and discriminatory concentrations of selected DMI fungicides in populations of *Venturia inaequalis* from previous studies

Sample collection site	Year	EC_{50} value / Discriminatory concentration	Active ingredient
United Kingdom	2010	2.016 μg/mL[1]	Myclobutanil
United Kingdom	2009	5.213 μg/mL[2]	Myclobutanil
Quebec, Canada	2007	3.079 μg/mL[3]	Myclobutanil
Ontario, Canada	2004	0.581 μg/mL[4]	Myclobutanil
Virginia, USA	2007	0.5 μg/mL*[5]	Myclobutanil
Michigan, USA	1997	0.1 μg/mL*[6]	Myclobutanil
Nova Scotia, Can.	1997	0.1 μg/mL*[6]	Myclobutanil
New York, USA	1997	0.1 μg/mL*[6]	Myclobutanil
United Kingdom	2009	0.065 μg/mL[1]	Fenbuconazole
Switzerland	1997	0.09 μg/mL[7]	Difenoconazole

*Discriminatory concentration; all others EC_{50} values.
1. Xu et al., 2009; 2. Gao et al., 2009; 3. Jobin and Carisse, 2007; 4. Errampalli, 2005; 5. Marine et al., 2007; 6. Koller et al., 1997; 7. Kunz et al., 1997

The development of widespread cross-resistance to DMI fungicides in *V. inaequalis* populations would be disastrous for many growers. With the highly effective nature of DMIs, many came to rely on the fungicides as their primary means of controlling apple scab. Though other products exist for orchard scab management, other positive attributes of DMI fungicides are their broad-spectrum nature and curative properties. In addition to apple scab, DMI fungicides control powdery mildew, rusts, flyspeck, and sooty blotch, as well as several peach and cherry fungal pathogens (PA Tree Fruit Production Guide, 2009). This broad spectrum activity eliminates the need to buy separate products for each disease, regardless of host. DMI fungicides are effective up to 96 hours after the start of an infection period, so growers have more flexibility with

timing their sprays (Ngugi, 2006; PA Tree Fruit Production Guide 2009). Loss of apple scab control, therefore, means that unless another equally effective chemistry is found to replace DMIs in spray programs, growers will need to use additional products and resources to control apple scab, with the possibility of a lower-quality product.

Areas with documented *V. inaequalis* resistance to DMI fungicides include New York, Michigan, and Virginia in the U.S.; Quebec and Ontario, Canada; Switzerland, and the United Kingdom (Errampalli, 2004; Gao et al., 2009; Jobin and Carisse, 2007; Koller et al., 1997; Kunz et al., 1997; Xu et al., 2009). However, an exhaustive study of Pennsylvania orchards has never been conducted. Commercial growers from the main apple-growing areas in the state have approached extension educators about the problem of reduced apple scab control in the past few years. Given the record of resistance development in other apple production regions, we hypothesize that a portion of the populations of *V. inaequalis* in Pennsylvania apple orchards is resistant to commonly used DMI fungicides. Moreover, because the introduction of the currently used DMI fungicides in the apple industry was staggered over a number of years, we suspect that cross resistance may also occur in some orchards. A previous study suggested that resistance to myclobutanil may be a diagnostic tool to evaluate resistance to other DMIs (Koller et al., 1997), so the present study may serve to test that claim.

Chapter 3

Materials and Methods

3.1 Sample collection:

Seven orchards were visited from June - early August 2008, and fourteen orchards were visited from late May – mid July 2009. Most of the sampled orchards were located around Adams County, which accounts for over 70% of the 21,000 acres of commercial apple production in Pennsylvania (USDA, 2009). Leaves and immature fruit with scab symptoms were collected from the cultivars 'Fuji,' 'Golden Delicious,' 'Delicious,' 'Rome Beauty,' and 'York Imperial.' In 2008, samples were taken from suspected problem areas of the blocks, while in 2009 samples were taken from five points (in a 'W' pattern) across blocks of 2-5 acres, to be representative of the entire block. Notes were taken on the general condition of the orchard (Table 2). The 2009 isolate cohorts equally represented each of the 5 sampling points from that block and cultivar, however, a master list remains so each isolate's origin is known. Samples were kept in brown paper bags in a 4°C cold room until further processing.

Table 2: General characteristics of orchards sampled in 2008 and 2009

Orchard	Sample Year(s)	Location (County)[v]	Cultivars sampled[w]	Overall scab pressure[y]	Site condition and characteristics[z]
AC-1	2008/2009	Southwest Adams	FJ, GD, YK	High	Average, at base of hill, some fireblight
AC-2	2009	Central Adams	GD, RD, RM	Low	Above average, on rolling hills
AC-3	2009	Central Adams	GD, RD, RM, YK	Average	Above average, near top of hill
BC-1	2009	East Berks	GD, RD, RM	High	Below average, very wet
AC-4	2008/2009	Central Adams	FJ, GD, RD, RM, YK	Average (RM high)	Above average, well mowed
AC-5	2009	Central Adams	FJ, GD, RD, RM, YK	Average	Above average, some weeds
BC-2	2009	East Berks	FJ, GD, RD, RM	Average (RD high)	Average, on side of mountain
AC-6	2009	Central Adams	RD (2)[x]	High	Average, on rolling hills
AC-7	2009	Northeast Adams	FJ, GD, RD, RM	Average	Average, on rolling hills
AC-8	2008/2009	Central Adams	FJ, GD, RD	High	Average, some weeds
YC-1	2009	Northwest York	GD, RM	Low	Above average, on gentle slope
AC-9	2009	Central Adams	GD (2)[x], YK	Average	Below average, wet, needed to be mowed
FC-1	2009	Southeast Franklin	GD, RD, RM, YK	Average (RM high)	Below average, wet, needed to be mowed
AC-10	2009	Southwest Adams	GD, RD, RM, YK	Average	Average, near top of hill

[v]All counties in Pennsylvania

[w]FJ = 'Fuji;' GD = 'Golden Delicious;' RD = 'Delicious;' RM = 'Rome Beauty;' YK = 'York Imperial'

[x]Two separate sampled plots of the same cultivar are indicated by (2)

[y]"Low" indicates one or a few scab lesions per leaf or fruit; "Average" indicates several distinct scab lesions on leaves or fruit; "High" indicates many scab lesions, sometimes converging, on leaves and fruit; cracked fruit

[z]Qualitative information about the condition of the orchard, taking into account pruning, weeds, fruit thinning, mowing status, incidence of other diseases

3.2 Grower survey on management strategies:

Commercial growers completed a survey on disease management tactics and orchard-related practices used in the sampled blocks (Appendix). This included general characteristics about their operations as well as information about their spray programs. Growers were surveyed on the overall size of the orchard, the number of cultivars grown, the age of the sampled trees, the cultivar and rootstock of the sampled trees, and the planting status of the sampled plot, that is, the arrangement of cultivars in the block. Growers were also surveyed on the history of DMI fungicide use in the sampled plots, whether applications were made as full sprays or alternate-row middle sprays, whether the grower used copper during dormant tree stages, and if the leaf litter was managed in any way, such as by shredding or through urea applications.

3.3 *Venturia inaequalis* isolation:

All isolations were carried out on quarter-strength potato dextrose agar (¼ PDA) prepared by suspending 5 g of PDA and 5 g of agar in 500 mL of deionized water and autoclaving for 15 minutes at 120°C. Using a scalpel, a single sporulating lesion was excised from a leaf or fruit, and streaked with the spore-side down onto 100 mm-diameter Petri-plates of ¼ PDA medium amended with either 100 parts per million (ppm) streptomycin, or 50 ppm tetracycline. The majority of isolations were performed using streptomycin-amended plates; the tetracycline-amended plates were used in the few cases where resistance to streptomycin in bacterial populations posed a problem to obtaining a clean culture.

Plates were incubated at 12 hours of light and 25°C within the laboratory. Twenty-four to 48 hours later, the plates were viewed under a Nikon or Olympus dissecting microscope at 100x. A single germinating conidium was chosen from a plate and aseptically transferred to a fresh plate containing ¼ PDA without antibiotics, then wrapped with parafilm. This procedure was repeated for 200 samples from 2008 and 950 samples from 2009.

3.4 Screening for resistance to DMI fungicides:

Isolates were screened for resistance to myclobutanil, fenbuconazole, and difenoconazole using the agar plug assay described by Stanis and Jones as well as Koller et al. (Koller et al., 1997; Stanis and Jones, 1985), with minor modifications as described below. Technical-grade myclobutanil (the active ingredient in Nova 40W or Rally 40W), fenbuconazole (Indar 75 WSP), and difenoconazole (Inspire) were obtained from the manufacturers (Dow Agrochemicals [Indianapolis, IN] for mycobutanil and fenbuconazole; Syngenta [Wilmington, DE] for difenoconazole). Fungicides were suspended in 70% ethanol and filter sterilized with a 0.2 µm polyethersulfone filter (VWR International). Media (¼ PDA) was amended with 0.125 µg/mL, 0.5 µg/mL, 1.0 µg/mL, and 2.5 µg/mL of myclobutanil, or with 0.05 µg/mL, 0.125 µg/mL, 0.25 µg/mL, 0.5 µg/mL, or 1.25 µg/mL of fenbuconazole or difenoconazole. Fungicide was added to the media at 55°C, followed by stirring for a minute or more before dispensing into culture plates (60 mm x 15 mm).

Cultures were transferred onto fungicide-amended plates after about 2 months of growth on non-amended ¼ PDA, when colonies were about 3 cm in diameter. Plug transfer was accomplished by using a 0.32 cm cork borer to remove medium out of the

center of the fungicide-amended plates, to create core holes for holding the mycelia plugs. A 0.32 cm diameter mycelia plug was then removed from the outer edge of an isolate colony and placed in the core hole at the center of the fungicide-amended plate. Three replicate plates of each fungicide concentration were prepared for each isolate tested. For each of the fungicide concentrations, a total of 435 isolates were screened on myclobutanil, 334 on fenbuconazole, and 356 on difenoconazole. A total of 288 individual isolates were screened on all three products, with the remaining isolates screened on one or more of the fungicides. With each plating, four isolates confirmed as sensitive or resistant (2 of each) to DMI fungicides from Dr. Kerik Cox (Department of Plant Pathology, Cornell University) served as positive and negative controls to confirm final concentrations of fungicide for each set of plates poured. The baseline isolates from Dr. Cox's lab were collected from New York orchards before DMI fungicides were introduced in the United States. A non-exposed, baseline population was not locally available.

After plating, cultures were incubated in the dark at 23°C in an incubator or in plastic containers kept in drawer cabinets of a 25°C laboratory. Ten days were allowed to pass before commencing weekly colony diameter measurements. Each fungal colony was measured across the same 2 perpendicular diameters each week, for a total of five measurements. An average diameter was calculated from three replicates at each fungicide concentration.

3.5 Long term storage of isolates:

After an isolate was tested on all three fungicides, a non-amended plate containing each isolate was placed under a UV light for 14 days to increase conidial

production. An aliquot of 1.5 mL sterile water was applied to the plate, the colony was agitated with a glass rod, and the mycelial-conidial suspension was placed in a 2 mL cryotube. Isolates were frozen in -20°C. As a backup, isolates were also maintained on 0.05 µg/mL myclobutanil ¼ PDA plates. This concentration of fungicide was used such that the resistant phenotype was not lost after subsequent culturing without fungicide challenge (K. Cox, personal communication).

3.6 Determination of resistant individuals:

For the screening process, isolates were initially evaluated at the 0.5 µg/mL fungicide concentration for all three fungicides. After 45 days of growth, relative growth (RG) values were calculated by dividing the average diameter of the colony on the 0.5 µg/mL plate by the average diameter of the colony on non-amended ¼ PDA plate and multiplying by 100. Isolates with RG values above 75 were classified as resistant; those with RG values less than or equal to 75 were classified as sensitive. The calculation of RG values and the threshold of RG = 75 used here are comparable to the methods used by Koller et al. (1997).

For closer comparison with previously published EC_{50} values (Kunz et al., 1997; Xu et al., 2010), isolates tested on fenbuconazole and difenoconazole were also evaluated at 0.25 µg/mL and 0.125 µg/mL concentrations, respectively. Isolates with RG values above 75 at these concentrations were classified as shifting toward resistance to these fungicides.

3.7 Statistical Analysis:

The effective concentration necessary to cause 50% inhibition (EC_{50}) was computed for each within-orchard population with the PROBIT procedure of SAS (SAS version 9.2; SAS Institute Inc., Cary, NC) as described by Hsiang et al. (1997). This was done by using RG values to determine the EC_{50} value and its corresponding 95% confidence limits with the PROBIT procedure. The effects of the cultivar EC_{50} values were evaluated using PROBIT analysis and orchards where populations from particular cultivars had significantly different ($P < 0.05$) EC_{50} values were noted. EC_{50} values and RG distributions from orchards sampled in both 2008 and 2009 were compared using a two-sample T-test and the Kolmogorov-Smirnov two-sample nonparametric statistic (SAS version 9.2; SAS Institute Inc., Cary, NC). Frequency distribution histograms of the RG values of isolates, evaluated at 0.5 μg/mL as described above, were plotted in SigmaPlot software (SigmaPlot for Windows Version 10; Systat Software Inc. San Jose, CA). To test the hypothesis that isolates were cross-resistant to the three fungicides, relative growth rates of the isolates at 0.5 μg/mL of each fungicide were subjected to Spearman's rank correlation analysis. Spearman's rank correlation coefficients adjusted for ties were computed using CORR procedure of SAS (SAS version 9.2; SAS Institute Inc., Cary, NC).

Chi-square categorical data analysis procedures (Stokes et al., 2000) were used to investigate the association between orchard-related factors and the status of sensitivity to DMI fungicides at the time of the survey. For each isolate and fungicide combination, binary variables describing the resistance status were constructed by assigning values of 0 when an isolate was scored as sensitive, and 1 for isolates designated as resistant or shifted toward resistance, based on the RG = 75 at the fungicide concentrations described

above. Orchard-related characteristics (Appendix) included in the survey were also coded as binary variables. To identify orchard-related factors associated with resistance, or shifts toward resistance to the fungicides, analysis of contingency tables was performed for data from each fungicide and Chi-square tests were used to assess the strength of the association (SAS version 9.2; SAS Institute Inc., Cary, NC; Stokes et al., 2000).

The relationship between orchard-related factors and the incidence of resistance, or shifts toward resistance, to DMI fungicides was further investigated with logistic regression analysis (Stokes et al., 2000). The binary variables describing isolate resistance status were modeled as the response variables, while the orchard-related factors identified as significantly associated with fungicide sensitivity were included as explanatory variables (SAS version 9.2; SAS Institute Inc., Cary, NC). The best logistic regression models were selected based on the Akaike information criterion (AIC; Hirotugu, 1974), the Bayesian Information criterion (formerly the Schwarz criterion [SC]), and the negative log likelihood, or deviance (McCallugh and Neider, 1989). AIC and SC adjust for the number of explanatory variables in the model while testing if the explanatory variables are significant. The deviance only tests if the explanatory variables are significant, without taking the number of explanatory variables into account (Stokes et al., 2000).

Chapter 4

Results

A total of 20 orchards in the major apple production regions of Pennsylvania were surveyed over the two years of the study. Out of these, over 900 single-conidial isolates of *V. inaequalis* were obtained. However, due to time constraints, only isolates from 14 of the orchards were screened in sufficient numbers at the levels of DMI concentration (at least five) needed to accurately compute EC_{50} values and their 95% confidence limits by probit analysis. For 2008, the number of isolates analyzed (*n*) was 69, and *n* = 479 for 2009.

4.1 Estimates of effective concentration (EC_{50}) values for Pennsylvania isolates

Overall, EC_{50} values were higher for myclobutanil than for fenbuconazole or difenoconazole; that is, isolates were generally less sensitive to the former fungicide than the latter. The EC_{50} values ranged from 1.203 to 2.843 µg/mL with a mean of 2.139 ± 0.090 µg/mL for myclobutanil, 0.517 to 1.135 µg/mL and a mean of 0.839 ± 0.053 µg/mL for fenbuconazole, and 0.098 to 0.205 µg/mL and a mean of 0.158 ± 0.053 µg/mL for difenoconazole (Table 3). Among the orchards, isolates from AC-7, 'Rome Beauty' cultivar had the highest EC_{50} on myclobutanil (3.593 µg/mL), and isolates from AC-3, AC-5, and AC-8 were also among the highest in EC_{50} values for each of the three fungicides (Table 3). Isolates from AC-6, 'Delicious' cultivar had the highest EC_{50} value on fenbuconazole, 2.422 µg/mL, and isolates from AC-5, 'York Imperial' cultivar had the highest difenoconazole EC_{50} value among orchards, 0.298 µg/mL (Table 3).

Table 3: Mean effective concentration for 50% inhibition (EC_{50}) values and corresponding limits of the 95% confidence interval (µg/mL) for *Venturia inaequalis* isolates from 14 Pennsylvania apple orchards tested on the sterol-demethylation inhibiting fungicides myclobutanil, fenbuconazole and difenoconazole, based on samples collected in 2009

		Myclobutanil				Fenbuconazole				Difenoconazole			
Orchard	**Variety**	***n*[w]**	**EC_{50}**	**95% C.I.[x]**		***n*[w]**	**EC_{50}**	**95% C.I.[x]**		***n*[w]**	**EC_{50}**	**95% C.I.[x]**	
AC-4	**FJ**	8	**2.362**	1.923	3.079	6	**1.033**	0.669	3.280	5	**0.164**	0.078	0.246
	GD	13	**2.238**	1.856	2.839	11	**1.093**	0.880	1.476	14	**0.166**	0.130	0.202
	RD	8	**2.627**	2.052	3.786	8	**1.163**	0.884	1.798	6	**0.181**	0.120	0.247
	RM	17	**2.560**	2.099	3.347	12	**1.244**	0.939	1.971	15	**0.207**	0.167	0.251
	YK	7	**2.366**	1.808	3.528	2	**0.535**	…[y]	…[y]	2	**0.176**	0.070	0.317
AC-1	**FJ**	4	**2.808**	1.937	6.335	8	**0.698**	0.505	1.200	8	**0.178**	0.101	0.253
	GD	7	**1.833**	1.425	2.535	8	**0.240**	0.133	0.380	8	**0.083**	0.017	0.134
	YK	3	**1.977**	1.191	6.065	13	**0.818**	0.584	1.600	12	**0.097**	0.057	0.133
AC-2	**GD**	7	**1.938**	1.501	2.681	2	**0.681**	…[y]	…[y]	7	**0.156**	0.089	0.223
	RD	13	**1.466**	1.195	1.851	8	**0.479**	0.346	0.753	7	**0.110**	0.043	0.168
	RM	10	**2.023**	1.604	2.746	1	**0.844**	0.387	2.716	6	**0.201**	0.122	0.288
AC-3	**GD**	11	**2.261**	1.843	2.956	6	**0.931**	0.642	2.024	7	**0.208**	0.143	0.280
	RD	11	**3.018**	2.146	5.729	10	**0.832**	0.638	1.217	9	**0.175**	0.111	0.240
	RM	13	**2.264**	1.671	3.639	10	**0.831**	0.660	1.096	10	**0.195**	0.141	0.254
	YK	12	**2.292**	1.800	3.254	5	**0.975**	0.664	2.190	6	**0.105**	0.004	0.179
BC-1	**GD**	9	**3.534**	2.497	7.206	9	**1.060**	0.765	1.969	9	**0.180**	0.119	0.245
	RD	8	**1.950**	1.474	2.891	5	**0.584**	0.344	1.409	6	**0.091**	0.006	0.154
	RM	9	**2.362**	1.842	3.415	10	**0.888**	0.620	1.821	9	**0.240**	0.171	0.328
AC-5	**FJ**	12	**2.656**	2.018	4.047	9	**0.890**	0.685	1.253	9	**0.195**	0.135	0.262
	GD	8	**2.248**	1.749	3.168	5	**1.001**	0.707	1.875	8	**0.192**	0.127	0.263
	RD	10	**2.339**	1.714	3.849	8	**0.619**	0.472	0.881	8	**0.170**	0.105	0.236
	RM	11	**2.203**	1.803	2.832	4	**1.699**	…[y]	…[y]	11	**0.189**	0.143	0.239
	YK	8	**3.315**	2.317	6.806	8	**0.928**	0.686	1.468	7	**0.298**	0.232	0.391
BC-2	**FJ**	8	**1.338**	1.020	1.829	6	**0.359**	0.253	0.615	6	**0.085**	-0.021	0.157
	GD	8	**1.328**	1.049	1.718	9	**0.416**	0.322	0.578	9	**0.067**	0.016	0.107
	RD	10	**2.389**	1.850	3.523	8	**0.698**	0.524	1.098	9	**0.161**	0.101	0.220
	RM	9	**1.120**	0.854	1.488	7	**0.514**	0.342	0.808	8	**0.089**	0.019	0.144
AC-6	**RDA**	6	**2.059**	1.513	3.282	1	**2.422**	…[y]	…[y]	1	**0.168**	…[y]	…[y]
	RDB	7	**1.361**	1.036	1.807	4	**0.230**	0.037	0.378	2	**0.074**	…[y]	…[y]
AC-7	**FJ**	7	**1.757**	1.374	2.370	7	**0.685**	0.485	1.040	7	**0.120**	0.041	0.185
	GD	8	**2.416**	1.893	3.408	8	**0.971**	0.730	1.494	8	**0.162**	0.094	0.227
	RD	5	**1.640**	1.169	2.528	5	**0.927**	0.621	1.816	6	**0.196**	0.114	0.288
	RM	7	**3.593**	2.417	9.292	3	**1.071**	0.714	2.431	3	**0.161**	0.092	0.247
AC-8	**FJ**	8	**3.327**	2.309	7.007	8	**1.044**	0.729	2.165	8	**0.237**	0.171	0.321
	RD	10	**2.545**	1.906	4.087	9	**1.169**	0.858	2.012	9	**0.175**	0.114	0.240
YC-1	**GD**	9	**2.035**	1.628	2.726	5	**0.752**	0.478	1.364	4	**0.065**	-0.066	0.140
	RM	10	**1.961**	1.527	2.717	8	**0.807**	0.646	1.047	7	**0.132**	0.070	0.187
AC-9	**GDL**	5	**2.679**	1.868	5.501	5	**1.030**	0.722	1.950	4	**0.204**	0.116	0.312
	GDU	10	**1.406**	1.139	1.789	8	**0.497**	0.339	0.720	10	**0.094**	0.026	0.146
	YK	9	**2.391**	1.779	3.824	9	**0.808**	0.598	1.260	8	**0.174**	0.114	0.236
FC-1	**GD**	6	**1.100**	0.766	1.627	5	**0.445**	0.237	0.698	5	**0.147**	0.073	0.220
	RD	8	**1.502**	1.132	2.093	8	**0.608**	0.443	0.836	8	**0.145**	0.087	0.202
	RM	9	**0.887**	0.621	1.254	0	…[y]	…[y]	…[y]	0	…[y]	…[y]	…[y]
	YK	4	**1.506**	0.984	2.617	8	**0.837**	0.502	1.985	3	**0.280**	0.196	0.412
AC-10	**GD**	12	**1.679**	1.326	2.257	9	**0.652**	0.423	1.312	7	**0.143**	0.077	0.206
	RD	8	**2.008**	1.493	3.161	5	**0.652**	0.423	1.313	7	**0.079**	-0.016	0.143
	RM	9	**2.149**	1.625	3.198	7	**1.078**	0.706	2.861	10	**0.180**	0.124	0.239
	YK	11	**1.758**	1.425	2.267	4	**0.688**	0.441	1.475	3	**0.125**	0.030	0.212
Total		435	…[y]	…[y]	…[y]	335	…[y]	…[y]	…[y]	356	…[y]	…[y]	…[y]
Mean ±SE		2.139 ± 0.090				0.839 ± 0.053				0.153 ± 0.008			

[v]EC_{50} values were calculated by probit analysis based on relative growth rates of three replicate plates of each isolate on ¼-strength potato dextrose agar medium amended with a range of concentrations of each fungicide. Estimates were

computed with the PROBIT procedure of SAS ver. 9.2.
[w]*n*= is the number of isolates from each cultivar that were screened.
[x]95% confidence interval for the EC_{50} value for each orchard.
[y]Yellow-shaded blocks indicate orchards and fungicides for which cultivars have statistically different EC_{50} values ($P < 0.05$).
[z]Dotted lines indicate missing values.

There were highly significant correlations among EC_{50} values for the different fungicides based on Spearman's rank correlation tests. The correlation between EC_{50} values for myclobutanil and fenbuconazole was 0.67 ($t = 6.049$; $P < 0.0001$), and the correlation between EC_{50} values for myclobutanil and difenoconazole was 0.605 ($t = 5.102$; $P < 0.0001$). The correlation between EC_{50} values for fenbuconazole and difenoconazole was 0.650 ($t = 5.743$; $P < 0.0001$; data not shown).

Orchards sampled in both 2008 and 2009 were compared in terms of their *V. inaequalis* populations' EC50 values (Table 4). The EC_{50} values from each year from each orchard were compared; there was no significant difference between the two sample years in any orchard, as determined by a two-sample *t*-test ($P > 0.05$). In addition, two-sample Kolmogorov-Smirnov tests for each orchard determined there was no difference in the distributions of RG values for any orchard between years ($P > 0.05$; Table 4).

Table 4: Comparison of the 2008 and 2009 EC_{50} and relative growth (RG) values for *Venturia inaequalis* isolates obtained from three Pennsylvania orchards sampled both years of the survey and tested on myclobutanil

	2008						2009							
			EC_{50} estimates		RG at 0.5 μg/mL myclobutanil				EC_{50} estimates		RG at 0.5 μg/mL myclobutanil		Probability for equality of estimates	
Orchard	n[u]	DMI[v]	**Mean EC_{50}[w]**	95% CI[x]	Median	Range	n[u]	DMI[v]	**Mean EC_{50}[w]**	95% CI[x]	Median	Range	**T-test P[y]**	**KS test P[z]**
AC-1	15	yes	**2.96**	1.93, 3.99	2.64	7.22	20	no	**2.34**	1.99, 2.70	2.57	3.16	**0.220**	**0.133**
AC-4	35	yes	**2.09**	1.59, 2.69	1.73	9.42	13	yes	**2.44**	2.00, 3.18	2.96	5.88	**0.388**	**0.064**
AC-8	19	yes	**3.70**	2.55, 4.85	3.66	9.17	18	yes	**3.68**	2.46, 4.90	3.22	9.90	**0.979**	**0.458**

[u] Number of isolates from cultivars sampled both years.
[v] Usage of DMI fungicides in orchard during that sample year.
[w] μg/mL, based on probit analysis.
[x] 95% confidence interval; in μg/mL.
[y] *P*-value from a two-sample t-test comparing the orchard EC_{50} values from both years.
[z] *P*-value from the nonparametric Kolmogorov-Smirnov test.

4.2 Distributions of PA isolates' RG values on myclobutanil, fenbuconazole, and difenoconazole

For a given DMI concentration, the relative growth (RG) values were determined by dividing the growth of an isolate on ¼ PDA plates amended with the specific concentration by the growth of the same isolate on nonamended ¼ PDA plates (Koller et al., 1997). Frequency distribution histograms demonstrated that the distribution of RG values for Pennsylvania isolates on 0.5 μg/mL myclobutanil or fenbuconazole were approximated a normal distribution with median values of 81.1 and 62.2, respectively (Fig. 1A and 1B). In comparison, at the 0.5 μg/mL concentration of these fungicides, the confirmed baseline isolates exhibited an average RG value of 21.7 and 6.8 for myclobutanil (n = 33) and fenbuconazole (n = 27), respectively (Fig. 1A and 1B). Approximately 64% of the 435 isolates tested on myclobutanil had RG values at or above 75, the threshold RG for designating an isolate as resistant (Fig. 1A), while approximately 24% of the 334 isolates tested on fenbuconazole were resistant (Fig. 1B).

By contrast, the distribution of the RG values for Pennsylvania isolates on 0.5 μg/mL difenoconazole was skewed to the right, typical of a log-normal distribution (Ngugi et al., 2002), with a median RG of 13.5 (n = 356; Fig. 1C). In comparison, the average RG value of the confirmed baseline isolates was only 1.2 (n = 25) at 0.5 μg/mL difenoconazole (Fig. 1C). Only 2 of the 356 isolates (i.e., 0.56%) analyzed were resistant to the 0.5 μg/mL difenoconazole, with the majority of isolates having an RG of 25 or less (Fig. 1C).

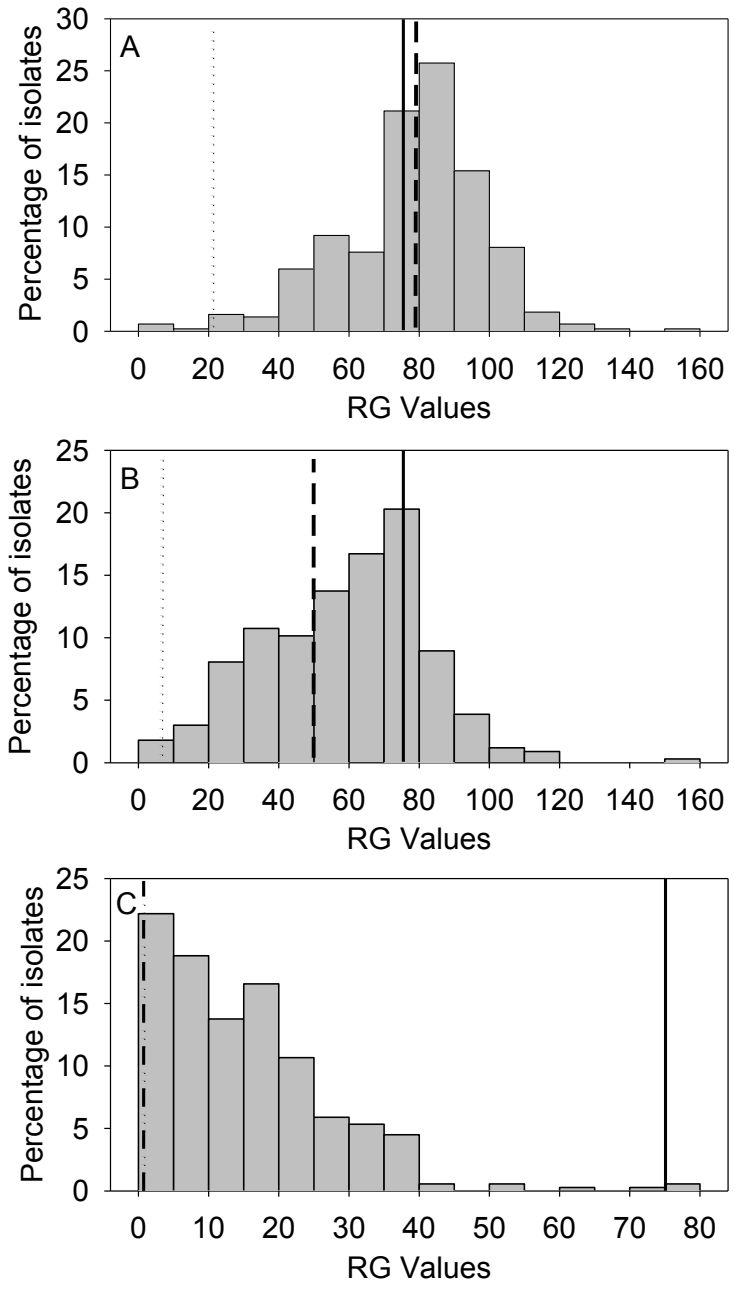

Fig. 1: Distributions of relative growth (RG) values of *Venturia inaequalis* isolates from Pennsylvania apple orchards on 0.5 µg/mL of myclobutanil (**A**), fenbuconazole (**B**), or difenoconazole (**C**), based on samples collected in 2008 and 2009. Bars represent percent of isolates grouped according to RG value and are based on sample sizes of 435, 334 and 356 for myclobutanil, fenbuconazole and difenoconazole, respectively. Dotted vertical lines indicate the average RG value of 2 confirmed baseline isolates (n = 31 [A], n = 27 [B], n = 25 [C]) and dot-dash vertical lines indicate the average RG value of 2 isolates shifted toward resistance (n = 33 [A], n = 27 [B], n = 25[C]). In C, both the baseline and shifted isolates had average RG = 1.17. Solid vertical lines indicate the RG = 75 threshold used to designate isolates as resistant or sensitive (**1A**: mean = 78, median = 81.1; **1B**: mean = 58.7, median = 62.2; **1C**: mean = 15.2, median = 13.5).

Since the distribution of isolates at 0.5 µg/mL fenbuconazole was not perfectly normal, and the distribution of isolates at 0.5 µg/mL difenoconazole was lognormal, RG values of the isolates were also evaluated at lower concentrations. Isolates were evaluated at 0.25 µg/mL for fenbuconazole and 0.125 µg/mL for difenoconazole. These concentrations are more comparable to those used in calculating EC_{50} values in other studies (0.065 µg/mL fenbuconazole [Xu et al., 2010], and 0.09 µg/mL difenoconazole [Kunz et al., 1997]). Isolates with RG values greater than 75 at these concentrations are hereafter considered to be shifted toward resistance to these fungicides. Approximately 44% of isolates were shifted on 0.25 µg/mL fenbuconazole (Fig. 2A), and 10% of isolates were shifted on 0.125 µg/mL difenoconazole (Fig. 2B). In comparison, confirmed baseline isolates had RG values of 14.8 on 0.25 µg/mL fenbuconazole (n = 27), and 3.5 on 0.125 µg/mL difenoconazole (n = 25) (Fig. 2A and 2B).

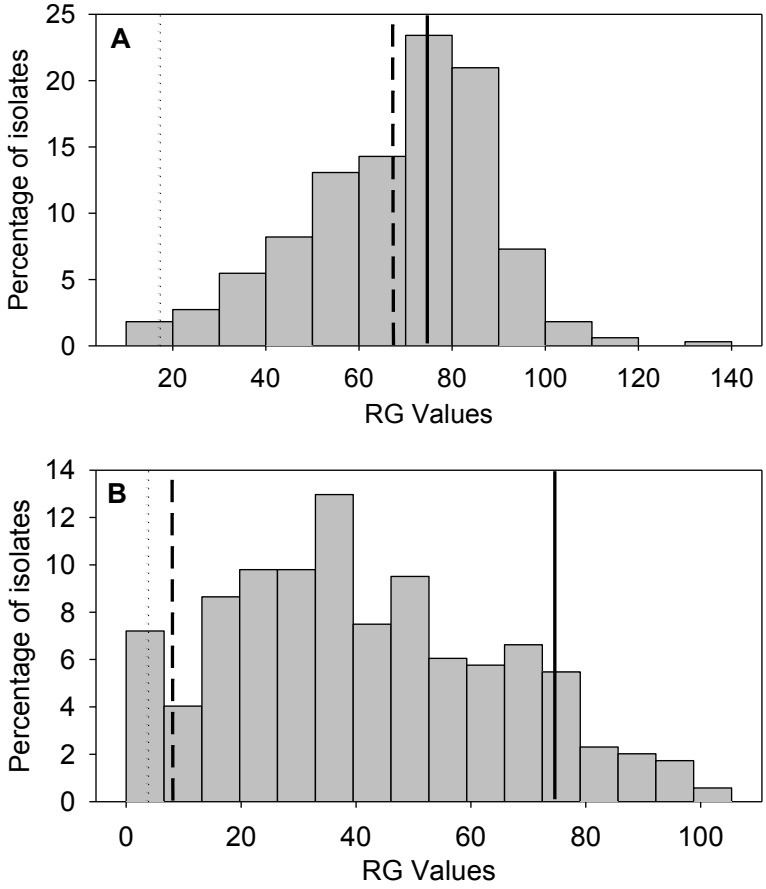

Fig. 2: Distribution of relative growth (RG) values *Venturia inaequalis* isolates from Pennsylvania apple orchards on 0.25 µg/mL fenbuconazole (**A)** and 0.125 µg/mL difenoconazole (**B**) based on samples collected in 2008 and 2009. Values represent percent of isolates in each RG category based on a sample of 334 isolates for fenbuconazole (A; mean = 68.7; median = 71.7), and 356 isolates for difencononazole (B; mean = 41.5; median = 37.7). Dotted vertical lines indicate the average RG value of 2 confirmed baseline isolates (*n* = 27 [A], *n* = 25 [B]) and dot-dash vertical lines indicate the average RG value of 2 isolates shifted toward resistance (*n* = 27 [A], *n* = 25[B]). Solid vertical lines indicate a threshold value (RG = 75); isolates at or above this threshold were considered to be shifted toward resistance to the fungicides.

4.3 Incidence of cross-resistance to DMI fungicides among isolates

A total of 288 isolates were tested on each of the three DMI fungicides. Of these, approximately 22% of isolates were resistant to both myclobutanil and fenbuconazole, based on relative growth on ¼ PDA amended with 0.5 µg/mL, while only 2 isolates were

resistant to all 3 active ingredients when evaluated at this concentration (Fig. 3). Of the 288 isolates, 42% were resistant to only myclobutanil, 2.8% only to fenbuconazole, and none were resistant to difenoconazole only (Fig. 3). Altogether, 64% of isolates tested were resistant to myclobutanil. Approximately 34% of isolates were not resistant to any DMI fungicide (*data not shown*). As noted in section 4.1, orchard EC_{50} values between isolates tested on myclobutanil and fenbuconazole are highly correlated ($r = 0.67$; $t = 6.049$; $P < 0.0001$), and the incidence of cross-shifted sensitivity to these two active ingredients was correlated to the orchard the isolates originated from ($\chi^2 = 46.3806$; $P < 0.0001$).

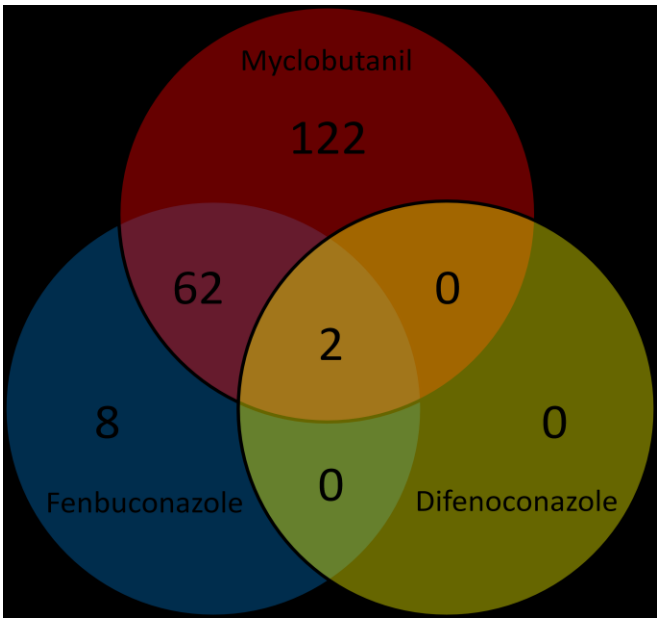

Fig. 3: Incidence of cross resistance among *Venturia inaequalis* isolates from Pennsylvania apple orchards tested on 0.5 µg/mL myclobutanil, fenbuconazole, and difenoconazole ($n = 288$) based on samples collected in 2008 and 2009.

4.4 Orchard management practices associated with *V. inaequalis* resistance to DMI fungicides

Orchard size, use of copper on dormant trees or debris, the number of cultivars grown in the orchard, and the number of DMI fungicide treatments in 2009 were highly correlated with the incidence of *V. inaequalis* isolates classified as resistant to myclobutanil at 0.5 µg/mL (Fig. 4). Approximately 65% of isolates obtained from large orchards (more than 20 acres) were resistant to myclobutanil, compared with 49% of isolates from orchards that were 20 or fewer acres in size (Fig. 4A). This relationship was confirmed by χ^2 tests ($\chi^2 = 5.542$; $P = 0.0188$; $n = 435$). There was also a strong correlation between the use of copper and the incidence of resistance to myclobutanil ($\chi^2 = 10.166$; $P = 0.0014$; $n = 435$), with only about 55% of isolates from orchards using dormant copper being resistant to myclobutanil, compared to over 70% of isolates from orchards not using copper (Fig. 4B). About 66% of isolates from orchards growing 10 or fewer cultivars were resistant to myclobutanil, compared with only 56% of isolates from orchards growing more than 10 cultivars; this result was also statistically significant ($\chi^2 = 4.0307$; $P = 0.0446$; $n = 435$) (Fig. 4C). Approximately 69% of isolates from orchards treated with more than 4 DMI fungicide sprays were resistant to myclobutanil, while 59% of isolates from orchards subjected to 4 or fewer sprays were resistant to the fungicide (Fig. 4D). This relationship was confirmed by χ^2 tests ($\chi^2 = 4.8229$; $P = 0.0281$).

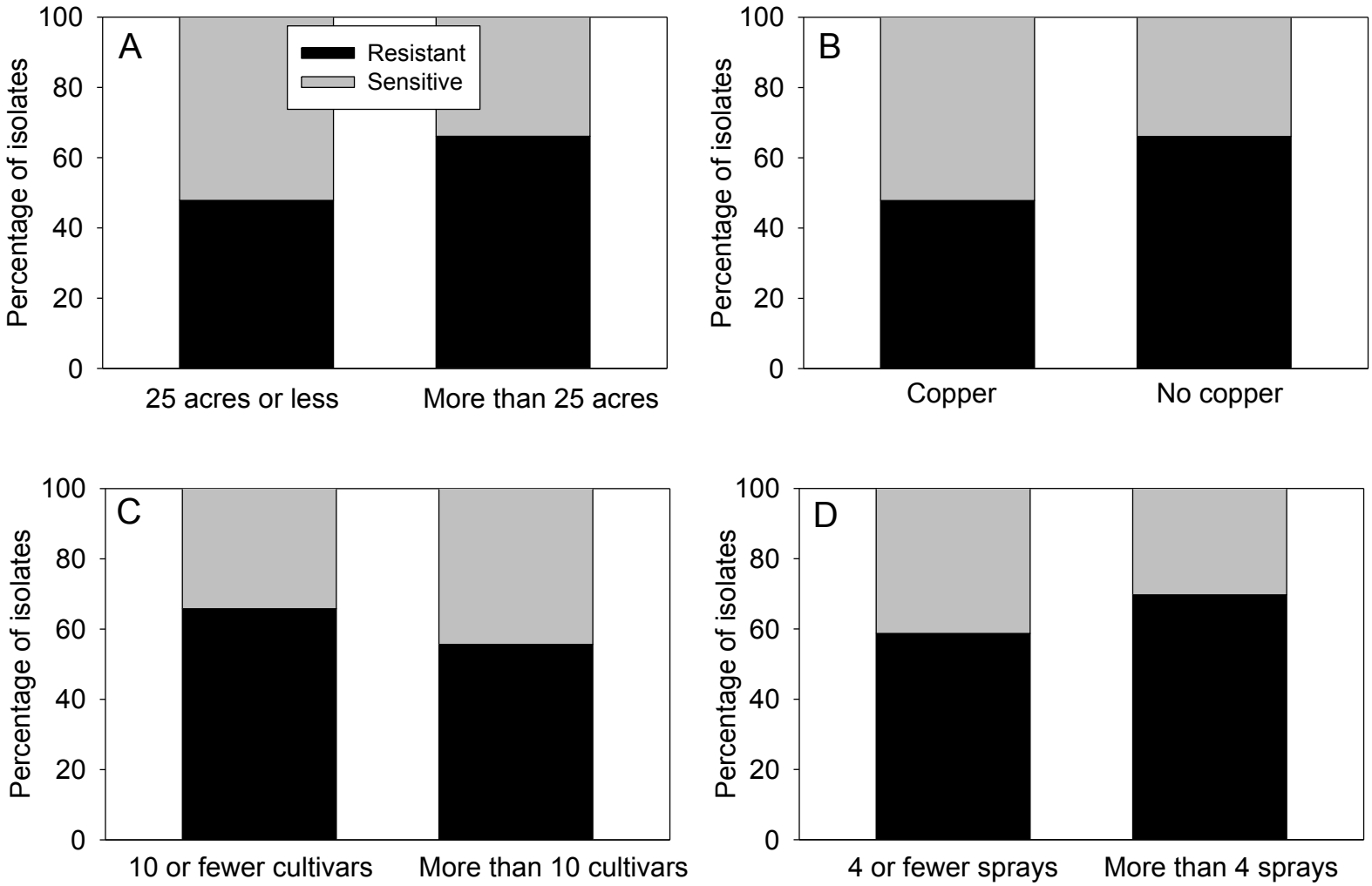

Fig. 4: Percent of *Venturia inaequalis* isolates from Pennsylvania apple orchards, collected in 2008 and 2009, with resistance or sensitivity to myclobutanil in each category of orchard size (**A**), use of dormant copper (**B**), number of cultivars grown in the orchard (**C**), and number of DMI fungicide applications in 2009 (**D**). Values were based on chi-squared tests based on the incidence of resistance to myclobutanil and differences are statistically significant (**A**: $\chi^2 = 5.542$, $P = 0.0188$; **B**: $\chi^2 = 10.166$, $P = 0.0014$; **C**: $\chi^2 = 4.0307$; $P = 0.0446$; **D**: $\chi^2 = 4.8229$; $P = 0.0281$).

Orchard size and planting trees in mixtures were significantly correlated with shifts toward resistance to fenbuconazole evaluated at 0.25 μg/mL (Fig. 5). Chi-squared analysis indicated that approximately 53% of isolates from large orchards (> 20 acres) exhibited shifts toward resistance to fenbuconazole, compared with isolates from small orchards (≤ 20 acres), for which only 30% of the isolates tested were shifted toward resistance to the fungicide ($\chi^2 = 5.4768$; $P = 0.0193$; $n = 334$; Fig. 5A). Surprisingly, a higher percentage of isolates from orchards of mixed plantings (about 64%) was shifted toward resistance to fenbuconazole, compared to the percentage of shifted isolates (51%) obtained from orchards with monoculture plantings ($\chi^2 = 5.2461$; $P = 0.0220$; $n = 334$; Fig. 5B). Interestingly, removing data from a single research orchard that was subjected to unusual management (trees planted as intra-row mixtures, and multiple fungicidal products applied on alternating groups of trees for many years) did not affect this result ($\chi^2 = 4.0695$; $P = 0.0437$; data not shown). As with the incidence of resistance to myclobutanil, the number of DMI fungicide sprays in 2009 was correlated with shifts toward resistance to fenbuconazole. Approximately 53% of isolates from orchards that were sprayed more than four times with DMI fungicides were shifted toward resistance in response to 0.25 μg/mL fenbuconazole, compared with 39% of isolates from orchards sprayed four times or less ($\chi^2 = 6.4622$; $P = 0.0110$; Fig. 5C).

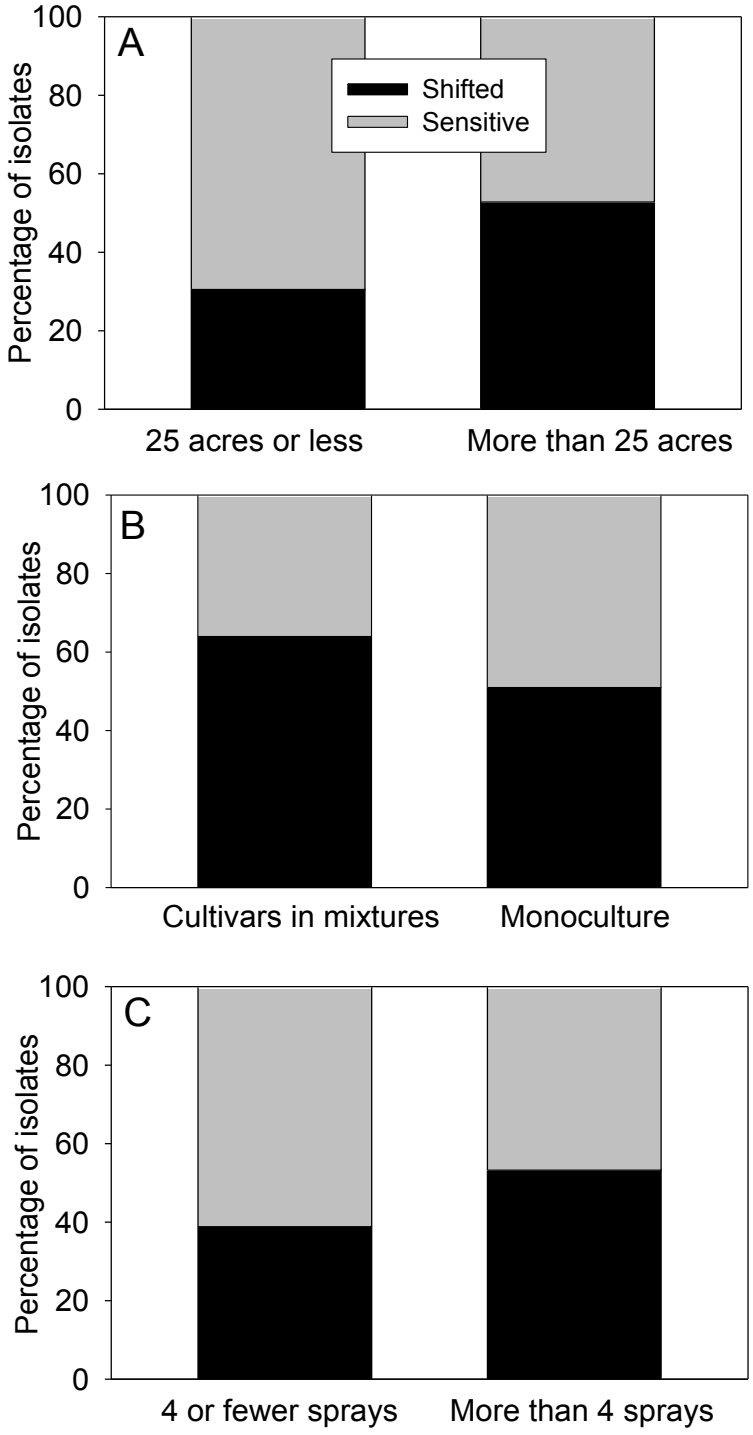

Fig. 5: Percent of *Venturia inaequalis* isolates from Pennsylvania apple orchards, collected in 2008 and 2009, shifted toward resistance to 0.25 μg/mL fenbuconazole in each category of orchard size (**A**), planting status (**B**), and number of DMI fungicide applications in 2009 (**C**). Differences are statistically significant (**A**: $\chi^2 = 5.4934$, $P = 0.0191$; **B**: $\chi^2 = 5.2461$, $P = 0.0220$; **C**: $\chi^2 = 6.4622$; $P = 0.0110$; $n = 334$).

Age, spray coverage (defined as spray application in alternate row middle or complete sprays), and apple cultivar type were practices associated with shifts toward resistance to difenoconazole, evaluated at 0.125 µg/mL (Fig. 6). Significantly more isolates (13.9%) from orchards with plantings older than 20 years were shifted toward resistance to difenoconazole, as compared to isolates from orchards younger than 20 years (6.6%; χ^2= 5.0495; *P* = 0.0246; *n* = 356; Fig. 6A). A surprising result was noted in which about 15% of isolates from orchards using complete sprays (both sides of the row) were shifted toward resistance to difenoconazole, while only 6% of isolates from orchards using alternate-row middle sprays were shifted (χ^2= 6.625; *P* = 0.0101; *n* = 356; Fig. 6B). Among the cultivars, approximately 24% of isolates from 'York Imperial' were shifted toward resistance to difenoconazole, compared to only 6-10% of isolates obtained from each of 'Fuji,' 'Golden Delicious,' 'Delicious,' and 'Rome Beauty' (χ^2= 9.7003; *P* = 0.0458; *n* = 356; Fig. 6C).

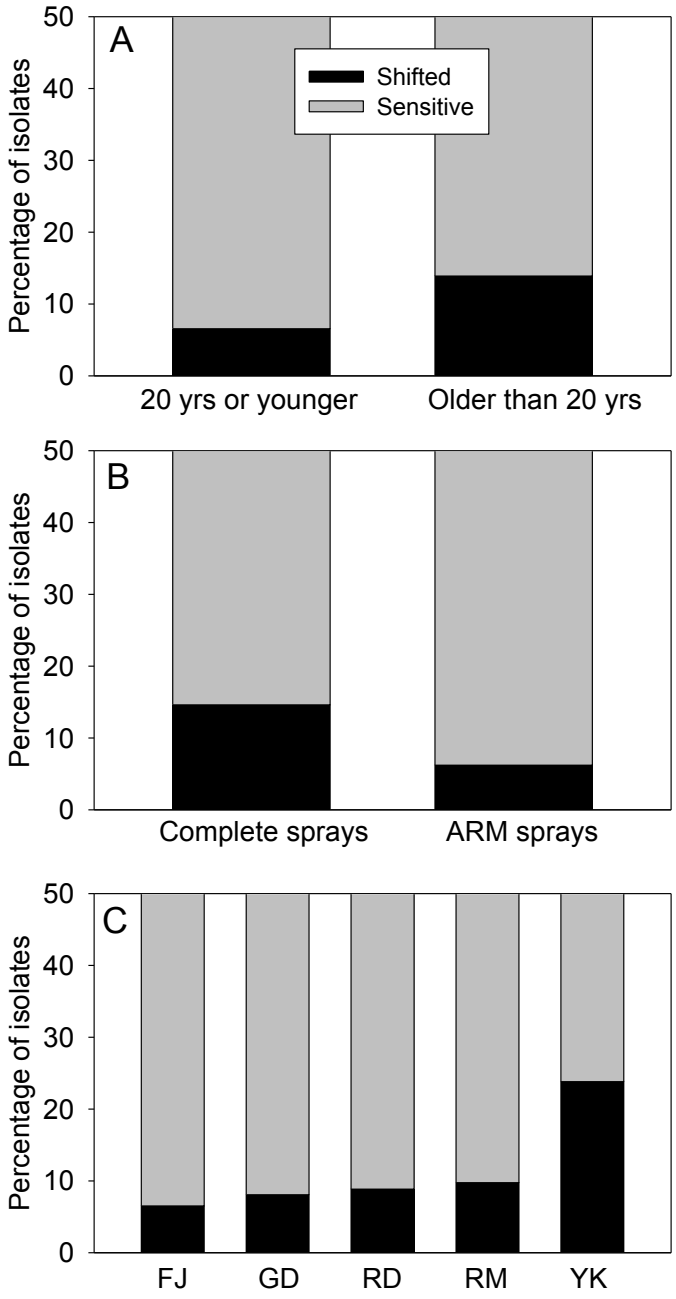

Fig. 6: Percent of *Venturia inaequalis* isolates from Pennsylvania apple orchards, collected in 2008 and 2009, shifted toward resistance to 0.125 µg/mL difenoconazole in each category of orchard age (**A**), spray status (**B**), and the cultivar it was isolated from (**C**). To emphasize differences, the *y* axes are scaled to a maximum value of 50% since none of the figures depict more than about 24% isolates with shifts toward resistance. Differences are statistically significant (**A**: χ^2= 5.0495, P = 0.0246, n = 356; **B**: χ^2= 6.625, P = 0.0101, n = 356; **C**: χ^2= 9.7003, P = 0.0458, n = 356). ARM implies alternate-row middle sprays, which are only applied to one side of the row of trees. FJ = 'Fuji,' GD = 'Golden Delicious,' RD = 'Delicious,' RM = 'Rome Beauty,' YK = 'York Imperial.'

4.5 Management strategies associated with cross-shifted isolates

Cross-shifted isolates are defined as those isolates with resistance or shifts toward resistance to each of the three DMI fungicides tested here. Cross-shifted isolates are defined here as having an RG value greater than 75 on 0.5 µg/mL myclobutanil, 0.25 µg/mL fenbuconazole, and 0.125 µg/mL difenoconazole. The use of copper-based fungicides when trees are in dormant stages (χ^2= 6.072; P = 0.0137; n = 288; Fig. 7A) and the age of the trees in orchards of isolate origin (χ^2= 4.595; P = 0.0321; n = 288; Fig. 7B) were correlated with the incidence of cross-shifted isolates. Orchards that used dormant copper had a lower percentage of individuals (1.4%) that were cross-shifted as compared with orchards that did not use dormant copper (5.8%) (Fig. 7A). Approximately 5.4% of isolates from trees that were older than 20 years were cross-shifted, compared with only 1.7% of isolates from orchards younger than 20 years (Fig. 7B).

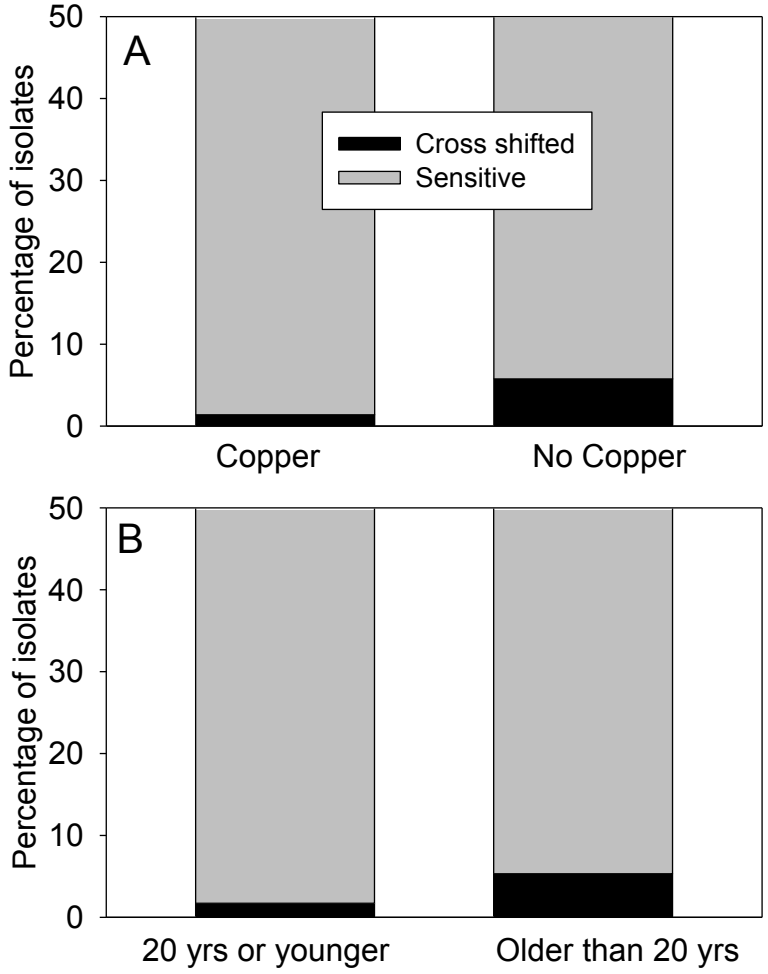

Fig. 7: Percent of *Venturia inaequalis* isolates from Pennsylvania apple orchards, collected in 2008 and 2009, cross-shifted toward resistance to three DMI fungicides in each category of copper use in dormant tree stages (**A**) and the age of the trees in the orchard (**B**). To emphasize differences, the *y* axes are scaled to a maximum value of 50% since none of the figures depict more than about 8% isolates with shifts toward resistance. Isolates were considered cross-shifted if RG >75 on each of 0.5 µg/mL myclobutanil, 0.25 µg/mL fenbuconazole, and 0.125 µg/mL difenoconazole. Differences were statistically significant (**A**: χ^2= 6.072; P = 0.0137; n = 288; **B**: χ^2= 4.595; P = 0.0321; n = 288).

4.6 Relationship between orchard management practices and *V. inaequalis* resistance to DMI fungicides

The relationship between orchard management practices and incidence of resistance or shift toward resistance to the DMI fungicides was further investigated with logistic regression analysis. The best-fitting regression model, based on AIC statistics, for the myclobutanil data is shown in Table 5. Most significantly, the use of copper in orchards was positively related to sensitivity to myclobutanil (Wald $\chi^2 = 10.998$; $P = 0.009$; Table 5). This model indicates that with other orchard management factors being equal, isolates from orchards that were not treated with copper were twice as likely (odds ratio = 2.015; 95% confidence interval (CI) = 1.332 to 3.050) to be resistant to 0.5 µg/mL myclobutanil than isolates from orchards using copper. Smaller orchards also were positively related to sensitivity to myclobutanil (Wald $\chi^2 = 4.2001$; $P = 0.0404$; Table 5). Other management factors being equal, isolates from orchards 25 acres or smaller were half as likely (odds ratio = 0.524; 95% CI = 0.283 to 0.972) to be resistant to 0.5 µg/mL myclobutanil than isolates from orchards larger than 25 acres.

Table 5: Parameter estimates from a logistic regression model relating the incidence of resistance to myclobutanil among *Venturia inaequalis* isolates from Pennsylvania apple orchards with selected orchard management factors, based on data collected in 2008 and 2009

Parameter	DF	Estimate[w]	SE	Wald χ^2	$P > \chi^2$
Intercept	1	0.3089	0.156	3.9187	0.0478
Copper[x]	1	0.3504	0.1057	10.998	0.0009
Orchard size[y]	1	-0.3227	0.1575	4.2001	0.0404
Age[z]	1	0.1825	0.1066	2.9315	0.0869

[w]Estimates based on 435 isolates screened at 0.5 μg/mL of myclobutanil.
[x]At least one application of a copper-containing product on dormant trees or leaf litter in the fall or spring preceding the sampled season.
[y]Orchards were grouped based on size (≤ 25 acres; or > 25 acres).
[z]Orchards were grouped based on age (≤ 20 years; or > 20 years).

Logistic regression analysis of the fenbuconazole data also showed that orchard size (Wald $\chi^2 = 4.2411$, $P = 0.0395$), and type of tree planting in the orchard, i.e. whether trees were planted as mixed cultivars or in monoculture blocks of single cultivars were significantly related (Wald $\chi^2 = 4.0278$, $P = 0.0448$) to shifts toward resistance to 0.25 μg/mL fenbuconazole (Table 6). Based on this analysis, isolates from orchards smaller than 25 acres were about half as likely to be resistant to fenbuconazole than isolates from larger orchards (odds ratio = 0.527; 95% CI = 0.286 to 0.970; Table 6). Additionally, isolates from orchards planted in mixtures by row were over 60% more likely to be resistant to 0.25 μg/mL fenbuconazole (odds ratio =1.609; 95% CI = 1.011 to 2.560).

Table 6: Parameter estimates from a logistic regression model relating the incidence of shifted sensitivity to fenbuconazole among *Venturia inaequalis* isolates from Pennsylvania apple orchards with selected orchard management factors based on data collected in 2008 and 2009

Parameter	DF	Estimate[x]	SE	Wald χ^2	P > χ^2
Intercept	1	-0.7475	0.2109	12.5633	0.0004
[y]Orchard size	1	-0.3205	0.1556	4.2411	0.0395
[z]Mix	1	0.4755	0.237	4.0278	0.0448

[x]Estimates based on 334 isolates tested on 0.25 µg/mL fenbuconazole.
[y]Orchards were grouped based on size (25 acres or smaller; or larger than 25 acres).
[z]Orchards were planted in either monoculture or mixture by row.

Chapter 5

Discussion and Conclusions

This study documents widespread shifts toward resistance to the DMI fungicides myclobutanil, fenbuconazole, and difenoconazole in populations of *Venturia inaequalis* from Pennsylvania apple orchards in 2008 and 2009. Based on a 0.5 μg/mL concentration and a threshold RG of 75, 64% of the 435 isolates screened were resistant to myclobutanil (Fig. 1A), and 24% of the 334 isolates were resistant to fenbuconazole (Fig. 1B). The confirmed baseline isolates grew at an average RG of 21.7 and 6.8 on 0.5 μg/mL myclobutanil and fenbuconazole, respectively (Fig. 1A and 1B). Although only 2 out of 356 isolates were resistant to difenoconazole at 0.5 μg/mL (Fig. 1C), this concentration is much higher than the EC_{50} value reported by another study (Kunz et al., 1997), and significant shifts towards resistance were also noted when the isolates were evaluated at the 0.125 μg/mL concentration (Fig 2B). Confirmed baseline isolates had average RG values of 1.17 and 3.46 at 0.5 μg/mL and 0.125 μg/mL difenoconazole, respectively (Fig. 1C and 2B). Resistance to DMI fungicides has been documented in most of the apple producing states in the eastern US, including New York and Michigan (Koller et al., 1997), as well as Virginia (Marine et al., 2008), but to the best of our knowledge, this is the first report from Pennsylvania.

Approximately 22% of tested isolates were cross-resistant to both myclobutanil and fenbuconazole at 0.5 μg/mL of each fungicide (Fig. 3). The 2 isolates that were resistant to 0.5 μg/mL difenoconazole were also resistant to myclobutanil and

fenbuconazole (Fig. 3). Cross-resistance to DMI fungicides has been documented in the United Kingdom, however, this result was based on conidial populations and individual isolates were not tested on each fungicide (Xu et al., 2009).

Another major goal of this study was to identify apple production-related factors that are associated with resistance to DMI fungicides. Larger orchards, refraining from use of copper during crop dormant stages, and growing 10 or fewer cultivars in an orchard were strongly and positively correlated with resistance to 0.5 μg/mL myclobutanil (Fig. 4). Larger orchards and planting cultivars in mixtures were positively correlated with shifts toward resistance to 0.25 μg/mL fenbuconazole (Fig. 5), while older plantings, the use of complete sprays (as opposed to alternate row middle) during cover applications, and isolates from the cultivar 'York Imperial,' were positively correlated with shifts toward resistance to difenoconazole evaluated at 0.125 μg/mL (Fig. 6). Most importantly, isolates from orchards not using copper, those with older trees, and those planted with fewer than 10 cultivars were positively correlated with cross-shifts toward resistance to the three DMI fungicides tested here (Fig. 7). An isolate was designated as being cross-shifted toward resistance if it had an RG value greater than 75 on concentrations of each of the three fungicides at the three test concentrations used, namely, 0.5 μg/mL myclobutanil, 0.25 μg/mL fenbuconazole, and 0.125 μg/mL difenoconazole.

Moreover, relationships between use of dormant copper spray and orchard size and resistance to myclobutanil was confirmed with logistic regression analysis (Table 5). Relationships between orchard size and planting mixtures of apple cultivars and the shift toward resistance to fenbuconazole were also established (Table 6). Many factors,

including the number of DMI applications (Gao et al., 2009) are thought to be related to changes in sensitivity of *V. inaequalis* populations to DMI fungicide, but this is the first study to provide quantitative evidence for relationships other than the number of DMI fungicides used (Gao et al., 2009).

Resistance to DMI fungicides in populations of *V. inaequalis* has been documented in many of apple-growing regions, including New York (Koller et al., 1997), Virginia (Marine et al., 2008), Michigan (Koller et al., 1997), Ontario and Quebec (Errampalli, 2004; Jobin and Carisse, 2007), the United Kingdom (Xu et al., 2009; Gao et al., 2009), Switzerland (Kunz et al., 1997), the Netherlands and Italy (Fiaccadori et al., 1987). The problems in these areas have ranged from shifts in sensitivity to complete resistance to specific DMI fungicides. The sample size of each of these studies ranged from 15 (Errampalli, 2004), to over 700 isolates (Koller et al., 1997), with EC_{50} value information readily used as the most informative measure of isolate or population response.

An EC_{50} value is defined as the mean concentration of active ingredient that is effective in inhibiting 50% of the growth of the sampled population. In the literature, EC_{50} values for *V. inaequalis* populations range from 0.09 µg/mL fenbuconazole to 5.213 µg/mL myclobutanil (Kunz et al., 1997; Gao et al., 2009). At the time of sample collection in this study, EC_{50} values for *V. inaequalis* populations in Pennsylvania apple orchards were within and above these estimates, ranging from 0.887 µg/mL to 3.593 µg/mL for myclobutanil; 0.230 µg/mL to 2.422 µg/mL for fenbuconazole; and 0.065 µg/mL to 0.298 µg/mL difenoconazole (Table 2). One study from the United Kingdom suggests that satisfactory scab control may only become a problem at EC_{50} values above

2.0 µg/mL for myclobutanil (Gao et al., 2009). Based on this estimate, twelve of the fourteen 95% confidence intervals of the EC_{50} values from Pennsylvania orchards sampled in this study exceed that level (Table 2). Our data, therefore, indicate that at the time of the survey, the level of resistance to myclobutanil in PA orchards was above the world average for this active ingredient, based on publicly available records.

Relative growth (RG) values determined at discriminatory doses are a straightforward way to determine sensitivity to fungicides at the individual level (Koller et al., 1997; Marine et al., 2007), and this study found 64% of tested isolates were resistant to 0.5 µg/mL myclobutanil (Fig. 1A). As a discriminatory concentration, 0.5 µg/mL is higher than the concentration previously used for determination of *V. inaequalis* resistance to DMI fungicides, 0.1 µg/mL (Koller et al., 1997). However, the 0.5 µg/mL value is comparable to an EC_{50} value and a discriminatory concentration found in more recent studies (Errampalli, 2004; Marine et al., 2007). Moreover, lower concentrations were not effective in inhibiting growth for the majority of these isolates. For example, only about 20% of *V. inaequalis* individuals from FC-1, the orchard with the lowest myclobutanil EC_{50} value, were sensitive at 0.125 µg/mL myclobutanil (*data not shown*), which is comparable to the previously-used discriminatory concentration of 0.1 µg/mL (Koller et al., 1997). Thus, the estimates given here for resistance on the individual level are likely more conservative than the actual problem of resistance to myclobutanil by *V. inaequalis* isolates from Pennsylvania.

Resistance to fenbuconazole was relatively less widespread, with 24% of isolates resistant to 0.5 µg/mL (Fig. 1B), and resistance to difenoconazole was rare, with less than 1% of tested isolates resistant to 0.5 µg/mL (Fig. 1C). These results may be attributed to

several factors. Myclobutanil, as an active ingredient, has been used for control of apple scab in the United States for 20 years or more (Koller et al., 2005; The National Pesticide Information Retrieval System [NPIRS Public], 2010), so it would be expected that populations would be most adapted to this fungicide. In contrast, fenbuconazole and difenoconazole had only been used in commercial apple orchards for 3 and 1 year(s), respectively at the time of the survey (PA Tree Fruit Production Guide, 2009; NPIRS Public, 2010), although products containing these active ingredients have been used on other crops. The breadth of resistance to fenbuconazole is surprising, given that it has been marketed for treatment of apple scab for a much shorter amount of time than myclobutanil. This may be the result of the strong cross-resistance among the fungicides documented in this study, whereby *V. inaequalis* individuals already exposed to myclobutanil develop resistance to fenbuconazole much faster than would be the case were fenbuconazole to be used on populations without a history of exposure to DMI fungicides. Nevertheless, other factors such as introduction of different inactive ingredients, changes in other products used in tank mixes with DMI fungicides over the years, the overall solubility of the products, the frequency of use of the products among growers, and the timing of the individual spray schedule cannot be ignored and require further investigation.

V. inaequalis cross-resistance was also determined among three the DMI fungicides tested in this study (Fig. 3). Only 2 isolates were cross-resistant to all three fungicides, whereas 22% of the isolates tested were resistant to both myclobutanil and fenbuconazole (Fig. 3). Other recent investigations into *V. inaequalis* resistance to these fungicides found that cross-resistance was prevalent, but that this was dependent on the

individual orchard (Xu et al., 2010), which concurs with the findings of this study. Cross resistance between myclobutanil and flusilazole, another DMI fungicide, has been documented in *V. inaequalis* populations from Quebec as well (Jobin and Carisse, 2007). A study on *V. inaequalis* resistance to difenoconazole in experimental orchards before the fungicide was released indicated full efficacy even when orchards had preexisting resistance to the DMI fungicides flusilazole and pyrifenox (Kunz et al., 1997). A different foliar pathogen, *Cercospora beticola,* had documented cross-resistance between some pairs of DMI fungicides, but none of the isolates were resistant to all eleven of the fungicides tested (Karaoglanidis and Thanassoulopoulos, 2003). This, along with the widely-held theory that *V. inaequalis* resistance to DMI fungicides is quantitatively inherited (Koller et al., 1997; Schnabel and Jones, 2001; Jobin and Carisse, 2007), suggests that shifts toward resistance to active ingredients within the same fungicide class may be conferred by different genetic changes. It may be that the two isolates with cross-resistance to all three DMI fungicides in this study possess multiple resistance mechanisms; this would require further investigation into protein levels and cellular mechanisms of resistant individuals in the presence of DMI fungicides.

Management factors contributing to the development of resistance to DMI fungicides were identified in this study through a survey distributed to the growers (Appendix). The only management factor that has been identified by others as being related to resistance to DMI fungicides is the number of DMI fungicide applications per year (Gao et al., 2009). The management factors investigated in the current study were compared to the incidence of shifts toward resistance to DMI fungicides at various levels, depending on the fungicide (0.5 µg/mL myclobutanil, 0.25 µg/mL fenbuconazole, and

0.125 µg/mL difenoconazole). Sensitivity to myclobutanil was correlated with younger trees, use of dormant copper, and growing ten or more cultivars in the orchard (Fig. 4).

The results on orchard age were expected, and support the hypothesis that *V. inaequalis* populations in older orchards have undergone more selection pressure due to the longer duration of exposure to DMI fungicides, which has been suggested in other studies (Kunz et al., 1997). It is estimated that a grower may have 30-40 DMI applications before shifts toward resistance to the fungicides occur (Koller et al., 2005; Gao et al., 2009), and most orchards in this study had been treated with DMI fungicides for at least 10 years, with several applications per year (J. Travis, personal communication) .

Growing ten or more cultivars in an orchard may provide an additional challenge for *V. inaequalis*, beyond that imposed by the use of fungicides. It has been shown that *V. inaequalis* isolates from one cultivar may be unable to infect other cultivars (Barbara et al., 2008). Additionally, it has been shown that growing cultivars in mixtures that include resistant cultivars reduces the overall apple scab incidence and severity recorded in the orchard (Didelot et al., 2007). It may be more difficult for individuals resistant to myclobutanil to arise in the orchard because those same individuals must also be genetically equipped to infect multiple cultivars, or be outcompeted by those that can infect multiple cultivars. Additionally, the number of cultivars grown in an orchard may be an indication of the market the particular grower is focusing on; growers focusing on fresh-market apples may be more likely to grow more cultivars than growers focusing on processing apple sales. Finally, target markets and associated profit margins may affect how growers manage their apple orchards. For example, processing apple markets have a

higher tolerance for apple scab, but processing crops also garner lower prices. This may result in the use of less efficacious but less costly fungicides initially, and once scab pressure nears the economic threshold for processing apples, the more costly DMI fungicides may be used as a sort of last resort to save the crop.

The relationship between the use of dormant copper application and resistance to myclobutanil (Fig. 4B and Table 3) is the most noteworthy result in terms of fungicide resistance management. Copper fungicides disrupt protein conformations nonspecifically in a number of organisms (Russell, 2005), so in addition to controlling the development of initial *V. inaequalis* inoculum, restricting applications to dormant tree stages reduces the risk of apple tree phytotoxicity. In a previous study, *V. inaequalis* isolates from leaf litter and trap trees were more sensitive to myclobutanil than isolates from the canopy (Gao et al., 2009). That study also noted that isolates from litter and trap trees were more variable in sensitivity than those obtained from the tree canopy (Gao et al., 2009). This may indicate that the effect of copper is mainly due to an overall reduction in mating individuals of *V. inaequalis*. Though little is known about the heritability of resistance to DMI fungicides, keeping the overall season-to-season populations low would slow the development of predominantly resistant populations; a hypothesis that warrants further investigation. In any case, existing equipment and personnel can be used for copper applications, making this an inexpensive choice for fungicide resistance management. However, this recommendation should be offered with caution because copper toxicity in apple orchard soil has recently become a concern as a result of reduced microbial activity in the soils of orchards with prolonged copper use (Wang et al., 2009).

For better comparison to previous studies, screenings with fenbuconazole and difenoconazole were also evaluated at the lower concentrations of 0.25 µg/mL and 0.125 µg/mL, respectively. Isolates with growth above 75 at these concentrations were deemed to be shifted toward resistance. On these concentrations, approximately 44% of isolates tested on fenbuconazole and 10% of isolates tested on difenoconazole were shifted toward resistance (Fig. 2). Shifts toward resistance to fenbuconazole was positively correlated with larger orchards (25 acres or more) and planting cultivars in mixtures, and both of these results were corroborated by logistical regression analysis (Table 4). The results on the effect of orchard size were not surprising because appropriate spray intervals and/or coverage can be more difficult to achieve for larger orchard operations than for smaller orchards. For example, field applicators may be covering the orchard at a faster pace, or applications may be interrupted by weather events, due to the simple fact that spraying a larger farm takes more time.

The outcome that planting apple cultivars in mixtures increases the likelihood of resistance to fenbuconazole was unexpected and surprising. Cultivar mixtures have been shown to reduce disease severity in many cropping systems (Garret and Mundt, 2000; Ngugi et al., 2001; Lannou, 2001; Pilet et al., 2006), including the *V. inaequalis* – apple system (Didelot et al., 2007), which in theory should result in a lower selection pressure for fungicide resistance from the reduced number of fungicide applications necessary to protect a mixed crop. However, a previous study demonstrated the best scab control occurred in orchards where cultivars were planted in mixtures and were also treated with a moderate schedule of fungicide applications (Didelot et al., 2007). In the case of resistance to DMI fungicides, it may be possible that individuals adapted to survival on a

variety of hosts may be more fit overall to withstand fungicides in the environment. Additional investigation would be required to determine if this were the case.

Resistance to difenoconazole was positively correlated with older orchards, complete spray coverage as opposed to alternate-row middle sprays, and individuals isolated from the York Imperial cultivar (Fig. 6). As indicated above, older orchards may have populations resistant to DMI fungicides as a result of more selection events, (i.e., longer duration of exposure which equates to more DMI fungicide applications). Complete spray coverage would be expected to decrease the incidence of resistance, however, it may be that growers chose to use products with difenoconazole as the active ingredient as a final effort to save the crop when disease pressure neared the economic threshold.

Isolates from the 'York Imperial' cultivar were more likely to be resistant to DMI fungicides than isolates from the other four cultivars tested here (Fig. 6C). Approximately 24% of isolates from 'York Imperial' were shifted toward resistance to difenoconazole, while only 6-10% of isolates from the other four cultivars were shifted toward resistance (Fig. 6C). 'York Imperial' is highly susceptible to apple scab, and is grown primarily for processing (PA Tree Fruit Production Guide, 2009). Because the tolerance for apple scab tends to be higher for processing apples, and they are generally sold for less than fresh market apples, growers may choose to spray the blocks of more lucrative apples first, leaving the less profitable apples for later. Such a choice may result in longer intervals between sprays for processing cultivars such as 'York Imperial.' Since the window of DMI curative action is 96 hours after the start of the infection period (PA Tree Fruit

Production Guide, 2009), it may be that the less profitable blocks of the orchard are occasionally overlooked until the next infection period.

Management factors correlated with the incidence of isolates cross-shifted toward resistance to all three DMI fungicides are the failure to use copper in orchards during dormant tree stages and orchards that were older than 20 years (Fig. 7). The reasons advanced above to explain the associations of these factors with the incidences of resistance to individual fungicides also apply to cross-resistance. Only five cultivars and three DMI active ingredients were tested here, warranting an expanded study, which could possibly include cultivars with resistance to *V. inaequalis* and other active ingredients within the DMI class. Little is known about the inheritance of resistance to DMI fungicides, so knowledge of seasonal or long-term changes in the population structure of *V. inaequalis* would be highly beneficial in developing strategies for resistance management. Investigating orchards with prior resistance to DMI fungicides that diversify their spray programs and change management tactics would be important to find out if DMI fungicides, as a class, have the potential to be reintroduced after several seasons of not using them. Additionally, it would be interesting to determine if cross-shifted individuals possess multiple mechanisms of resistance to DMI fungicides, which could result in the abandonment of the class of fungicides for apple scab management.

Conclusions

After testing a large collection of *V. inaequalis* isolates on myclobutanil, fenbuconazole, and difenoconazole, resistance to myclobutanil and fenbuconazole was widespread in Pennsylvania apple orchards at the time of the survey, with incidences of

64% and 24%, respectively, based on a conservative discriminatory dose of 0.5 μg/mL. Nearly all *V. inaequalis* isolates were sensitive to difenoconazole at 0.5 μg/mL. Approximately 44% and 10% of isolates were shifted toward resistance to lower concentrations of fenbuconazole and difenoconazole, respectively. Cross-resistance to myclobutanil and fenbuconazole occurred in 22% of the *V. inaequalis* population, while only 2 isolates were cross-resistant to all three fungicides. We conclude that myclobutanil is no longer an effective compound for control of apple scab in most Pennsylvania orchards, and that the effectiveness of fenbuconazole and difenoconazole will quickly be lost.

Copper use on trees or litter during dormant season, the age of the apple trees in the orchard, the number of cultivars grown in the orchard, planting status, the number of DMI fungicide applications in 2009, the size of the orchard, spray coverage, and the cultivar isolated from were factors affecting the likelihood of resistance to DMI fungicides in the orchards surveyed. Application of copper fungicide during dormant sprays should be encouraged to reduce the risk of resistance to DMI fungicides developing in commercial apple orchards. Limiting the number of DMI fungicide treatments per season and practices that improve spray coverage and shorten between-spray intervals will slow the rate of resistance development to DMI fungicides. Cross-shifted sensitivity to DMI fungicides in Pennsylvania warrants additional research into new chemistries and altered approaches to managing apple scab.

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Appendix

PSU-FREC Grower Management Practices Survey

Penn State Fruit Research and Extension Center DMI Fungicide Resistance Survey

Name of orchard: _____

How large is the orchard: _____

How many cultivars do you grow: _____

Are cultivars in mixtures: Yes / No

Cultivar	Age	Rootstock

Do you use DMIs or SIs : Yes / No
(Nova, Indar, Inspire, Rally, Procure, Scala, Vanguard, Rubigan)

How many DMI sprays this year: _____

Complete or half-row sprays: Complete / Half-row

How long have you used DMIs/SIs? Less than 5 yrs 5-10 yrs 10+ yrs

Do you use dormant copper: Yes / No 1 or 2 applications?

Do you use leaf shredding, urea, or other applications to leaf debris: Yes / No

Who manages this orchard: _____

Do you think you have a problem with resistance: Yes / No

* * *