Characterization of Fungicide Resistance in *Venturia inaequalis* Populations in Virginia

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Plant Pathology, Physiology, and Weed Science

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March 28, 2012
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Keywords: *Venturia inaequalis*, apple scab, fungicide resistance, sterol-inhibitor, strobilurin, seasonal variability, microclimate, Virginia

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ABSTRACT

Apple scab (causal organism: *Venturia inaequalis*) is an economically devastating disease of apples that is predominantly controlled with fungicides. Of the chemical classes currently available, the sterol-inhibiting (SI) and strobilurin (QoI) fungicides are the most commonly used. Recent observations indicate that *V. inaequalis* populations in Virginia have developed resistance to myclobutanil and other SIs. However, little is known about the frequency and distribution of SI and QoI resistance in Virginia's scab populations. The first objective of this research was to evaluate *V. inaequalis* populations in Virginia for SI (Chapter 2) and QoI resistance (Chapter 5). We hypothesized that *V. inaequalis* populations would have various levels of SI and QoI resistance. Fungal isolates were collected from experimental orchards at the Alson H. Smith Jr., Agricultural Research and Extension Center (AHS AREC) and from commercial orchards in Virginia and Maryland. Sensitivities were determined by assessing colony growth at 19°C on potato dextrose agar (PDA) amended with 0 or 1.0 μg ml^{-1} of myclobutanil (SI) (N=87) or trifloxystrobin (QoI) (N=25) at 28 days. A range of fungicide sensitivity was observed for both chemical classes. The second objective of this research was to monitor the temporal dynamics of SI resistance over five sequential field seasons (Chapter 3). We hypothesized that levels of SI resistance would vary within a year, but would exhibit a shift toward increased resistance over multiple years. To monitor shoot growth, neon rubber bands were placed over actively growing shoot tips following myclobutanil application or sample collection. Fungal isolates were collected from the same trees from

2007 through 2010 (N=176) and compared with isolates collected from wild apple seedlings (N=3). A continuum of SI resistance was observed for each year, and the *V. inaequalis* population exhibited a baseline shifted toward reduced sensitivity. The third objective of this research was to examine the spatial distribution of SI fungicide resistance within the tree canopy in a lower-density orchard (less than 150 trees A^{-1}) (Chapter 4). We hypothesized that spray deposition and SI resistance would not be homogeneous throughout the tree canopy. Leaves collected from larger trees (>8m) in a lower-density orchard at the AHS AREC were analyzed for manganese deposition, pre- and post-mancozeb application. Fungal isolates (N=105) were collected from several locations within the canopy in replicated trees in the same orchard. Weather sensors also monitored the microclimates within those tree canopies. Spray deposition, microclimate and SI resistance were influenced by canopy location. The fourth objective of this research was to investigate potential SI resistance mechanisms. We hypothesized that resistant isolates would segregate from sensitive isolates when examined genetically or chemically. Previously classified isolates were screened for point mutations within the *CYP51A1* gene (Appendix C), differences in polymorphic bands (alleles) (Appendix D), and differences in metabolism of myclobutanil (Appendix E). The consensus sequences for the *CYP51A1* gene were identical for all isolates tested (N=9), and results from amplified fragment length polymorphism experiment (N=82) were inconclusive. There were, however, significant differences among incubation time and myclobutanil concentration in the bioassay (N=11). Our results indicate that myclobutanil is still an effective compound for control of apple scab in many areas of Virginia.

Acknowledgements

I wish to sincerely thank my doctoral committee for their enthusiasm, suggestions and tireless editing of this document. Thank you Dr. Keith Yoder for being a patient and generous mentor. I am lucky to have known you and will always have a soft spot in my heart for fruit with faces. Thank you Dr. David Schmale III for sharing your passion for undergraduate instruction and publishing. Thank you Dr. Anton Baudoin for asking challenging questions and keeping me on my toes. Thank you Dr. Erik Stromberg for funding the weather sensors and being an outspoken advocate for field research. Thank you Dr. Mizuho Nita for providing statistical expertise and a fresh perspective.

Thank you to Allen Cochran, Billy Royston and Scott Kilmer for maintaining the apple trees and teaching me how to apply fungicides. You were my second family and made Winchester feel like home. Thanks for the camaraderie and all the memories – bungee birds, Billy-isms, Dave impressions, gourmet dipping sauce, the little blue suit, and yellow safety glasses.

Thank you to the Virginia Agricultural Council and the Virginia Apple Research Program for reliably funding this research.

Thank you to my friends and fellow VT graduate students Melissa Keller, Brenna Traver, Julie Keating, Taylor Jones, Alicia Wood-Jones, Grace Engelman, David Jones and John Cianchetti. If we can survive this, we can survive anything!

Thank you to the fruit growers, extension agents and chemical reps, who asked about my research and welcomed me at meetings. Thank you to Drs. Alan Biggs and Henry Ngugi for treating me like a colleague, to Dr. Shawn Askew for talking to me about C^{14}-labeled chemicals, and to Mary Ann Hansen for allowing me to intern in the Plant Disease Clinic.

Thank you to my undergraduate adviser Dr. Lakshmaiah Sreerama for having faith in me as a future scientist. I am so grateful for all the opportunities you gave me during my time at St. Cloud State University.

Thank you to my friends Ashlee Bekish, Kimberly Perry, Linda Krueger and Elaine Chu, who listened to me ramble on for six years about the highs and lows of graduate school.

And finally, thank you to my parents (Kim and Vera Marine) and my brother (Zach Marine) for your endless love, support and playful teasing. I couldn't have done this without you. And yes, Dad, I'm finally graduating.

The terminal Ph.D. [living the dream] a poem by SCM

There is no clear path in education,
for every fork yields to turns.
Success is not measured in completion,
but in maintaining the desire to learn.

So research is never finished.
It is simply put on a shelf.
Data analyzed and papers written,
in hopes the project will be picked up by someone else.

So the time has come to reminisce,
of the blood, sweat, and tears.
Let us celebrate the joy of finishing
and the start of my scientific career.

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Chapter 1. Introduction

The domesticated apple (*Malus domestica* Borkh.) is a fruit tree that has been cultivated and dispersed by humans for several thousand years (16,36). World apple production is currently dominated by eight cultivars: Delicious, Golden Delicious, McIntosh, Jonagold, Braeburn, Gala, Granny Smith, and Fuji (16). Within the United States, 4.5 million tons of apples are produced annually with an estimated value of $1.6 billion (47). Virginia has been a major apple-producing state since the early twentieth century, and currently ranks sixth in apple production (2%), behind Washington (58%), New York (11%), Michigan (8%), Pennsylvania (5%) and California (4%) (47). Although the majority of Virginia's apples are processed, a growing proportion are intended for the domestic fresh market, where fruit appearance is closely associated with fruit quality.

Apple scab is an economically devastating disease that occurs wherever apples are grown (4). It is caused by the fungus *Venturia inaequalis* (Cooke) G. Winter, a hemibiotrophic ascomycete. On leaves, *V. inaequalis* produces velvety olive-green lesions that become cracked and torn as the leaves age. On fruit, *V. inaequalis* produces scabby and blistered dark lesions, which significantly reduce the fruit's fresh market value. *V. inaequalis* infects a range of Rosaceous hosts including: apples (*Malus* spp.), hawthorn (*Crataegus* spp.), mountain ash (*Sorbus* spp.) and firethorn (*Pyracantha* spp.) (36).

V. inaequalis mostly overwinters in fallen leaves but also in infected buds as mycelium. During this time, the fungus undergoes sexual reproduction and produces pseudothecia. In spring, rain causes the asci in the mature pseudothecia to expand and

forcibly discharge ascospores, which are then wind-dispersed onto blossoms and leaves (40). On a growing shoot, the five youngest leaves are the most susceptible. Ascospore germination is dependent on temperature and the leaf surface remaining wet. As the ascospores colonize the tissue, they form circular olive-green lesions that distort and pucker the leaves. These primary lesions produce asexual conidia, which are rain-dispersed onto other leaves. Secondary lesions continue to produce conidia throughout the growing season. Severe scab infections will defoliate a tree, and that physiological stress predisposes it to other diseases and significantly reduces its return bloom and yield.

Management strategies for apple scab have predominantly relied on three approaches: sanitation, resistant cultivars, and chemical application (36). Sanitation involves hastening leaf decomposition via degrading compounds, plowing beneath the soil surface (5), or raking and controlled burning. While these methods reduce inoculum sources, they are not commonly used in large commercial orchards due to expense, time and environmental constraints. Scab-resistant cultivars, like Prima, Jonafree and Liberty, reduce disease intensity a great deal, but still require additional scab control measures during seasons of high disease pressure. Unfortunately, these cultivars have yet to achieve major commercial importance (16). Additionally, their lack of resistance to other early season apple diseases (such as powdery mildew and rusts) only saves a few sprays from the overall disease management program.

Of the management strategies, chemical application is the most common and successful control method (4,22,36). Within the last 100 years, advancements in chemistry have led to the development of several classes of fungicides including: carbamates, ethylenebisdithiocarbamates (EBDCs), phthalimides, dodine,

benzimidazoles, anilino-pyrimidines, sterol-inhibitors (SIs), and strobilurins (QoIs). Currently, SIs and QoIs are the dominant locally systemic fungicides used in commercial apple production.

SI fungicides inhibit targets within the fungal sterol biosynthesis pathway. This group is composed of four classes that differ in mode of action: demethylation inhibitors (DMIs), hydroxyanilides, amines, and squalene-epoxidase inhibitors (18). DMIs are the only class of practical importance in apple scab management. They are also the most extensively studied class of SI fungicides, as they are highly potent antifungal agents with significant structural flexibility and patentability (27). DMIs work by inhibiting the sterol C-14 α-demethylation of 24-methylenedihydrolanosterol, an ergosterol precursor (34). Ergosterol is a key component in fungal cell membranes, maintaining membrane fluidity and integrity. There is also evidence that suggests ergosterol may have regulatory functions in growth regulation and appressoria development (27). DMI fungicides can be further divided into four groups based on stereochemistry: piperazines, pyrimidines, imidazoles and triazoles (45). Although commercial formulations are mixtures of two or more isomers (compounds with the same number of atoms but differing in their arrangement), one isomer is generally more active (27).

Myclobutanil is one of the main SI fungicides used to manage apple scab. It was first described in 1986 and was registered for use in apple production in 1989 (27,38). Myclobutanil is classified as a DMI triazole and is sold as a racemic mixture of two isomers (50:50 ratio) (13) under the tradename Rally (and formerly Nova) for use on apple. It is often applied with a broad-spectrum fungicide like mancozeb (an EBDC). In addition to controlling apple scab, myclobutanil is highly effective against powdery

mildew, cedar-apple rust and quince rust. Myclobutanil's broad spectrum of disease control and its after-infection activity have established it as a powerful tool in apple scab management throughout the United States.

QoI fungicides inhibit mitochondrial respiration by binding to the Qo site of cytochrome b. This, in turn, blocks electron transfer between cytochrome b and cytochrome c_1 and halts ATP production. QoIs were first described in the early 1990's and include: azoxystrobin, kresoxim-methyl, metominostrobin, trifloxystrobin, picoxystrobin, orysastrobin, and pyraclostrobin (2,19). In Virginia, growers typically use kresoxim-methyl (tradename Sovran) or trifloxystrobin (tradename Flint) to control apple scab. Like myclobutanil, both have a broad spectrum of activity and exhibit after-infection activity. Unfortunately, the high specificity of QoI fungicides also means that a single nucleotide polymorphism in the cytochrome b gene will confer resistance. QoI resistance has been documented in several fungi including *Erysiphe*, *Blumeria* and *Mycosphaerella* species (2,15).

Fruit tree architecture and orchard layout have paralleled the reliance on chemical applications. Dwarfing rootstocks reduce extension shoot growth and promote a more horizontal branch orientation (16). When these smaller, more compact trees are planted in rows, growers obtain higher density orchards (more than 200 trees A^{-1}) that are less costly to harvest and easier to manage, in terms of achieving spray coverage and pruning. Despite this growing trend, however, less than half of Virginia's apple acreage is planted to that density (46). Studies have shown that in lower density plantings with larger trees (less than 150 trees A^{-1}; tree-row-volume of 40,600 m^3 ha^{-1}), spray deposition can vary widely due to the inverse relationship between foliar coverage and tree size (6,44). In

addition, growers using airblast sprayers to apply chemicals in lower density plantings may need to adjust nozzle placement and throughput more often as the field season progresses than growers with higher density plantings (33). Since apple scab is managed with frequent fungicide applications, insufficient spray coverage in larger trees is a serious concern.

Fruit tree architecture also impacts the microclimate and the distribution of inoculum within the tree canopy. A study in the Midwest found that the upper eastern portion of the apple tree canopy had the longest leaf wetness duration, while the lower western portion has the shortest (3). Other studies have shown the airborne ascospore concentration is not uniformly distributed within an orchard, within a block of the same cultivar, or within an individual tree canopy (7,9). During rain events, the airborne ascospore concentration and deposition decrease with increasing tree height, but both are greater in the tree center (7).

An even greater problem is that, within the last twenty years, some *V. inaequalis* populations have developed resistance to the SI and QoI fungicides. Published reports have detailed SI resistance in the United States (8,28,39,49) as well as in several countries around the world (12,23,24,32). Similarly, QoI resistance has been widely documented in *V. inaequalis* (14,17,30,34,41). SI and QoI fungicide resistance are a serious concern for three reasons: (i) past experience with dodine and benomyl resistance, (ii) the potential for resistance across different chemical classes, and (iii) the lack of knowledge concerning the SI resistance mechanism.

Dodine was developed in the 1950's and became an integral part of spray programs due to its after-infection activity. Its continued and exclusive use over several

years exerted selection pressure on populations of *V. inaequalis*, and by the 1970's, dodine resistance was widespread (25,26,48). Benomyl was developed in the 1960's and registered in 1973. Because of dodine resistance and the range of activity benzimidazoles had against other fungal diseases, benomyl was heavily used early on (27). Within four years, benomyl resistance was being reported across the northeastern United States (25). In both cases, inheritable resistance prompted the discontinuation of the fungicides.

Resistance across different chemical classes is also a concern. Isolates of *V. inaequalis* demonstrating resistance to one class of fungicides have been shown to develop resistance to an unrelated class at an accelerated rate (29). In addition, recent evidence suggests that SI resistance compromises the effectiveness of strobilurins in controlling apple scab post-infection (29,39). The phenomenon of multi-drug resistance (MDR) has not been widely associated with plant pathogens, although distinct MDR phenotypes of *Botrytis cinerea* have been identified in field populations for over twenty years (11,31). Not surprisingly then, the developments of dodine resistance and benomyl resistance in *V. inaequalis* populations were initially considered separate entities, implying that the mechanisms of resistance were specific to each fungicide class. As a result, management practices relied on the introduction of a new class to counteract resistance to an existing one (29). Resistance across different chemical classes reduces the lifetime efficacy of all fungicide classes involved. In the case of SIs and QoIs, it will reduce growers' chemical options for after-infection control of *V. inaequalis*.

Finally, phytopathogenic fungi developing resistance to SI fungicides are a serious concern because the genetic cause remains unresolved. In contrast to dodine and benomyl resistance, in which a single mutation resulted in tolerance, the mechanisms by

which *V. inaequalis* acquires resistance to SI fungicides do not appear to be as clear-cut. In some field strains, the presence of an upstream insertion and overexpression of the cytochrome P450 gene (which encodes an enzyme involved in the synthesis of ergosterol) were correlated with SI fungicide resistance (42). In other filamentous fungi, the upregulation of ATP-binding cassette transporters (which form energy-dependent efflux pumps in cell membranes) confers fungicide resistance (1,21,37,43). With *V. inaequalis*, DMI resistance is likely to be polygenic (20,35), as no single anomaly has been identified in all SI-resistant field isolates. Elucidating the genetic cause of SI resistance is important as certain mechanisms (such as upregulated ATP-binding cassette transporters) are more frequently associated with MDR (10, 31).

At the present time, apple scab is the most economically important disease of apples. Management programs rely heavily on SI and QoI fungicides, but populations of *V. inaequalis* are developing resistance to these chemical classes. The ultimate goal of this research is to increase our knowledge of the frequency, timing and mechanisms of fungicide resistance in order to improve existing and/or develop alternative management strategies.

Research Objectives

1. Evaluate the *V. inaequalis* populations in Virginia for SI and QoI fungicide resistance,

2. Monitor the temporal dynamics of SI fungicide resistance over multiple field seasons,

3. Examine the spatial distribution of SI fungicide resistance within the tree canopy in a lower density orchard, and

4. Investigate potential SI fungicide resistance mechanisms.

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Chapter 2. Resistance to myclobutanil in populations of *Venturia inaequalis* in Winchester, Virginia

The following chapter was formatted to facilitate publication in *Plant Health Progress*. This work was originally published by Marine, Schmale and Yoder online (November 2007). DOI:10.1094/PHP-2007-1113-01-RS of *Plant Health Progress*, a journal of Plant Management Network.

Resistance to myclobutanil in populations of *Venturia inaequalis* in Winchester, Virginia

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Marine, S.C., Schmale III, D.G., and Yoder, K.S. 2007. Resistance to myclobutanil in populations of *Venturia inaequalis* in Winchester, Virginia. Online. Plant Health Progress doi:10.1094/PHP-2007-1113-01-RS.

ABSTRACT

Sterol-inhibiting (SI) fungicides are widely used to manage apple scab, caused by *Venturia inaequalis*. However, recent observations indicate that populations of *V. inaequalis* in orchards in Virginia have developed resistance to myclobutanil and other SI fungicides. Little is known about the frequency and distribution of fungicide resistance in apple scab populations in Virginia. Isolates of *V. inaequalis* were collected from three different apple orchards in Winchester, VA in 2006. Orchards were treated with myclobutanil on 12 April, 19 April, 1 May, 30 May, and 7 July. The sensitivity of 87 single-spored isolates of *V. inaequalis* to myclobutanil was determined by monitoring their growth on agar dishes amended with 0, 0.1, 0.5, or 1.0 µg/ml myclobutanil. A relative continuum of fungicide resistance was observed: 16 isolates were resistant, 40 isolates were moderately resistant, and 31 isolates were sensitive to myclobutanil. After 28 days, the mean growth of isolates collected from trees treated with myclobutanil was significantly greater than that of isolates collected from non-treated trees at all concentrations of myclobutanil tested in vitro. High levels of fungicide resistance found in populations of *V. inaequalis* suggest that replacement programs may need to be developed to manage apple scab in Virginia.

INTRODUCTION

Fungicide resistance poses a significant threat to crop production worldwide (20). This stable, inheritable adjustment in pathogen populations to a fungicide limits the efficacy and lifetime use of the fungicide (5,6,7,11). Fungicide resistance can lead to an entire class of fungicides being abandoned (6), with replacement programs usually leading to higher control costs or decreased efficacy.

Apple scab, caused by *Venturia inaequalis* (Cooke) G. Winter, is a devastating disease of apple (*Malus domestica*) worldwide (13). The disease results in unsightly lesions on fruit, which significantly decrease their commercial value (13). Myclobutanil, a sterol-inhibiting (SI) fungicide, has been one of the common fungicides used to control apple scab because it provides adequate control of apple scab when applied within several days following infection (23). In Virginia, SI fungicides have been used commonly in commercial orchards for 15 or more years as pre-bloom and immediate post-bloom sprays to manage scab, powdery mildew (25), cedar-apple rust, and quince rust (24). The number of applications made annually in individual orchards is related to varietal susceptibility to these diseases and the intended market of the fruit as fresh or processed. In some cases these have been routine applications, planned as part of the early season disease management program; in other situations they are applied only on an "as needed" basis as supplements for after-infection control.

There have been published reports of resistance to myclobutanil in populations of *V. inaequalis* in New York (9,23) and Michigan (9). There is also evidence of resistance to myclobutanil in Canada (9) and other regions of the world (3,15,19). Recently, Yoder (26) observed that myclobutanil and other SI fungicides were losing effectiveness for

controlling apple scab in Virginia. An increased knowledge of the frequency and distribution of fungicide resistance in populations of *V. inaequalis* in Virginia is important for developing innovative, rational, and informed approaches to managing apple scab (10,18,20,21). Should resistance to myclobutanil persist in apple scab populations, alternative disease management strategies for apple scab must be developed.

Based on data collected by Yoder (26) and reports of apple scab resistance to the SI fungicides in the northeastern United States (9), we hypothesized that i) populations of *V. inaequalis* in Winchester, VA orchards show various levels of resistance to myclobutanil, and ii) isolates of *V. inaequalis* collected from apple trees treated with myclobutanil are less sensitive to myclobutanil than to isolates collected from non-treated trees. The specific objective of this study was to characterize the relative frequency of resistance to myclobutanil in *V. inaequalis* populations collected in Winchester, VA in 2006. An abstract on a portion of this work has been published (14).

MATERIALS AND METHODS

Experimental fields. Studies were conducted at the Virginia Tech Agricultural Research and Extension Center (AREC), near Winchester, VA in May, July, and August 2006. The AREC is located within 2 km of 300 ha of commercial apple orchards, most of which have been treated with SI fungicides for 15 years or more. Ethylene-bis-dithiocarbamate (EBDC) fungicides were usually combined with the SIs to broaden the disease spectrum and lengthen residual activity in these orchards. For more than 30 years, experimental and registered SI fungicides have been included in AREC fungicide tests directed at early season diseases, broad spectrum season-long management, or specific tests such as post-

infection control of scab, rusts, or powdery mildew. This test pattern resulted in SI fungicide applications in some of the AREC orchard blocks nearly every year for 15 to 20 years, although the applications were made to just a limited number of trees totaling less than a hectare. Approximately 15 ha of additional AREC plot areas used for entomological and horticultural research were treated with SIs in combination with an EBDC fungicide, as in a commercial schedule (16), since 1988.

Mature test trees were selected from three orchard blocks on AREC property. Each of the blocks was represented by the following single apple cultivars: Ramey York/M.9 rootstock trees planted in 1999, Fuji/M.9 rootstock trees planted in 1995, or Gala/M.26 rootstock trees planted in 2000. The Fuji block was located 100 m west of the York block, and the Gala block was located 500 m to the south of the York and Fuji blocks. Some trees within these test blocks had SI usage prior to 2006, but this was relatively minimal compared to usage in nearby research and commercial orchards. The Fuji block had received 12 SI applications in 2003-2005, the Gala block had received six SI applications 2001-2005, and the Ramey York block had been treated with SIs only four times, all in 2005. In 2006, all three blocks were treated with the same rate of myclobutanil (Nova 40W) on the same days. The untreated trees were either part of the same row of trees or were included in rows immediately adjacent to treated trees. Myclobutanil was applied with a high pressure handgun sprayer to runoff at a rate of 1.25 oz/100 gal (9.36 g/100 liters, at approximately 935 liters/ha), on 12 April (tight cluster-pink), 19 April (50% bloom), 1 May (petal fall), 30 May (first cover), and 7 July. Standard maintenance materials (16), applied to all trees in the test blocks on each application date, included esfenvalerate (Asana) oil, streptomycin (Agri-mycin),

methoxyfenozide (Intrepid), methomyl (Lannate), phosmet (Imidan), imidacloprid (Provado), thiacloprid (Calypso), and carbaryl (Sevin XLR) and ethephon (Ethrel). No fungicides were applied to the Fuji block from 1995 to 2003.

Collection of apple scab lesions and culturing of *V. inaequalis*. Four to five leaves containing at least one scab lesion were collected from individual non-treated or treated trees in each block on 16 May, 6 July, and 4 August 2006. Leaves were placed in individual plastic bags, stored temporarily in a cooler, and transferred to the laboratory where they were maintained in refrigerated storage until further processing. In total, leaves were collected from 45 treated and 26 non-treated trees.

One lesion per leaf was removed with a razor blade and streaked across 2% water agar containing 50 µg/ml tetracycline, chloramphenicol, and streptomycin sulfate. Dishes were incubated at 25°C for 24 h, at which time individual germinated conidia were transferred to ¼-strength potato dextrose agar (PDA) dishes. Single-spored isolates were incubated on ¼ PDA at room temperature for 4 to 5 weeks or until the colony was 1.5 cm or larger. A total of 87 single-spored isolates of *V. inaequalis* was collected and used in this study.

Assays for fungicide resistance. Sterile 5 ml pipet tips were used to remove 2 mm diameter agar plugs from the margins of fungal colonies growing on ¼ PDA. Individual agar plugs were transferred to the centers of ¼ PDA dishes amended with myclobutanil at 0, 0.1, 0.5, and 1.0 µg/ml. Isolates were incubated at 19°C and the radial growth of colonies was measured weekly for 4 weeks. The diameter of each isolate was measured

in two directions at a 90° angle, and the measurements were averaged. The test was replicated three times, and the colony growth for each isolate for each treatment was averaged across all three replications.

Statistical analyses. Analysis of variance (ANOVA) was used to test for significant differences among fungicide treatments, isolates collected from treated and non-treated trees, and isolate collection date. Data were analyzed using PROC GLM in SAS (Windows, release 8.02, SAS Institute Inc., Cary, NC).

RESULTS

Mean colony growth on PDA amended with myclobutanil was greater for isolates collected from treated trees than isolates collected from non-treated trees (Table 1). Most of the isolates grew less at higher concentrations of myclobutanil (Table 1). The mean colony growth of isolates was significantly different among fungicide rates measured at 7 ($P < 0.001$), 14 ($P < 0.001$), 21 ($P < 0.001$), and 28 days ($P < 0.001$). The mean colony growth of isolates collected from treated and non-treated trees was also significantly different when measured at 7 ($P = 0.013$), 14 ($P < 0.001$), 21 ($P < 0.001$), and 28 days ($P < 0.001$). However, the mean colony growth of isolates was not significantly different among collection dates when measured at 7 ($P = 0.764$), 14 ($P = 0.703$), 21 ($P = 0.401$), and 28 days ($P = 0.169$).

Isolates were classified as resistant, moderately resistant, or sensitive based on the percent growth reduction defined as the difference between colony growth on media amended with 0 and 1.0 µg/ml myclobutanil at 28 days. Isolates with equal growth across

all fungicide treatments were classified as resistant, and isolates with minimal growth at 0.1, 0.5, and 1.0 µg/ml were sensitive (Fig. 1). Isolates were classified as resistant if the percent growth reduction was less than 25%, moderately resistant if the percent growth reduction was between 25 and 50%, and sensitive if the percent growth reduction was greater than 50% (Table 2). Thirty-one isolates were classified as sensitive, 40 isolates were moderately resistant, and 16 were resistant (Table 2). The frequency of resistant isolates was greater from treated trees (87.5%) than non-treated trees (12.5%).

DISCUSSION

The findings of this study demonstrate that isolates of *V. inaequalis* from the AREC near Winchester, VA vary in their resistance to myclobutanil. Though we observed a continuum of fungicide resistance in our populations, the majority of the isolates were classified as either moderately resistant or resistant to myclobutanil, with the remainder classified as sensitive. Colony growth varied among isolates, fungicide rates, and between replicate growth tests. This is the first detailed report of fungicide resistance to myclobutanil in populations of *V. inaequalis* in Virginia, and to our knowledge, the first report of SI resistance in apple scab from orchards in the southeastern United States. This study represents an important baseline for monitoring fungicide resistance to myclobutanil in apple scab populations in Virginia, and can contribute to the knowledge of fungicide resistance in apple scab populations on a global basis (3,9,15,19,23).

Our data support our hypothesis that populations of *V. inaequalis* in Virginia show varying levels of fungicide resistance to myclobutanil. The percent growth

reduction of 87 isolates ranged from 6 to 82 percent on 1.0 µg/ml myclobutanil. Our data also support our hypothesis that isolates of *V. inaequalis* collected from apple trees treated with myclobutanil have higher levels of resistance compared to isolates collected from non-treated trees. The majority of isolates classified as resistant to myclobutanil (87.5%) were collected from treated trees; 12.5% of isolates from non-treated trees were classified as resistant.

Fungicide resistance has been determined using sensitivity assays on agar media amended with varying levels of fungicide (8,9,10), using microscopic analysis of conidiophore formation, and measurements of germ tube length (12). Kunz et al. (12) compared microscopic analysis to other methods frequently used to evaluate fungicide sensitivity, and concluded that all of the methods were indistinguishable from each other when baseline sensitivities were determined. Thus, our results can be compared to others that are using similar methodologies to assay for fungicide resistance.

The frequency of myclobutanil-resistant isolates of *V. inaequalis* collected from treated trees was higher than in isolates collected from non-treated trees. This might be due, at least in part, to the exposure of isolates from treated trees to treatments of myclobutanil over the course of the growing season. Köller and Wilcox (10) observed a similar trend with the SI fungicide fenarimol. They showed that the frequency of fenarimol-resistant isolates significantly increased over three consecutive growing seasons in a New York apple orchard following 12 fungicide applications. If the selection pressure for myclobutanil resistance is similar to fenarimol resistance, then 12 or more applications of myclobutanil or myclobutanil-based sprays may select for higher levels of fungicide resistance. As part of our ongoing research program, we are examining the

most appropriate time to select scab lesions that have been treated with myclobutanil, but were least controlled. This would be the most likely way to detect a resistant population, given our limited ability to forecast the onset of scab fungicide resistance beyond an individual growing season.

We found no evidence that the collection date influenced fungicide resistance in our populations. Consequently, resistance assays may be conducted at any time of the year. The lack of an observed increase in fungicide resistance may be due to the aerial transport of spores from adjacent orchard blocks (1,2), or recirculation of spores from baseline inoculum within the sampled block (4). In addition, it is possible each collection date consisted of both older and newer lesions (13), since all lesions were not removed from a tree and older sampled lesions were not labeled as such. The selection pressure also may not have been high enough to favor one resistant genotype over another.

The majority of the *V. inaequalis* isolates collected for this study were classified as either moderately resistant or resistant to myclobutanil. Since fungicide resistance can persist in apple scab populations, alternative disease management strategies for apple scab may be needed. Restricting the use of myclobutanil and other SIs for a period of years may help lower *V. inaequalis* resistance in orchards with an extensive myclobutanil history, but to our knowledge, detailed studies examining this phenomenon have not been conducted. However, SIs have also been highly effective and widely used in Virginia for control of powdery mildew, cedar-apple rust and quince rust, and these uses would continue to exert selective pressure on the fungal population.

Future research may help to identify new tools (e.g., on-site real-time PCR) to rapidly detect and monitor fungicide resistance in populations of *V. inaequalis* (17,22).

Additional knowledge about the frequency, timing, and selective processes contributing to SI resistance will be necessary to identify effective and appropriate management strategies for apple scab in the future.

ACKNOWLEDGEMENTS

We thank the Virginia Agricultural Council and Virginia Apple Research Program for financial support; and A. E. Cochran II, W. S. Royston, Jr., and S. W. Kilmer for technical support. We thank A. Baudoin for a critical review of the manuscript.

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Table 2.1. Average colony diameter (cm) of 87 isolates of *Venturia inaequalis* on PDA amended with varying levels of myclobutanil

Measurement of colony growth[x]	**Myclobutanil concentration and source of isolates**							
	0 µg/ml		0.1 µg/ml		0.5 µg/ml		1.0 µg/ml	
	Treated							
	No[y]	Yes[z]	No[y]	Yes[z]	No[y]	Yes[z]	No[y]	Yes[z]
7 days	0.588	0.458	0.495	0.422	0.479	0.442	0.432	0.417
14 days	1.034	0.850	0.693	0.658	0.685	0.517	0.542	0.583
21 days	1.514	1.438	0.955	0.992	0.937	0.833	0.710	0.742
28 days	2.000	2.338	1.312	1.483	1.263	1.175	0.986	1.067

[x] The radial growth of colonies of *V. inaequalis* was measured at 7 days, 14, 21, and 28 following dish inoculation.

[y] Isolates were collected from 26 trees that had not been treated with myclobutanil during the sampling year.

[z] Isolates were collected from 45 trees that had been treated with myclobutanil (Nova 40W) at a rate of 1.25 oz/100 gal dilute, applied to runoff. Fungicide treatments were applied on 12 April (tight cluster-pink), 19 April (50% bloom), 1 May (petal fall), 30 May (first cover), and 7 July.

Table 2.2. Classification of fungicide resistance of 87 isolates of *Venturia inaequalis* based on percent growth reduction between 0 and 1.0 µg/ml myclobutanil at 28 days

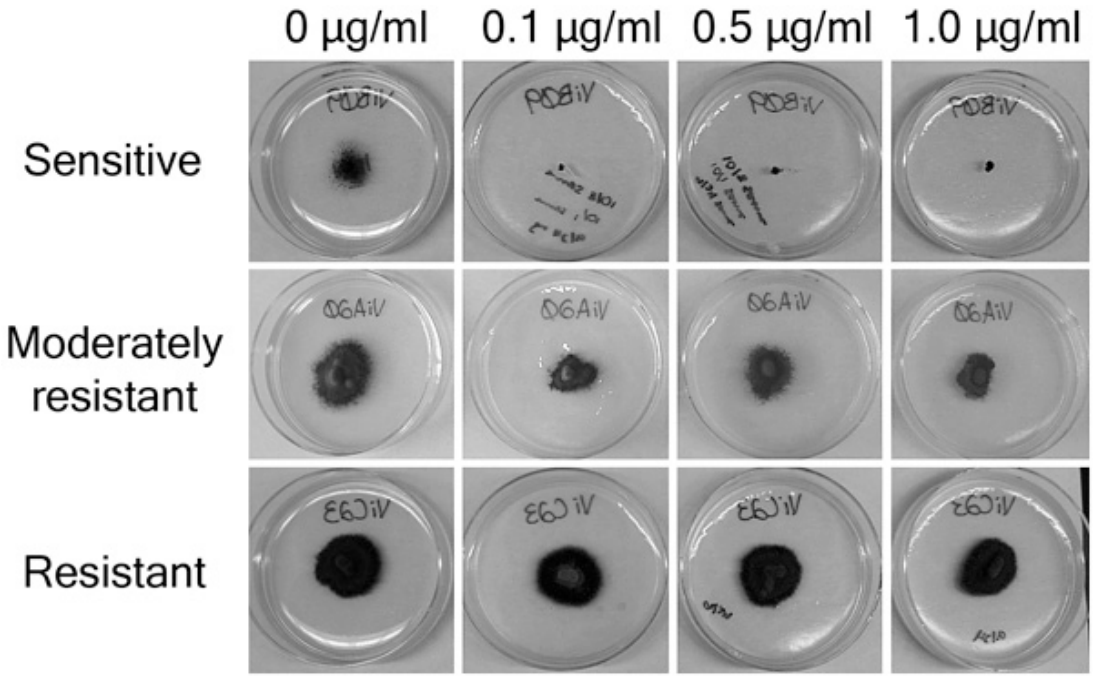
Classification[x]	Total number of isolates	Percentage of isolates from treated trees[y]	Percentage of isolates from non-treated trees[z]
Sensitive	31	28% (17/61)	54% (14/26)
Moderately resistant	40	49% (30/61)	38% (10/26)
Resistant	16	23% (14/61)	8% (2/26)

[x] Isolates were classified as resistant if the percent growth reduction was less than 25%, moderately resistant if the percent growth reduction was between 25 and 50%, and sensitive if the percent growth reduction was greater than 50%.

[y] Isolates were collected from 45 trees that had been treated with myclobutanil (Nova 40W) at a rate of 9.36 g/100 liters dilute at approximately 935 liters/ha (1.25 oz/100 gal dilute at approximately 100 gal/A), applied to runoff. Fungicide treatments were applied on 12 April (tight cluster-pink), 19 April (50% bloom), 1 May (petal fall), 30 May (first cover), and 7 July.

[z] Isolates were collected from 26 trees that had not been treated with myclobutanil during the sampling year.

Figure 2.1. Classification of fungicide resistance to myclobutanil in three representative isolates of *Venturia inaequalis* from Virginia. Isolates were classified as sensitive (top row), moderately resistant (middle row), or resistant (bottom row) to myclobutanil at four different myclobutanil concentrations in agar.



Chapter 3. Seasonal distribution of sterol inhibitor (SI) fungicide resistance and isolate recovery in populations of *Venturia inaequalis* in Winchester, Virginia

The following chapter was formatted to facilitate publication in *Plant Disease*. This work was originally submitted by Marine, Schmale and Yoder on March 8, 2012 to *Plant Disease*, a journal of the American Phytopathological Society.

Seasonal distribution of sterol inhibitor (SI) fungicide resistance and isolate recovery in populations of *Venturia inaequalis* in Winchester, Virginia

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Marine, S.C., Schmale III, D.G., and Yoder, K.S. 2012. Seasonal distribution of sterol inhibitor (SI) fungicide resistance and isolate recovery in populations of *Venturia inaequalis* in Winchester, Virginia. Plant Disease XX: XXX-XXX.

ABSTRACT

Sterol-inhibiting (SI) fungicides are widely used to manage apple scab, caused by *Venturia inaequalis*. Long-term reliance on chemical applications has resulted in populations of *V. inaequalis* developing resistance to myclobutanil and other SI fungicides. Little is known about the frequency and distribution of SI fungicide resistance in populations of *V. inaequalis* in Virginia over sequential years. Isolates of *V. inaequalis* were collected from SI-treated and non-treated trees in three orchard blocks in Winchester, Virginia from 2006 through 2010. Fungicide plate assays with a discriminatory dose of 1.0 µg ml^{-1} myclobutanil were used to evaluate resistance. A range of sensitivities was seen at each collection year, and the average percent growth suppression was similar for *V. inaequalis* isolates collected from treated and non-treated trees of the same cultivar. Isolate recovery (defined as the percent of viable conidia that produced colonies) exhibited a declining pattern of seasonality (May = June > July > August) but pure cultures were obtained consistently in the late season. There was a significant association between the number of resistant isolates and the year, with 2006 having a higher frequency of resistant isolates than all other years combined. Of the 255 isolates tested, only 8% (20/255) were classified as resistant. The adjustment in sampling method in 2007 (i.e. selecting fresh scab lesions based on shoot growth) did coincide with *V. inaequalis* collections that contained a greater proportion of sensitive isolates. The

seasonal variability of isolate recovery and the use of mixed populations in sensitivity tests have important implications for disease management. This is the first report on the distribution of SI fungicide resistance over sequential growing seasons in Virginia.

INTRODUCTION

Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cooke) G. Winter, is an economically devastating disease of apples that occurs wherever apples are grown (3). The disease causes unsightly lesions on foliage and fruit, which not only undermines the health of the tree, but also significantly decreases the apples' fresh market value (15). Management strategies for scab have predominantly relied on chemical applications, and advancements in chemistry have led to the development of several classes of fungicides. Currently, two locally systemic fungicide classes (sterol-inhibiting (SI) fungicides and strobilurin (QoI) fungicides) are commonly used for apple scab management in commercial apple production.

In Virginia, SI fungicides have been used in commercial orchards for more than 20 years as pre-bloom and immediate post-bloom sprays to manage a variety of diseases including scab, powdery mildew (22), cedar-apple rust, and quince rust (21). Due to this broad spectrum of disease control and after-infection activity, SI fungicides such as myclobutanil (Nova/Rally) have established themselves as powerful tools in scab management.

Unfortunately, the extensive, long-term use of this class of fungicides has led to the development of resistance within the United States (4,11,16,18) and worldwide (6,8,9,14). In Virginia, initial reports showed SI fungicides were losing effectiveness for

controlling scab (23) and that the majority of isolates collected in a given season were resistant to standard rates of myclobutanil (16).

Surprisingly, the development of SI-resistance in Virginia was delayed more than a decade as compared to other apple-producing regions such as Michigan (11) and New York (11), despite the high scab pressure. Studies examining fungicide efficacy in the same location over multiple years provide valuable data on local disease pressure, spray programs and future pathogen concerns. Köller's group in New York, for example, modified their control recommendations to advocate mixing SIs with dodine to manage SI-resistant scab populations (13), based on more than twenty years of data from experimental and commercial orchards. Similarly, increased knowledge of the seasonal distribution of SI fungicide resistance in Virginia would aid us in our control recommendations.

The overall goal of this study was to characterize SI fungicide resistance in the *V. inaequalis* population in Virginia and adjust grower-recommended management strategies accordingly. We hypothesized that 1) even at a single geographic location, levels of resistance would vary from year to year, and 2) the temporal change at the location would be a gradual shift toward increased resistance, evident over time. The specific objectives of this study were: i) to document the frequency of SI fungicide resistance in fungal isolates in Winchester, Virginia, in orchard blocks exposed to the same concentration of myclobutanil over sequential years and to ii) identify potential environmental and artificial factors related to those observed patterns of SI fungicide resistance.

MATERIALS AND METHODS

Experimental orchards. Three orchard blocks located at the Alson H. Smith, Jr., Agricultural Research and Extension Center (AHS AREC) in Winchester, Virginia were used for this study (20). The AREC is located within 2 km of 300 ha of commercial apple orchards, and the orchard blocks used in this study were surrounded by approximately 15 ha of entomological and horticultural experimental orchards (Figure 3.1). A total of 55 mature trees (37 treated, 18 non-treated) were selected from each block, and each block represented a single apple cultivar: Fuji, Ramey York, or Gala. All three blocks were treated with 1.25 oz 100 gal^{-1} (9.36 g 100 $liters^{-1}$) of myclobutanil (Nova/Rally 40W; Dow AgroSciences, Indianapolis), applied to runoff. Myclobutanil application was based on growth stage, and all trees received the fungicide application on the same days from 2006 through 2010 (Table 3.1). The intervals between fungicide applications were adjusted based on recorded scab infection periods to allow some infection for collection of isolates. The environmental conditions were recorded by a weather station 150m from the Gala block. The non-treated trees in each block were either part of the same row of trees or were included in rows immediately adjacent to treated trees. Standard maintenance materials to control other diseases and insects were applied to all trees in each test block, as needed (17).

Monitoring of shoot growth. In order to identify potential infection events for a cohort of lesions, shoot growth was monitored on several branches of treated and non-treated trees within each block starting in 2007. It allowed us to select lesions from newly grown leaves since the previous sample collection. Neon rubber bands were placed on the

actively growing shoot tips following myclobutanil application or sample collection (Figure 3.2). Leaves sampled during a collection were selected based on the mean number of new leaves for that cultivar and the location of those leaves on the reference shoots.

Sampling of orchards. Diseased leaves were collected from individual treated and non-treated trees on: 16 May, 6 July and 4 August in 2006; 24 May, 13 June, 11 July and 14 August in 2007; 8 May, 6 June and 10 July in 2008; 18 May, 2 and 16 June, and 1 and 30 July in 2009; and 4 and 21 May and 30 June in 2010. A total of 34 treated and 20 non-treated trees at the AHS AREC were used. Diseased leaves collected in May 2010 from a group of isolated wild apple seedling trees in Sand Patch, Pennsylvania, provided *V. inaequalis* isolates (N=3) that had never been treated with fungicides and were representative of a wild-type population.

Sensitivity tests. Previously described methods of culturing monoconidial isolates of *V. inaequalis* and conducting sensitivity tests based on percent growth suppression (PGS) were used (16). PGS was defined as the difference in colony growth on media amended with 0 and 1.0 μg ml^{-1} myclobutanil at 28 days and was determined with at least two replicates per isolate. Isolates were classified as resistant if they had a PGS less than 25%, moderately resistant if they had a PGS between 25% and 50%, and sensitive if they had a PGS greater than 50%. Additional doses of myclobutanil (0.1 and 0.5 μg ml^{-1}) were tested with each isolate. Isolates were incubated at 19°C and colony diameters were measured bi-directionally every week for 4 weeks. The number of isolates tested per year

ranged from 9 to 87. Mycelium for sensitivity tests originated from monoconidial isolates from one lesion per sampled leaf. A total of 255 single-spored isolates of *V. inaequalis* was used in this study.

Data analysis. Prior to analysis, growth measurement data and percent growth suppression data were transformed using a square-root transformation and an angular transformation (arcsine$\sqrt{proportion}$), respectively. For the analysis to examine the effect of amended media on mycelium growth, a mixed model (JMP for Macintosh, version 9.0, SAS Institute Inc., Cary, NC) was used. Assay treatment factor (myclobutanil concentration), location (orchard) and collection year were considered as fixed factors, and replication was considered a random factor. For the analysis to determine the effect of environmental and artificial factors on the growth of isolates on the amended media (1.0 µg ml^{-1} myclobutanil), a stepwise regression model (*P*-value threshold = 0.25, mixed direction) was used to examine potential combinations of independent variables to explain the variability of the dependent variable (growth measurement or percent growth suppression) using JMP. Two-way interaction of the independent variables was also examined. The resulting models were then examined by fitting the model to the dataset, and the final linear regression model for each dependent variable was selected based on: the degree of model fit to the data (R^2, BIC and AIC), examination of residuals, and biological soundness. Then the final models were fit to the data using a linear least square regression.

RESULTS

Within season SI sensitivities. Generally, a range of resistance was seen at each collection date and each collection year, regardless of tree treatment. The mean colony growth of each isolate was significantly different ($P < 0.001$) among assay treatments (0, 0.1, 0.5 or 1.0 μg ml^{-1} myclobutanil) and assay times (7, 14, 21 or 28 days) within a given season. PGS values ranged from: 6.4 to 81.8 (average 41.7) in 2006, 24.4 to 86.4 (average 54.3) in 2007, 18.4 to 90.8 (average 70.9) in 2008, 44.3 to 85.8 (average 65.4) in 2009, and 10.9 to 83.8 (average 58.1) in 2010. All years were more resistant (i.e. had lower PGS values) to the discriminatory dose of myclobutanil than the wild-type isolates, which had a narrow PGS range of 86.3 to 91.2 (average 86.2).

SI sensitivities across all seasons. When pooled data were analyzed, the mean colony growth on 1.0 μg ml^{-1} myclobutanil at 28 days was significant different among cultivars ($P = 0.006$), whether the isolate was collected from a treated or non-treated tree ($P = 0.012$), and collection year ($P < 0.001$). Tukey's HSD test indicated isolates collected from York trees had the highest mean colony growth on 1.0 μg ml^{-1} myclobutanil while those from Gala trees had the lowest (Table 3.2). Isolates collected from treated trees tended to grow better on myclobutanil-amended plates than isolates collected from non-treated trees. The mean colony growth on the discriminatory dose of myclobutanil was also significant different among years, with 2006 having the highest and 2008 having the lowest (Table 3.2).

Generally, the population of *V. inaequalis* isolates had a right-skewed distribution, and the frequency of PGS values for isolates collected from treated trees was

similar to those collected from non-treated trees (Figure 3). In 2006, when any diseased leaf was sampled, about one-fifth of isolates had PGS values ranging from 0 to 30 (Figure 3.2). The population also exhibited a bimodal distribution with the two peaks representing the highest frequency of isolates collected from non-treated trees (PGS value of 30) and treated trees (PGS value of 70). After implementation of shoot growth monitoring, the frequency of isolates with the PGS values ranging from 0 to 30 decreased, the frequency of isolates with PGS values ranging from 80 to 100 increased, and the population normalized (Figure 3.3).

Characterization of isolates. Of the total 255 *V. inaequalis* isolates evaluated, approximately 63% (160/255) were classified as sensitive, 29% (75/255) as moderately resistant and 8% (20/255) as resistant. Each classification category was composed of a similar proportion of isolates collected from each cultivar (Table 3.3) and from treated and non-treated trees (Table 3.4).

PGS was significantly different among cultivars ($P = 0.023$) and year ($P < 0.001$). Tukey's HSD test indicated isolates collected from Gala trees had the highest PGS (i.e. were more sensitive) while those from York trees had the lowest (i.e. were more resistant) (Table 3.5). In terms of collection year, Tukey's HSD test indicated 2006 had the lowest PGS values and 2008 had the highest (Table 3.5). The interaction between year and tree treatment (i.e. whether or not the isolate originated from a treated or non-treated tree) was tested and found to be positive ($P = 0.008$). The interaction of collection month and tree treatment was also tested and found to be positive ($P = 0.033$). Further examination of these interactions is needed.

Plotting the mean colony growth on 1.0 μg ml^{-1} myclobutanil at 28 days against the PGS for each isolate indicated the three populations (sensitive, moderately resistant and resistant) were generally well separated (Figure 3.4). Outliers with higher mean colony growth tended to originate from treated trees and late season (July or August) collections.

Isolate recovery. Isolate recovery exhibited a pattern of seasonality (Figure 3.5). Although an average of 258 leaves were sampled per month per year, the percent of viable conidia that produced colonies was highly variable. Isolate recovery was greatest in May and June at 51% (703/1382 and 696/1370, respectively) and lowest in August at 14% (75/554) (Table 3.6). Although similar success was seen for leaves collected from SI-treated and non-treated trees (Figure 3.4), overall isolate recovery increased with each subsequent year from a low of 20% in 2006 to a high of 55% in 2009 (Figure 3.6).

DISCUSSION

This 5-year study showed that SI fungicide resistance in *V. inaequalis* populations in Winchester, Virginia, is both diverse and randomly distributed over time. A range of sensitivities was seen at each collection month and year, and colony growth rate on myclobutanil amended media varied among isolates, fungicide rates and assay replications. The majority of the isolates were classified as sensitive based on the discriminatory dose of myclobutanil (1.0 μg ml^{-1}), and we suspect that the continued presence of non-treated trees within each orchard block and throughout the AHS AREC may serve as a reservoir for sensitive inoculum.

However, when the average PGS values of these isolates were compared to wild-type *V. inaequalis* isolates, it was apparent the baseline sensitivity at the AHS AREC had shifted and isolates from that population were less sensitive to the fungicide. The scab population at the AHS AREC has been exposed to the SIs for more than 30 years, and some orchard blocks have received fungicide applications nearly every year for 20 years. Other studies have found a positive correlation between the number of SI applications and the sensitivity of *V. inaequalis* to those fungicides (7,12), and we have documented that resistant isolates are present in our populations (16).

Isolates originating from York and Fuji trees were significantly different from isolates from Gala trees in regards to mean colony growth on 1.0 $\mu g\ ml^{-1}$ myclobutanil and PGS. Isolates collected from York and Fuji trees grew better on the discriminatory dose of myclobutanil and had lower PGS values, whereas isolates collected from Gala trees had lower mean colony growth and higher PGS values. The difference amongst cultivars is probably more related to the geographical location of the trees, as the York and Fuji trees are bordered by apple trees in a standard management program (i.e. fungicides applied on a 10-14 day schedule) so the selection pressure on that scab population is greater. In contrast, the Gala trees are relatively isolated (being located 500m to the south of the York and Fuji trees) and are immediately adjacent to an orchard block in which alternate rows remain non-treated the duration of the season.

Percent growth suppression – referred to as relative growth (RG) values in other publications (9,11) – was used to determine fungicide sensitivity, with classification based on a discriminatory dose of myclobutanil. Previous work by Köller et al. demonstrated that RG values could be used to quantify fungicide sensitivity because of

their correlation with ED_{50} values (11). Although this study used a higher discriminatory dose of myclobutanil than previously published studies (11, 18), it separated the population into three distinct groups (sensitive, moderately resistant, and resistant isolates).

V. inaequalis isolates within each classification that had greater colony growth on 1.0 μg ml^{-1} myclobutanil tended to originate from treated trees and late season collections. The prevalence of competing leaf microflora later on in the field season (1,20) and the decline in favorable environmental conditions (15) may have contributed to these observed outliers. Though research in other pathogen-fungicide combinations has found insensitive isolates may have a lower (10), similar (5) or higher fitness (2) than sensitive isolates, fitness parameters measured usually do not include heat tolerance. To our knowledge, no published reports have determined the fitness cost of SI-resistant *V. inaequalis* isolates.

Of the isolates classified as resistant to 1.0 μg ml^{-1} myclobutanil, the majority (16 of 20) was collected in 2006. We suspect the discrepancies in SI fungicide resistance between 2006 and the following years may be due to sampling technique and changes in commercial grower management practices in nearby orchards. Collections in 2006 consisted of a mixture of older and newer lesions, as all lesions were not removed from a tree and older sampled lesions were not labeled as such. In contrast, collections in 2007 through 2010 consisted only of newer lesions, as leaf selection was based on the average shoot growth for each cultivar since the last collection. Because the spray interval was extended to allow scab infection to occur, myclobutanil may have had more of a fungistatic effect on newer lesions (which would have only been exposed to a single

fungicide application), thereby allowing sensitive isolates to remain in the population and sporulate between applications. Additionally, commercial growers in the vicinity had stopped using the SIs following scab control failures in 2004-05, and this change in management practices reduced selection pressure and may have further diluted the resistant scab inoculum drifting into the nearby research trees in subsequent years.

We found evidence that collection date influenced the success rate of isolate recovery. Although more than a hundred leaves were sampled each month, isolate recovery rate was highest early in the field season and sharply declined after June, regardless of year or tree treatment. Isolate recovery rate was almost identical in May and June, and we suspect this was due to continued tree growth and frequent secondary infections. In contrast to studies in the Midwest (19), which showed unsuccessful isolation at the end of the season, we were able to reliably culture 28% of isolates in July and 14% of isolates in August. We observed an increase in leaf contaminants later in the season, but were able to successfully isolate *V. inaequalis* isolates with the inclusion of antibiotics in the media and by reducing the time between streaking leaves on water agar and transferring germinated conidia. The difference in isolate recovery rate may also be due to differences in environmental factors (daily temperature fluctuations, humidity levels, and frequency and length of rain events) and disease development (ability of primary inoculum to survive winter conditions, susceptibility of planted apple varieties, and number of secondary infections) between the Midwest and the Mid-Atlantic.

Although we included only newer lesions in our 2007-2010 sensitivity tests, scabby leaves collected for evaluation typically represent mixed populations due to random sampling. This has important implications for disease management as fungicide

recommendations are based on population estimates. Evaluations based on newer lesions allow researchers to monitor the selection pressure of each fungicide application on the population's sensitivity, while evaluations based on mixed populations allow researchers to examine the overall impact of a spray program on fungicide resistance. We should note, however, that the development of SI resistance is incremental and further investigation on the influence of sampling technique is needed.

Our results indicate that myclobutanil is still an effective compound for control of apple scab in many areas of Virginia. The population's baseline has shifted toward increased resistance, but this gradual progression is masked in the variability from year to year. Growers must continue to alternate different chemical classes during the season to successfully manage scab and minimize the selection for SI fungicide resistance. A recent study also found that orchards using dormant copper applications were less likely to have scab populations with resistance to multiple fungicides in the SI chemical class (18). Growers may want to consider this practice for orchards with a history of SI control failures.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Virginia Agricultural Council and the Virginia Apple Research Program. We thank Dow AgroSciences for providing the commercial and technical grade fungicide. We thank A.K. Wood-Jones, A.E. Cochran II, W.S. Royston, Jr., and S.W. Kilmer for technical support. We thank A. Baudoin for a critical review of the manuscript, and M. Nita for statistical analysis.

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Table 3.1. Spray schedule for treated trees at AHS AREC in Virginia from 2006-2010.

	Myclobutanil Spray Schedule[b]				
Growth Stage[a]	**2006**	**2007**	**2008**	**2009**	**2010**
Tight cluster-pink	April 12	April 18	April 17	April 17	
50% bloom	April 19				
Petal fall	May 1	May 2		May 5	April 20
First cover	May 30	June 7	May 13	May 20	May 5
Second cover	July 7	July 23	May 29	June 3	May 25
Third cover			June 12	June 18	June 8
Fourth cover			June 25	July 2	July 22
Fifth cover			July 11	July 17	
Sixth cover			August 1	August 3	

[a] Growth stage refers to the chronological development of deciduous fruit trees and is used to time chemical applications. At tight cluster-pink, the blossom buds are exposed but tightly grouped. First cover is approximately 2 weeks after petal fall, and subsequent cover sprays occur at 10-20 day intervals thereafter.

[b] Myclobutanil was applied to runoff at a rate of 1.25 oz 100 gal^{-1} (9.36 g 100 $liters^{-1}$). Treatments were applied on the same day to all treated trees.

Table 3.2. Mean colony growth of *Venturia inaequalis* isolates by cultivar, tree treatment and year.

Factor	Level	Mean colony growth[a]
Cultivar	Fuji	0.993 ab
	York	1.034 a
	Gala	0.799 b
Tree treatment[b]	Treated	0.972 a
	Non-treated	0.933 b
Collection year	2006	1.081 a
	2007	1.021 ab
	2008	0.682 d
	2009	0.832 bcd
	2010	0.885 c

[a] Mean colony growth on 1 μg ml^{-1} myclobutanil at 28 days. Data transformed prior to analysis using a square-root transformation. Mean separation by Tukey's HSD test (p=0.05).

[b] Myclobutanil was applied to runoff at a rate of 1.25 oz 100 gal^{-1} (9.36 g 100 $liters^{-1}$). Treatments were applied on the same day to all treated trees.

Table 3.3. Characterization of *Venturia inaequalis* isolates from different years and cultivar locations in Virginia.

Year	Cultivar and field location at AHS AREC[a]	No. of isolates from: Treated trees[b]	Non-treated trees[c]	Total	Isolates classified as (%)[d] Sensitive	Moderately resistant	Resistant
2006	Fuji, Block 8	18	8	26	35	42	23
	York, Block 7	37	12	49	31	53	16
	Gala, Block 30 South	5	7	12	58	25	17
2007	Fuji, Block 8	11	9	20	70	30	0
	York, Block 7	31	9	40	58	40	2
	Gala, Block 30 South	9	6	15	60	40	0
2008	Fuji, Block 8	4	4	8	75	25	0
	York, Block 7	2	8	10	90	0	10
	Gala, Block 30 South	29	3	32	100	0	0
2009	Fuji, Block 8	3	3	6	100	0	0
	York, Block 7	0	0	0	-	-	-
	Gala, Block 30 South	3	0	3	33	67	0
2010	Fuji, Block 8	10	0	10	100	0	0
	York, Block 7	13	7	20	78	11	11
	Gala, Block 30 South	2	2	4	75	25	0
Total		177	78	255	63	29	8

[a] AHS AREC is located in Winchester, Virginia. Block 8 was located 100 m west of Block 7, and Block 30 South was located 500m to the south of Blocks 7 and 8.

[b] Myclobutanil was applied to runoff at a rate of 1.25 oz 100 gal^{-1} (9.36 g 100 $liters^{-1}$). Treatments were applied on the same day to all treated trees.

[c] Non-treated trees were either part of the same row of trees or were included in rows immediately adjacent to treated trees.

[d] Classification was based on the percent growth suppression, which was defined as the difference in colony growth on media amended with 0 and 1 μg ml^{-1} myclobutanil at 28 days. Sensitive isolates had a PGS >50%, moderately resistant isolates had a PGS of 25-50%, and resistant isolates had a PGS <25%.

Table 3.4. Classification of *Venturia inaequalis* isolates by tree treatment.

Classification[a]	% isolates from Treated trees[b]	Non-treated trees
Sensitive	61% (108/178)	68% (52/77)
Moderately resistant	30% (54/178)	27% (21/77)
Resistant	9% (16/178)	5% (4/77)

[a] Classification was based on the percent growth suppression, which was defined as the difference in colony growth on media amended with 0 and 1 μg ml^{-1} myclobutanil at 28 days. Sensitive isolates had a PGS >50%, moderately resistant isolates had a PGS of 25-50%, and resistant isolates had a PGS <25%.

[b] Myclobutanil was applied to runoff at a rate of 1.25 oz 100 gal^{-1} (9.36 g 100 $liters^{-1}$). Treatments were applied on the same day to all treated trees.

Table 3.5. Mean percent growth suppression of *Venturia inaequalis* isolates by cultivar and year.

Factor	Level	Mean PGS[a]
Cultivar[b]	Fuji	0.819 ab
	York	0.757 b
	Gala	0.939 a
Collection year	2006	0.695 a
	2007	0.831 b
	2008	1.001 b
	2009	0.948 b
	2010	0.885 b

[a] Percent growth suppression (PGS) based on the difference in colony growth on media amended with 0 and 1 μg ml^{-1} myclobutanil at 28 days. Data transformed prior to analysis using an angular transformation (arcsine$\sqrt{proportion}$). Mean separation by Tukey's HSD test (p=0.05).

[b] Fuji trees located 100 m west of York trees, and Gala trees located 500m to the south of both Fuji and York trees.

Table 3.6. Recovery of *Venturia inaequalis* isolates from different sampling months and years.

Year	Sampling date	Leaves collected[a]	Viable conidia[b]	Colonies obtained[c]	Percent isolation success from: Treated tree leaves[d]		Non-treated tree leaves[e]		Total	
2006	May 16	165	115	22	33	(15/45)	10	(7/70)	19	(22/115)
	July 6	230	210	40	16	(25/155)	27	(15/55)	19	(40/210)
	August 4	276	276	57	18	(34/192)	27	(23/84)	21	(57/276)
2007	May 24	278	240	131	48	(74/155)	67	(57/85)	55	(131/240)
	June 13	306	300	180	63	(121/192)	55	(59/108)	60	(180/300)
	July 11	306	240	19	9	(17/192)	4	(2/48)	8	(19/240)
	August 14	306	278	18	5	(10/186)	9	(8/92)	6	(18/278)
2008	May 8	94	85	68	85	(44/52)	73	(24/33)	80	(68/85)
	June 6	318	317	100	32	(61/191)	31	(39/126)	32	(100/317)
	July 10	312	263	80	28	(43/154)	34	(37/109)	30	(80/263)
2009	May 18	330	306	231	73	(153/209)	80	(78/97)	76	(231/306)
	June 2	360	299	212	65	(130/201)	84	(82/98)	71	(212/299)
	June 16	360	350	166	51	(110/216)	42	(56/134)	47	(166/350)
	July 1	360	350	189	56	(119/212)	51	(70/138)	54	(189/350)
	July 30	354	198	24	14	(13/92)	10	(11/106)	12	(24/198)
2010	May 4	326	316	157	47	(101/213)	54	(56/103)	50	(157/316)
	May 21	325	320	94	33	(71/214)	22	(23/106)	42	(94/320)
	June 30	149	104	38	35	(21/60)	39	(17/44)	37	(38/104)
Total		5155	4567	1826	40	(1162/2931)	40	(653/1636)	40	(1826/4567)

[a] Leaves with apple scab lesions were collected from SI-treated and non-treated trees at the AHS AREC in Winchester, Virginia.

[b] One lesion per leaf was used to obtain conidia. Viable conidia germinated on antibiotic-amended water agar after 24 hours at 21°C.

[c] One viable conidium per leaf was transferred onto potato dextrose agar. Assessment of colonies was done within four months of sampling. Due to arrested growth and contamination from leaf microflora, not all viable conidia produced colonies.

[d] Myclobutanil was applied to runoff at a rate of 1.25 oz 100 gal^{-1} (9.36 g 100 $liters^{-1}$). Treatments were applied on the same day to all treated trees.

[e] Non-treated trees were either part of the same row of trees or were included in rows immediately adjacent to treated trees.

Figure 3.1. Image of AHS AREC orchard blocks and nearby commercial orchards. *Venturia inaequalis* isolates collected from York, Fuji and Gala orchard blocks (hatched boxes) from 2006-2010. The AHS AREC is within 300 km of nearby commercial orchards (white X's). Image courtesy of Google Earth (Google Inc., Mountain View, CA).

Figure 3.2. Image of apple shoot with neon rubber bands used to monitor growth during the field season. Starting in 2007, several shoots were selected in a subset of SI-treated and non-treated Fuji, York and Gala trees. Neon rubber bands (white circles) were placed over the actively growing shoot tip following myclobutanil application or sample collection. Leaves sampled during a collection were selected based on the mean number of new leaves for that cultivar and the location of those leaves on the reference shoots.

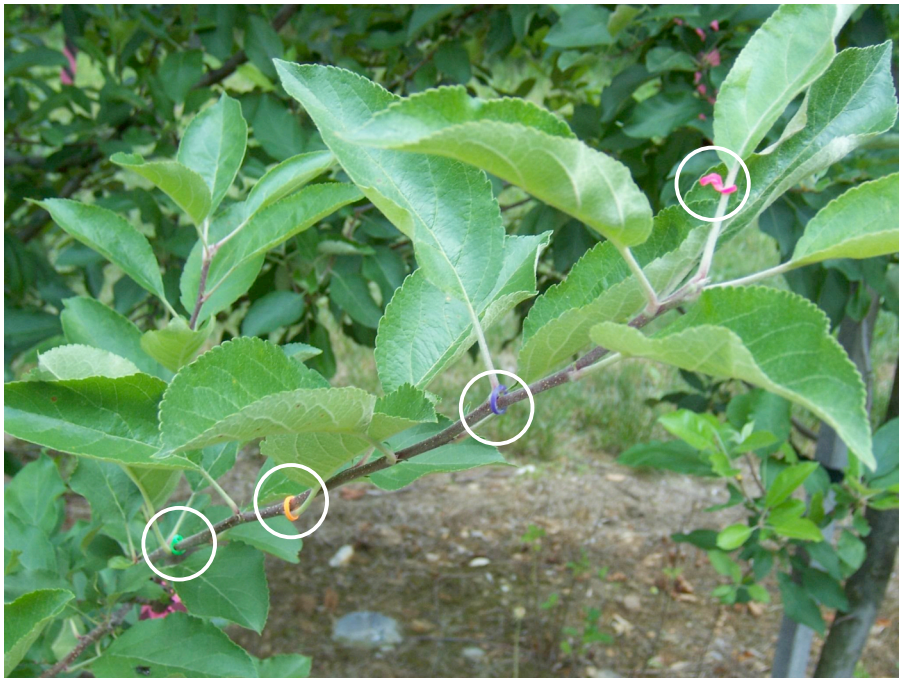


Figure 3.3. Frequency distribution of myclobutanil sensitivity in *Venturia inaequalis* isolates in 2006 (N=87) and 2007-2010 (N=168). Isolates sampled from SI-treated (white bars) or non-treated trees (black bars) at the AHS AREC in VA. Growth suppression determined using 1.0 μg ml^{-1} myclobutanil.

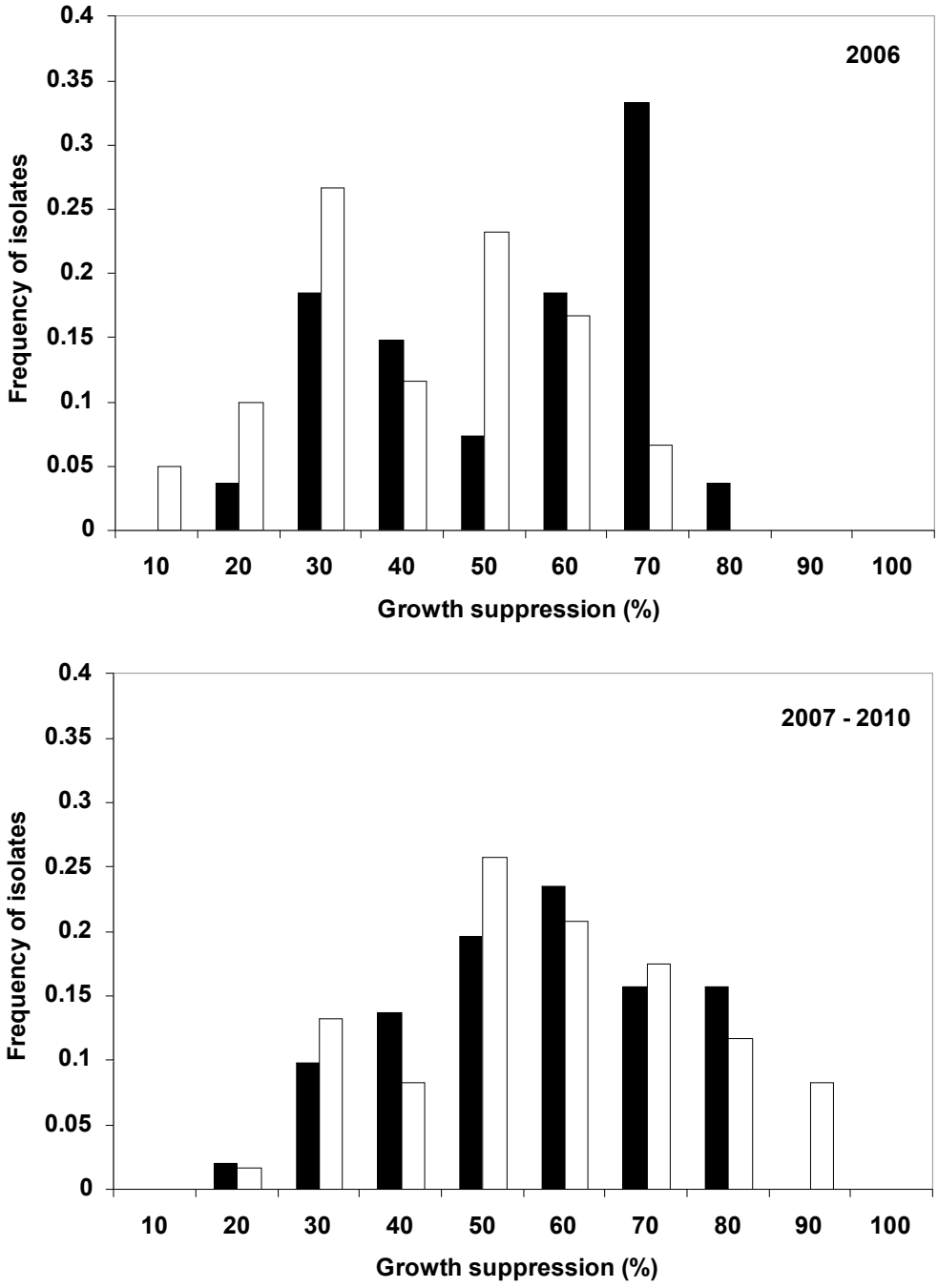


Figure 3.4. Comparison of percent growth suppression and mean colony growth at 1.0 µg ml^{-1} myclobutanil at 28 days. *Venturia inaequalis* isolates classified as resistant (red; N=20), moderately resistant (blue; N=75) or sensitive (green; N=160). Growth suppression based on the difference in growth at 0 and 1.0 µg ml^{-1} myclobutanil at 28 days.

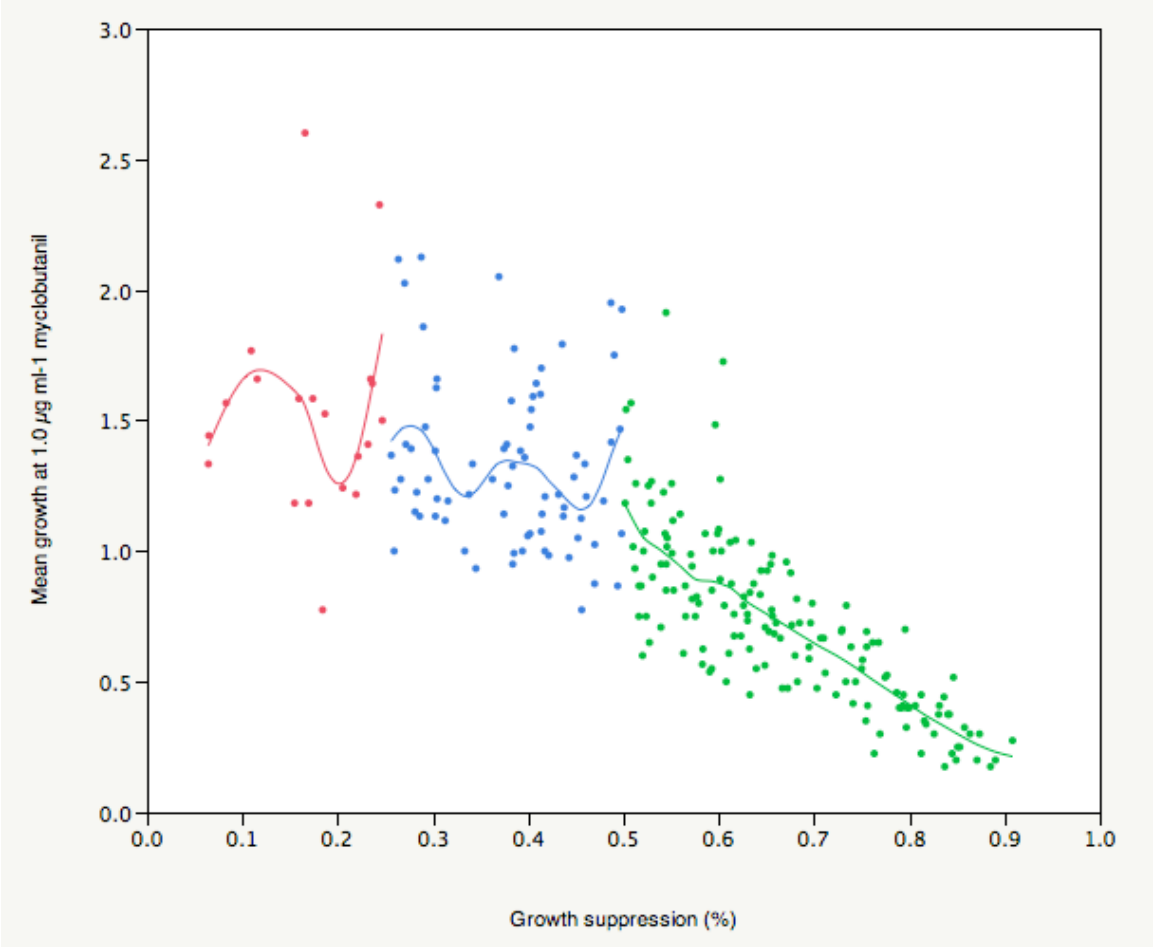


Figure 3.5. Average percent isolation success by sampling month and tree treatment. Isolates of *Venturia inaequalis* sampled from SI-treated (gray dashed line) and non-treated (black line) trees at the AHS AREC in VA from 2006 through 2010.

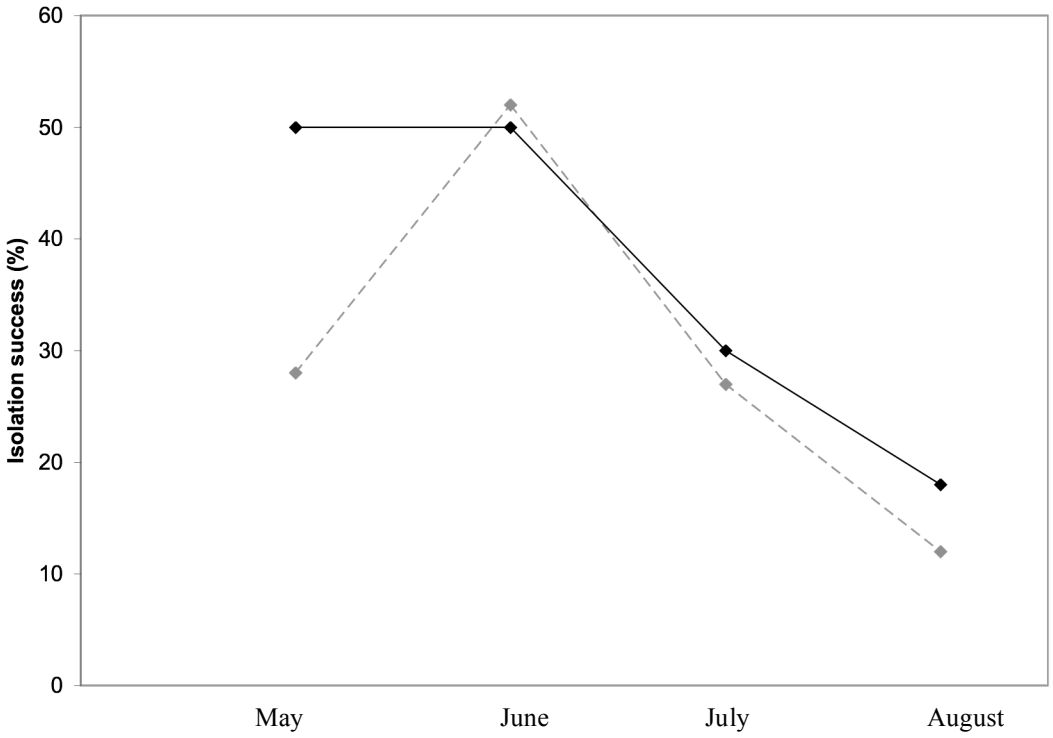
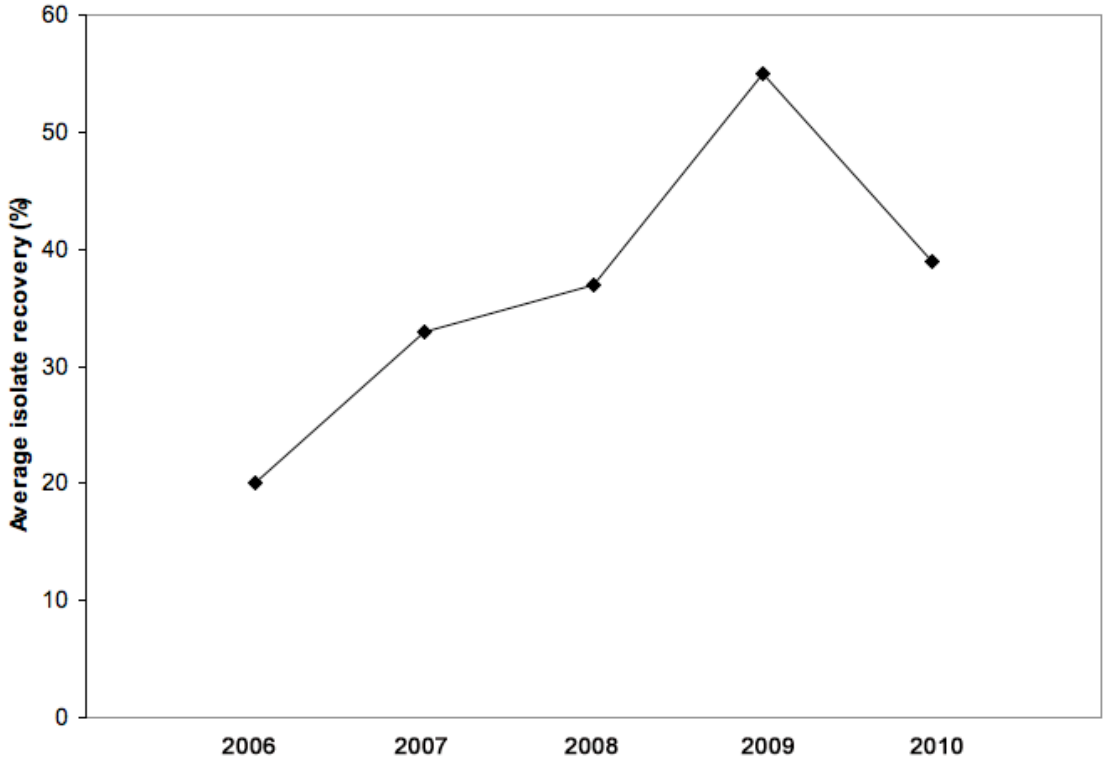


Figure 3.6. Average percent isolate recovery by year. Isolates sampled from 2006 through 2010 at the AHS AREC in VA. Isolate recovery calculated by dividing the number of *Venturia inaequalis* colonies obtained by the number of viable conidia from individual leaves collected from SI-treated and non-treated trees during each year.



Chapter 4. Influence of canopy location and microclimate on SI fungicide resistance in *Venturia inaequalis* populations

ABSTRACT

Management of apple scab (causal organism *Venturia inaequalis*) relies primarily on fungicide use, and fruit tree training and orchard layout have paralleled this trend. However, studies have found that in lower-density plantings (less than 150 trees A^{-1}), leaf moisture and ascospore concentration are not uniformly distributed within an orchard or an individual tree. The impact this may have on secondary infections and SI fungicide resistance in scab has not been studied. In 2011, leaves collected pre- and post-mancozeb application from trees in a lower density block were analyzed for manganese deposition. *V. inaequalis* isolates were collected from myclobutanil and mancozeb treated and non-treated trees, and fungicide plate assays with a discriminatory dose of 1.0 μg ml^{-1} myclobutanil were used to evaluate resistance. Weather sensors monitored the microclimate within tree canopies, and infection periods prior to a sampling interval were analyzed as a group. We found that manganese levels (post-mancozeb application) and microclimate were significantly different within the tree canopy. The interaction of tree treatment and canopy height was significant, and the majority of isolates tested were sensitive to myclobutanil at 1.0 μg ml^{-1}. The results from this study show that spray deposition, microclimate and SI fungicide resistance are influenced by canopy location.

INTRODUCTION

Venturia inaequalis is the causal organism of apple scab, an economically devastating disease of apples that occurs wherever apples are grown (2). Although management programs recommend sanitation and planting scab-resistant cultivars, chemical application is the most commonly used control method. Fruit tree architecture and orchard layout have paralleled the reliance on chemical applications. For example, dwarfing rootstocks reduce extension shoot growth and promote a more horizontal branch orientation (7), which produces a more compact tree. For the last thirty years, commercial growers have been steadily replacing more traditional orchards with these higher density plantings (more than 180 trees A^{-1}) because of the benefits of early production and higher returns (14). Although spray coverage depends on canopy density and leaf surface area, amongst other factors (10), growers can also achieve more uniform spray coverage with higher density plantings.

Despite the shift toward higher density plantings, less than half of Virginia's apple acreage is planted to that density (16). Studies have shown that in lower density plantings with larger trees (less than 150 trees A^{-1}; tree-row-volume of 40,600 m^3 ha^{-1}), spray deposition can vary widely due to the inverse relationship between foliar coverage and tree size (3,15). In addition, growers using airblast sprayers may need to more frequently adjust nozzle placement and throughput as the field season progresses (10). Since scab is managed with frequent fungicide applications, insufficient spray coverage is a serious concern, especially with fungicide resistance to the sterol inhibitors (SIs) documented throughout the U.S. (5,9,12). At the Alson H. Smith, Jr., Agricultural

Research and Extension Center (AHS AREC), SI fungicide resistance was first documented in a lower density orchard block comprised of trees up to 8m (17).

The tree architecture in lower-density plantings may also play a role in disease epidemiology. Recent studies have found that airborne *V. inaequalis* ascospore concentration is not uniformly distributed within an orchard, within a block of the same cultivar, or within an individual tree canopy (4,6). Leaf moisture also varies within the tree, with the upper, eastern portion of the canopy having up to three more wet hours per day than the lower, western portion (1). Since ascospore germination is dependent on temperature and the leaf surface remaining wet, variations within the canopy may result in scab "hot spots".

The impact of tree architecture, particularly in larger trees, on secondary infections and resistance in scab has not been studied. Our preliminary investigation in 2010 yielded mixed results (Appendix A), and we suspect that was due to incomplete microclimate data (collected from 15 June to 13 October 2010) and small sample sizes for the fungicide assay.

The overall goal of this study was to examine the influence of canopy location and microclimate on SI fungicide resistance in Virginia's scab population. We hypothesized that 1) spray deposition would not be homogeneous throughout an apple tree canopy, and 2) the presence of fungicide resistant isolates would vary from east to west and from top to bottom within the tree canopy. The specific objectives of this study were to: i) determine the spray deposition within the tree canopy, ii) characterize SI fungicide resistance in *V. inaequalis* isolates collected from different locations within the tree canopy, and iii) monitor the microclimate within trees.

MATERIALS AND METHODS

Experimental orchard. A lower-density orchard block (less than 150 trees A^{-1}) located at the AHS AREC in Winchester, Virginia was used for this study. Mature Stayman Winesap trees were selected from three-tree-sets comprised of Stayman, Idared and Ginger Gold. Myclobutanil (Rally 40W, Dow AgroSciences, Indianapolis, IN) was applied to both sides of the tree with an airblast sprayer at a rate of 5.0 oz 100 gal^{-1} A^{-1} on 7 April, 14 April, 20 April, 2 May, 20 May, 3 June, 17 June and 11 July 2011. Mancozeb (Penncozeb 75DF, United Phosphorous, Inc., King of Prussia, PA) was tank-mixed and applied with myclobutanil at a rate of 3 lb 100 gal^{-1} A^{-1}. Myclobutanil application was based on growth stage and disease pressure with the interval extended to allow some infection for collection of isolates. The non-treated trees were replicated within the same area as treated trees and were bordered by non-treated Idared and Ginger Gold trees. Standard maintenance materials were applied to all trees in each test block as needed (14), and included: petroleum oil (Damoil), methoxyfenozide (Intrepid), methidathion (Supracide), phosmet (Imidan), imidacloprid (Provado), thiacloprid (Calypso), acetamiprid (Assail), ziram, spinetoram (Delegate), captan, carbaryl (Sevin), and naphthalene acetic acid (NAA) with nonionic surfactant (Regulaid).

Spray deposition. 25 leaves were collected from each of seven locations (lower west, middle west, upper west, lower east, middle east, upper east, or middle by the trunk) within four Stayman trees that were not included in any other fungicide trials. Lower canopy was less than 1.5m above the ground, middle canopy was from 1.5-3m above the

ground, and upper canopy was greater than 3m above the ground. East and west canopy locations were sampled from the periphery into the tree 1.5m, and from the middle by the trunk outwards 1.5m. Mancozeb (Manzate Pro-Stick 75WG, DuPont, Wilmington, DE) was applied to both sides of the tree with an airblast sprayer at a rate of 3 lbs 100 gal^{-1} A^{-1} on 25 May 2011, after trees were fully foliated. Mancozeb contains a high proportion of manganese (15% active ingredient by weight in Manzate Pro-Stick), which can be readily quantified in nutrient analysis. After the residue had dried, twenty-five leaves were collected from the same seven locations. All leaves were oven dried at 72°C for three hours and then finely ground with a coffee grinder. Samples were mailed to A&L Eastern Laboratories in Richmond, VA, for manganese analysis.

Monitoring of shoot growth. Shoot growth was monitored on several branches of two treated and two non-treated trees within the block. Neon rubber bands were placed on the actively growing shoot tips following fungicide application or sample collection. Leaves sampled during a collection were selected based on the mean number of new leaves and the location of those leaves on the reference shoots.

Sampling of orchards. Ten leaves with apple scab lesions were collected from each of three canopy heights (lower, middle and upper) and two zones (interior or exterior) within individual trees on 12 May, 13 June and 8 July 2011. A total of four SI-treated and four non-treated trees were sampled.

Sensitivity tests. Previously described methods of culturing monoconidial isolates of *V. inaequalis* and conducting sensitivity tests based on percent growth suppression (PGS) were used (11). PGS was defined as the difference in colony growth on media amended with 0 and 1.0 μg ml^{-1} myclobutanil at 28 days. Isolates were classified as resistant if they had a PGS less than 25%, moderately resistant if they had a PGS between 25% and 50%, and sensitive if they had a PGS greater than 50%. Additional doses of myclobutanil (0.1 and 0.5 μg ml^{-1}) were tested with each isolate. Isolates were incubated at 19°C and colony diameters were measured bi-directionally every week for 4 weeks. Sensitivity tests were conducted in triplicate. The number of isolates tested per collection ranged from 34 to 36. Mycelium for sensitivity tests originated from monoconidial isolates from one lesion per sampled leaf. A total of 105 single-spored isolates of *V. inaequalis* was used in this study.

Monitoring the microclimate. Seven EL-USB-1 data loggers (Lascar Electronics, Erie, PA) monitoring temperature and relative humidity were positioned at three canopy heights (lower, middle and upper) and facing in three directions (East, west or by the trunk) within the four SI-treated and non-treated trees mentioned previously. An additional EL-USB-1 data logger was placed about 2m above the ground in the open space between trees within the row in each of the replications. Four WatchDog A-series data loggers (Spectrum Technologies, Plainfield, IL) monitoring temperature and leaf wetness were positioned in the middle (1.5-3m above the ground) by the trunk of two treated and two non-treated trees. Weather data was recorded from 4 April until 26 August 2011.

Data analysis. For spray deposition, data were averaged from the four single-tree replications and analyzed using PROC GLM in SAS (Windows, release 9.2, SAS Institute Inc., Cary, NC). For sensitivity tests, growth measurement data was transformed using a square-root transformation prior to analysis. A stepwise regression model (p-value threshold = 0.25, mixed direction) in JMP (Macintosh, version 9.0, SAS Institute Inc., Cary, NC) was used to examine potential combinations of independent variables to explain the variability of the dependent variable (growth measurement). Two-way interaction of the independent variables was also examined. The resulting models were then examined by fitting the model to the dataset, and the final linear regression model was selected based on: the degree of model fit to the data (R^2, BIC and AIC), examination of residuals, and biological soundness. For monitoring the microclimate, infection periods or secondary wettings prior to a sampling interval were analyzed as a group using PROC MIXED where tree replication and assay replication were considered as random variables, and responses (temperature, relative humidity and leaf wetness) on different days were considered as fixed factors.

RESULTS AND DISCUSSION

Spray deposition. Prior to mancozeb application, the within-leaf manganese concentration was not significantly different amongst the seven locations (lower west, middle west, upper west, lower east, middle east, upper east or middle by the trunk) within the tree (Table 4.1). The mean manganese concentration (32.29 ppm, range of 25

to 44 ppm) was below the normal range for apple trees (50-101 ppm), according to analysis by A&L Eastern Laboratories.

Following mancozeb application, there were significant differences among the seven locations (Table 4.1). The middle by the trunk had the lowest manganese deposit (137.25 ppm), and we suspect this was because that location was the furthest from the spray nozzles at any given time and the trunk may have partially blocked the spray's movement through the canopy. The lower east portion of the canopy had the highest manganese deposit (271.25 ppm), and this may be attributed to wind speed and direction (1.6 mph, SW) at the time of mancozeb application. In addition, the effects of pruning to a topped-off central leader result in greater foliage distribution in the lower portion of the tree. A previous study on apple tree microclimate (8) had also shown considerable variation in percent leaf area from north to south and east to west. Although the results from our 2011 spray deposition experiment differ from our preliminary assessment in 2010 (in which the middle west portion of the canopy had the highest manganese deposit; Appendix B), we suspect this may be due to the late timing of the mancozeb application in 2010. Comparisons between manganese deposition and myclobutanil deposition can be readily made as mancozeb was tank-mixed and applied with myclobutanil throughout the season.

Characterization of isolates. When pooled data were analyzed, the mean colony growth of *V. inaequalis* isolates was significant different among assay treatments, assay times and collection dates ($P < 0.001$) but not among tree treatment (i.e. whether or not the tree had been treated with myclobutanil) ($P = 0.633$) (Table 4.2). Tukey's HSD test indicated the mean colony growth of July isolates on 1.0 μg ml^{-1} myclobutanil was

significantly lower (i.e. were more sensitive) than that for May and June isolates. This may be due to differences in wind speed and direction during infection periods early in the season. SI-treated trees were bordered to the East and West by non-treated rows, and windier conditions in May and June (average wind speed 8 mph) may have dispersed more conidia from the non-treated rows onto the treated trees.

The interaction of collection date and tree treatment was tested and found to be positive ($P < 0.001$). Tukey's HSD test indicated the mean colony growth of isolates collected from non-treated trees in July was significantly lower than that of isolates collected from treated or non-treated trees in May or June. The interaction of tree treatment and canopy height was also tested and found to be positive ($P < 0.001$). Tukey's HSD test indicated the mean colony growth on 1.0 μg ml^{-1} myclobutanil of isolates collected from the upper canopy of non-treated trees was higher (i.e. were more resistant) than that of isolates collected from the same canopy zone in treated trees (Figure 4.1). Turbulent air movement during driving rain events may have dispersed conidia from treated trees onto non-treated trees. The contribution of primary inoculum concentration within different canopy locations may also be a factor, as a previous study found that the ascospore concentration in rainwater was greater in the upper portions of the canopy (4).

Isolates were classified based on differences in colony growth, using a discriminatory dose of 1.0 μg ml^{-1} myclobutanil. PGS values ranged from: 29.7 to 81.8 (average 61.2) for 12 May, 16.4 to 84.2 (average 55.6) for 13 June, and 36.0 to 90.1 (average 64.3) for 8 July. Of the isolates evaluated, the majority (85 of 105) was classified as sensitive and only two isolates were classified as resistant. Sensitive isolates

were collected from treated and non-treated trees, and the average PGS was similar for treated and non-treated trees of the same collection date. We suspect the continued presence of non-treated trees within the orchard block and the high incidence of scab on the non-treated trees provided sensitive inoculum throughout the season. The inclusion of mancozeb in each myclobutanil application would have also effected the scab population, as mancozeb is a highly effective broad-spectrum fungicide. We suspect the fitness cost of resistance may limit the survival of resistant isolates when they are exposed to mixtures containing an SI and a protectant compound.

Our PGS data did not support our hypothesis, as *V. inaequalis* isolates collected from the upper portion of treated trees did not differ in SI sensitivity as compared to isolates collected from the lower portion. In fact, the average PGS for each canopy height (upper = 62.0, N=23; middle = 61.3, N=40; lower =57.0, N=42) and zone (interior = 61.2, N=40; exterior = 58.5, N=42) was not significantly different from one another.

Monitoring the microclimate. A total of 24 infection periods or secondary wettings (eight between 4 April and 12 May, nine between 13 May and 13 June, and seven between 14 June and 8 July) occurred during the microclimate assessment (Table 4.3). Our data show that the apple trees canopies in a lower density orchard block in Virginia display substantial spatial heterogeneity in temperature and relative humidity. Within the tree canopy, the average duration and rainfall intensity of an infection period decreased as the season progressed (20.6 hrs > 12.3 hrs > 11 hrs; 0.68 in > 0.44 in > 0.18 in), while the average temperature increased (10.62°C < 15.73°C < 19.75°C). Not surprisingly, temperature and relative humidity were significant ($P < 0.001$) amongst canopy height, direction, and infection period. The interaction of canopy height and

direction was tested and found to be positive. Generally, the upper eastern portion of the canopy had the highest temperature early in the field season (4 April–13 June) while the lower western portion had the highest temperature late in the field season (14 June–8 July) (Table 4.4). Relative humidity fluctuated more within the tree canopy, but generally was higher in the lower eastern and middle eastern portions of the canopy.

These fluctuations may be due to several factors including sun exposure, air movement, tree architecture and ground cover management. As mentioned previously, trees pruned to a topped-off single leader have more foliage at the bottom than at the top, so sensors placed in the upper canopy would be exposed to more direct sunlight and wind. Later in the season, however, the temperature does not fluctuate much from day to evening, high relative humidity sets in, and the air tends to become stagnant. Consequently, the ground and ground cover become sources of ambient heat and humidity for the lower canopy. Since sensors were placed before the trees were fully foliated, changes in shoot growth during the season may have resulted in varying degrees of exposure.

Temperature and relative humidity recorded in the open space between trees within the row were significantly lower ($P < 0.001$) from those recorded within the trees. We suspect this is because the sensors within the orchard row were 2m above the ground, distancing them from potential heat sinks and wind disturbances.

This investigation showed that in a conventional orchard block spray deposition and microclimate are influenced by canopy location, but SI fungicide resistance does not appear to be. Future work may involve additional field seasons, larger *V. inaequalis* sample sizes and different types of ground cover. In terms of scab management, growers

should adjust their airblast sprayers as the season progresses to ensure adequate and uniform spray coverage. Thorough pruning is also important with larger trees, as it facilitates better light penetration and air movement.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Virginia Agricultural Council and the Virginia Apple Research Program. We thank Dow AgroSciences and DuPont for providing the fungicides. We thank Erik Stromberg for funding the weather sensors and Mizuho Nita for analyzing the data. We thank A.E. Cochran II for designing and constructing the shelters for the EL-USB-2 sensors. We thank S.W. Kilmer for technical support. We thank Zach Marine for reformatting the weather sensor data.

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Table 4.1. Distribution of mancozeb within the tree canopy.

	Manganese Concentration (ppm)		
Sampling Zone[a]	**Before**[b]	**After**[c]	**Foliar Deposit**[d]
Lower West	34.25 a	277.25 ab	243.00 ab
Middle West	30.25 a	287.25 ab	257.00 ab
Upper West	32.25 a	240.00 bc	207.75 bc
Lower East	32.75 a	304.00 a	271.25 a
Middle East	30.00 a	239.00 bc	209.00 bc
Upper East	31.00 a	196.00 cd	165.00 cd
Middle by trunk	35.50 a	172.75 d	137.25 d

[a] Lower zone was less than 1.5m above the ground, middle zone was 1.5-3m above the ground, and upper zone was greater than 3m above the ground.

[b] Background levels of manganese before mancozeb application. Mean separation by Waller-Duncan K-ratio t-test (p=0.05). Data averaged from four single-tree reps.

[c] Mancozeb (Manzate Pro-Stick 75WG) was applied with an airblast sprayer to both sides of the tree at a rate of 3.0 lbs A^{-1} on 25 May 2011.

[d] Foliar deposit equals the difference in manganese concentration prior to and following mancozeb application for that canopy location.

Table 4.2. ANOVA for differences between factors.

	Effect tests, P-value
Factor	**All (N=105)**
Tree treatment[a]	0.633
Collection date[b]	<0.001
Collection x tree treatment	<0.001
Tree treatment x canopy height[c]	<0.001
Canopy height x canopy zone[d]	0.082

[a] Whether myclobutanil was applied to the tree the *V. inaequalis* isolate originated from.

[b] Leaves collected on 12 May, 13 June and 8 July 2011.

[c] Canopy heights: lower (<1.5m above the ground), middle (1.5-3m above the ground) or upper (>3 m above the ground).

[d] Canopy zone: interior (outward from the trunk 1.5m) or exterior (inward from the periphery 1.5m).

Table 4.3. Summary of infection periods or secondary wettings that occurred from 4 April through 26 August 2011.

# Inf Period[a]	Onset	End	Duration (hrs)	Avg Temp (°C)[b]	Rainfall (in)[c]
1	6:00 April 8	11:00 April 9	29	6.32	0.43
2	6:00 April 12	9:00 April 13	27	11.00	1.65
3	2:00 April 16	20:00 April 16	20	10.87	2.15
4	21:00 April 19	8:00 April 20	12	9.42	0.01
5	7:00 April 22	8:00 April 23	25	6.32	0.31
6	22:00 April 24	9:00 April 25	11	15.80	0.03
7	6:00 May 1	8:00 May 2	26	12.45	0.08
8	6:00 May 3	9:00 May 4	15	12.51	0.75
9	1:00 May 13	14:00 May 13	14	16.71	0.43
10	21:00 May 14	8:00 May 15	14	16.43	1.26
11	15:00 May 15	8:00 May 16	17	14.56	0.25
12	20:00 May 16	15:00 May 17	19	15.87	1.60
13	21:00 May 17	8:00 May 18	12	15.18	0.28
14	21:00 May 18	11:00 May 19	15	12.46	0.02
15	2:00 May 23	9:00 May 23	7	17.42	0.06
16	2:00 May 27	8:00 May 27	6	16.41	0.01
17	1:00 May 28	8:00 May 28	7	16.47	0.01
18	16:00 June 18	8:00 June 19	17	21.24	0.16
19	23:00 June 19	10:00 June 20	12	19.14	0.61
20	23:00 June 20	7:00 June 21	8	19.91	0.01
21	0:00 June 22	7:00 June 22	7	20.73	0.26
22	1:00 June 27	11:00 June 27	10	18.45	0.05
23	19:00 July 4	8:00 July 5	14	18.82	0.01
24	21:00 July 7	7:00 July 8	9	19.99	0.16

[a] Earliest two infection periods (March 30-April 1 and April 3-4) missing from list as sensors placed in trees on April 4.

[b] Temperature averaged from fifty-six EL-USB-1 data loggers within the block.

[c] Rainfall measured with a Pestcaster (Neogen Corporation, Lansing, MI).

Table 4.4. Detailed analysis of microclimate data.

Infection periods prior to a sampling interval[a]						
	4April-12May (N=8)[b]		**13May-13June (N=9)**		**14June-8July (N=7)**	
Factor	Temp[c]	RH	Temp	RH	Temp	RH
Upper canopy[d]	10.07 a	87.88 c	15.54 a	93.49 b	19.71 c	91.72 c
Middle canopy	9.88 b	89.07 b	15.48 a	94.04 a	19.79 b	92.17 b
Lower canopy	9.89 b	89.56 a	15.39 b	92.98 c	19.83 a	92.62 a
East side	10.03 a	88.75 b	15.61 a	93.85 a	19.77 b	92.24 a
West side	9.88 b	88.96 ab	15.36 c	93.23 b	19.81 a	92.05 b
Middle by trunk	9.85 b	89.05 a	15.44 b	93.97 a	19.71 c	92.28 a

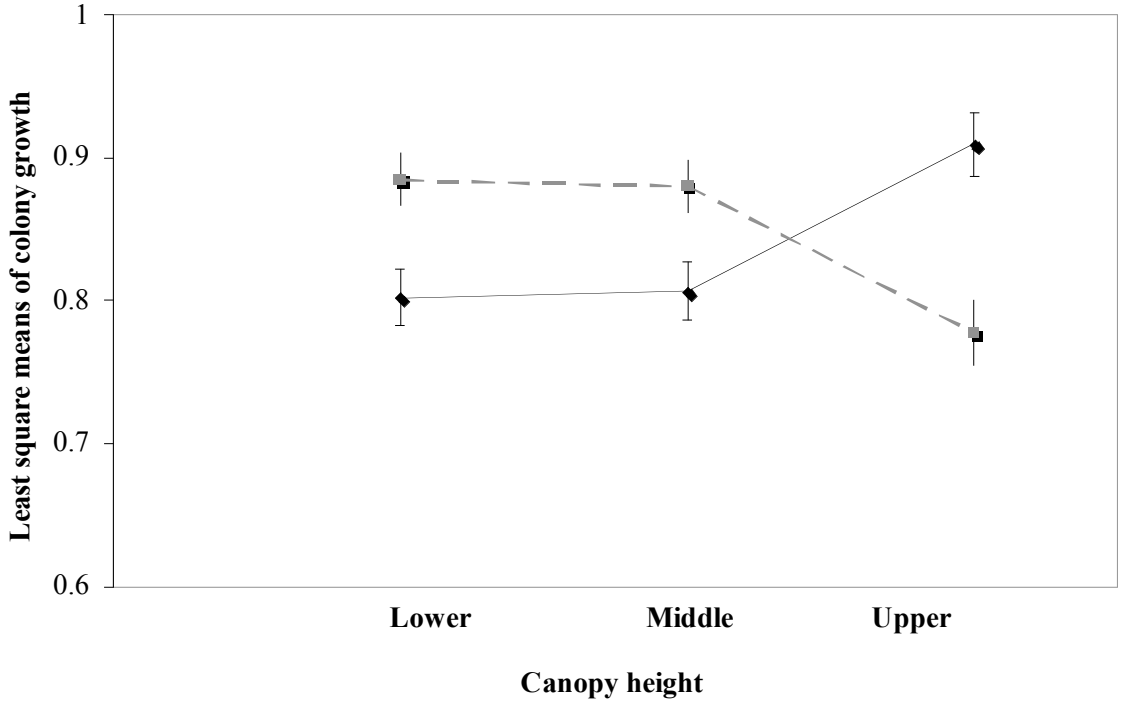
[a] Scabby leaves collected on 12 May, 13 June and 8 July 2011.

[b] Number of observations used in data analysis: 17861 (4April–12May), 11813 (13May–13June) and 8415 (14June–8July).

[c] Temperature in degrees Celsius.

[d] Upper canopy greater than 3m above the ground, middle canopy between 1.5-3m above the ground, and lower canopy less than 1.5m above the ground.

Figure 4.1. LS means plot of colony growth versus tree treatment and canopy height. Isolates of *Venturia inaequalis* sampled from SI-treated (gray dashed line) and non-treated (black line) trees in a lower density planting at the AHS AREC in VA in 2011. Standard error bars shown.



Chapter 5. First report of reduced sensitivity to a QoI fungicide in isolates of apple scab (*Venturia inaequalis*) in Virginia and Maryland

The following chapter was formatted to facilitate publication in *Plant Disease*. This work was originally submitted by Marine, Schmale and Yoder to *Plant Disease*, a journal of the American Phytopathological Society. This work was accepted on 27 March 2012.

First report of reduced sensitivity to a QoI fungicide in isolates of apple scab (*Venturia inaequalis*) in Virginia and Maryland

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Marine, S.C., Schmale III, D.G., and Yoder, K.S. 2012. First report of reduced sensitivity to a QoI fungicide in isolates of apple scab (*Venturia inaequalis*) in Virginia and Maryland. Plant Disease XX: XXX.

DISEASE NOTE

Apple scab caused by *Venturia inaequalis* (Cooke) Winter continues to be a significant concern for apple growers in Virginia and Maryland. Management of apple scab has relied on foliar fungicides including strobilurins (QoIs) such as trifloxystrobin. In recent years, populations of *V. inaequalis* with reduced sensitivity to the QoIs have been reported in other apple-growing regions of the United States (1,2). Although QoI fungicides generally remain effective in the Mid-Atlantic, concerns about the development of resistance in some Virginia and Maryland orchards prompted this study. Twenty-five isolates of *V. inaequalis* were obtained from scabby leaves from commercial and experimental orchards in Virginia in 2010 (N=6) and 2011 (N=14) and from a commercial in Maryland (N=5) in 2011. Orchards had previously been treated with QoI or sterol-inhibiting (SI) fungicides. Isolates of *V. inaequalis* were grown on potato dextrose agar (PDA) amended with 0, 0.1 or 1.0 trifloxystrobin with 100 μg ml^{-1} salicylhydroxamic acid (SHAM) and incubated at 19°C. Colony growth was measured every week for four weeks. To account for the SI use at some orchards, isolates of *V. inaequalis* were also evaluated on PDA amended with 0, 0.1, 0.5 or 1.0 μg ml^{-1} myclobutanil. Fungicide sensitivities were expressed as a percentage of the difference in colony growth using a discriminatory dose of 1.0 μg ml^{-1} trifloxystrobin with SHAM or 1.0 μg ml^{-1} myclobutanil at 28 days. Isolates with <25% growth suppression were

classified as fully resistant whereas those with >70% growth suppression were classified as sensitive. Isolates with 25-70% growth suppression were classified as partially resistant. Effective concentration (EC_{50}) values (trifloxystrobin concentration inhibiting colony growth by 50%) were also calculated for a subset of fully resistant and sensitive isolates. Of the 25 isolates tested, six were fully resistant to trifloxystrobin (mean EC_{50} value greater than 10.0 μg ml^{-1}) and 10 were sensitive (mean EC_{50} value of 0.04 μg ml^{-1} ± 0.05 μg ml^{-1}). Nine isolates were classified as partially resistant. Some isolates had more than a 200-fold increase in resistance to trifloxystrobin, and one isolate grew almost as well on 10.0 μg ml^{-1} of trifloxystrobin as on the unamended control (growth suppression of 2.4%). Current-season use of QoI fungicides on isolate source trees was significantly associated with a lack of sensitivity χ^2 (1) = 3.72 ($P < 0.06$). All six fully resistant isolates originated from QoI-treated commercial orchards, which had shown control failures. Seven of 10 isolates sensitive to QoIs originated from trees which had been treated with SIs during the isolation year. Resistance to myclobutanil was not significantly associated with resistance to trifloxystrobin χ^2 (1) = 1.220 ($P < 0.5$), and only one isolate was resistant (i.e. >25% growth suppression) to both. Despite the long history of QoI use at the experimental orchards, no isolates fully resistant to trifloxystrobin were identified there. To our knowledge, this is the first report of *V. inaequalis* isolates with resistance to trifloxystrobin in Virginia and Maryland. Since SI resistance has been documented in Virginia (3) and resistance to both the SI and QoI chemical classes is a concern in the Mid-Atlantic region (4), tank-mixing or alternating QoIs with broad-spectrum fungicides having different modes of action is recommended.

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Table 5.1. Characterization of *Venturia inaequalis* isolates from different locations in Virginia and Maryland

Isolate (Location, #)[a]	State	Year	Recent QoI use? [b]	QoI use history[c]	QoI growth suppression (%)[d]	SI growth suppression (%)[e]
Madison 1	VA	2010	no	0 appl	100	49
Rappahannock 1	VA	2011	no	2 appl	76	67
Frederick 1	VA	2011	yes	20-25 appl	40	26
Frederick 3	VA	2011	yes	20-25 appl	2	50
Frederick 4	VA	2011	yes	20-25 appl	22	-
Frederick 5	VA	2011	yes	20-25 appl	30	48
Frederick 6	VA	2011	yes	20-25 appl	22	74
Frederick 7	VA	2011	yes	20-25 appl	100	70
Frederick 8	VA	2011	yes	20-25 appl	27	45
Washington 1	MD	2011	yes	12-15 appl	18	17
Washington 2	MD	2011	yes	12-15 appl	34	45
Washington 3	MD	2011	yes	12-15 appl	7	66
Washington 4	MD	2011	yes	12-15 appl	26	54
Washington 5	MD	2011	yes	12-15 appl	24	54
Virginia Tech 1	VA	2010	yes	50-52 appl	72	22
Virginia Tech 2	VA	2010	yes	50-52 appl	54	57
Virginia Tech 3	VA	2010	yes	50-52 appl	48	65
Virginia Tech 4	VA	2010	yes	50-52 appl	75	43
Virginia Tech 5	VA	2010	yes	50-52 appl	-	78
Virginia Tech 6	VA	2011	no	80-100 appl	46	36
Virginia Tech 7	VA	2011	no	80-100 appl	70	85
Virginia Tech 8	VA	2011	no	80-100 appl	79	22
Virginia Tech 9	VA	2011	no	80-100 appl	80	90
Virginia Tech 10	VA	2011	no	80-100 appl	91	100
Virginia Tech 11	VA	2011	no	80-100 appl	100	16

[a] Location refers to the county the orchard is located in. Virginia Tech isolates from AHS AREC in Frederick county. Commercial orchards listed above the black line; experimental orchards below.

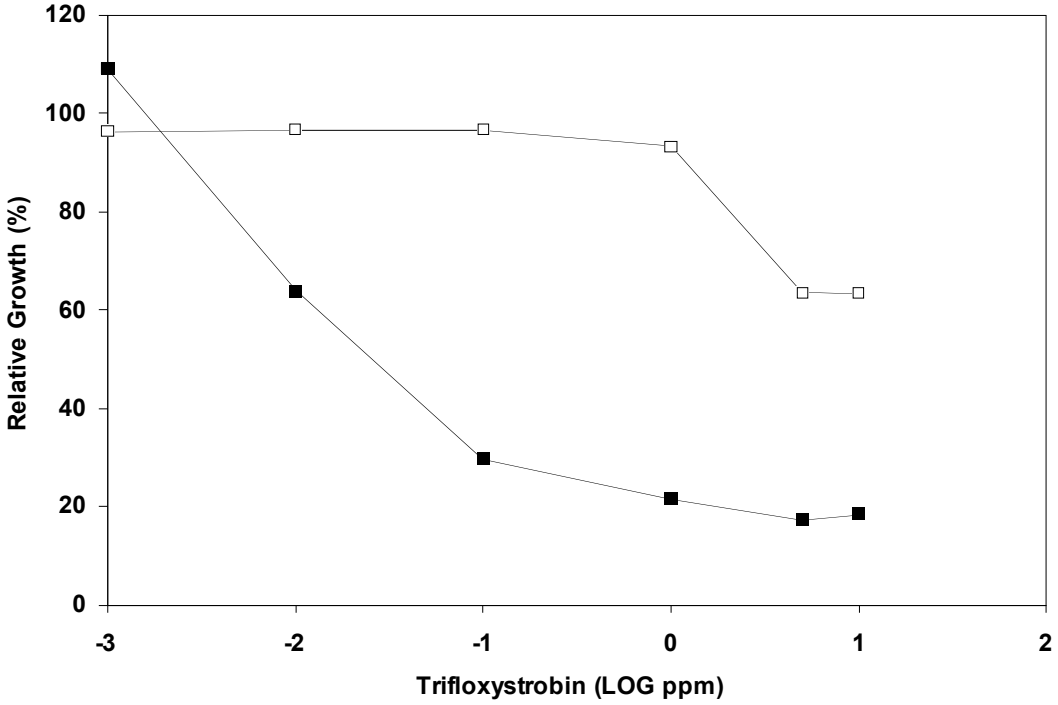
[b] QoI fungicide applied within four weeks of foliage collection.

[c] Total number of QoI fungicide applications (full and half rates) since orchard was planted.

[d] Percent growth suppression (PGS) based on the difference in colony growth on 0 and 1.0 μg ml^{-1} trifloxystrobin (QoI) with SHAM at 28 days. Isolates with <25 PGS were classified as fully resistant, 25-70 PGS were classified as partially resistant, and >70 PGS were classified as sensitive. Dash indicates isolate omitted from assessment.

[e] PGS based on the difference in colony growth on 0 and 1.0 μg ml^{-1} myclobutanil (SI) at 28 days. Dash indicates isolate omitted from assessment.

Figure 5.1. Relative growth for a subset of *Venturia inaequalis* isolates on various QoI concentrations. Isolates previously classified as sensitive (black squares) or fully resistant (white squares) based on the percent difference in colony growth on 0.0 and 1.0 μg ml^{-1} of trifloxystrobin with 100 μg ml^{-1} salicylhydroxamic acid at 28 days.



Chapter 6. Summary

Venturia inaequalis (Cooke) G. Winter is the causal agent of apple scab, a devastating disease that is predominantly controlled with SI and QoI fungicides. Unfortunately, populations of *V. inaequalis* have been developing resistance to these chemical classes. The goal for this research was to characterize SI and QoI resistance in Virginia scab populations. A range of SI resistance was observed at each collection month and year, and mean colony growth was significantly different amongst cultivars, collection years, and whether or not the tree was treated with myclobutanil. The majority of isolates collected in 2006 were classified as moderately resistant or resistant to myclobutanil, whereas the majority of isolates collected in 2007 through 2010 were classified as sensitive. Isolate recovery exhibited a pattern of seasonality, regardless of year or tree treatment. Spray deposition, microclimate and SI resistance were influenced by canopy location. A genetic mechanism for SI resistance was not found, as no mutations were identified within the *CYP51A1* gene and AFLP results were inconclusive. The time *V. inaequalis* conidia were incubated with myclobutanil was a significant factor in the bioassay, but metabolic breakdown could not be examined as TLC plates failed to develop. No QoI-resistant isolates were observed at the AHS AREC, but resistant isolates identified from commercial orchards had a mean EC_{50} value greater than has previously been published. SI resistance was not significantly associated with QoI resistance. Our results indicate that myclobutanil and trifloxystrobin are still effective compounds for control of scab in some Virginia orchards, but longevity of this is uncertain and scab populations should be monitored.

Chapter 2 Summary

A previous report detailed SI resistance in Virginia, and some *V. inaequalis* samples had a 10X increase in resistance to myclobutanil (39). Using poison plate assays, we found that the majority of the *V. inaequalis* isolates were classified as either moderately resistant or resistant to myclobutanil. We observed a continuum of resistance in our populations, but found no evidence that the collection date influenced resistance in our populations. Colony growth varied among isolates, fungicide rates, and between replicate growth tests, but the frequency of resistant isolates was greater from treated than non-treated trees. This is the first detailed report of fungicide resistance to myclobutanil in populations of *V. inaequalis* in Virginia, and to our knowledge, the first report of SI resistance in apple scab from orchards in the southeastern United States.

Chapter 3 Summary

Despite high scab pressure, development of SI resistance in Virginia has been delayed more than a decade compared to other apple-producing regions (21,22). Studies examining fungicide efficacy in the same location over multiple years provide valuable data on local disease pressure, spray programs and future pathogen concerns. Using neon rubber bands to monitor shoot growth, recently infected leaves were collected from 2007 through 2010, and cultured *V. inaequalis* isolates were evaluated for SI resistance. In contrast to our previous study (Chapter 2), we found that the majority of isolates were classified as sensitive. Although each classification category had a similar proportion of isolates from SI-treated and non-treated trees, mean colony growth was significantly different among collection years and cultivars. We also found that isolate recovery (i.e.

the percent of viable conidia that produced colonies) exhibited a pattern of seasonality (May = June > July > August), regardless of tree treatment. This is the first report on the distribution of SI fungicide resistance over sequential growing seasons in Virginia.

Chapter 4 Summary

Previous studies have shown that in lower density plantings with larger trees (less than 150 trees A^{-1}), spray deposition can vary widely (2,35), and that airborne *V. inaequalis* ascospore concentration is not uniformly distributed within the orchard or within an individual tree canopy (4,5). Leaves collected from different canopy locations (upper, middle or lower canopy; eastern or western canopy) pre- and post-mancozeb application were analyzed for manganese deposition, and *V. inaequalis* isolates collected from similar canopy locations in myclobutanil- and mancozeb- treated and non-treated trees were evaluated using poison plate assays. Weather sensors monitored the within-canopy microclimate from 4 April until 26 August, and infection periods prior to a fungal collection were analyzed as a group. Background levels of manganese were homogeneous throughout the tree canopy, but spray deposition was not. Although SI resistance varied among canopy heights and canopy zones, the interaction of tree treatment and canopy height was positive. We found that the majority of the *V. inaequalis* isolates were classified as sensitive, and the average PGS was similar for treated and non-treated trees of the same collection date. We observed substantial spatial heterogeneity in temperature and relative humidity, though variations were correlated with early or late season.

Chapter 5 Summary

Growers with SI-resistant *V. inaequalis* populations may rely more heavily on the QoIs for scab control. Previous reports detailed QoI resistance in Michigan (25) and New York (8), but little was known about the baseline sensitivity in Virginia or Maryland. Using poison plate assays, we found that the majority of the *V. inaequalis* isolates from the AHS AREC were classified as sensitive to trifloxystrobin, while the majority of isolates from two commercial orchards reporting control failures were classified as partially or fully resistant. Fully resistant isolates had a mean EC_{50} value greater than 10.0 μg ml^{-1} which, to our knowledge, is the highest to be published. In contrast, sensitive isolates had a mean EC_{50} value of 0.04 μg ml^{-1}. Current season use of QoIs was significantly associated with a lack of sensitivity, but resistance to myclobutanil (an SI) was not significantly associated with resistance to trifloxystrobin. This is the first report of *V. inaequalis* isolates with reduced sensitivity to trifloxystrobin in Virginia and Maryland.

Appendices C-E Summary

Despite extensive study, the genetic cause for SI resistance remains unresolved. A previous report detailed SI resistance in some field strains of *V. inaequalis* was correlated with overexpression of the *CYP51A1* gene and the presence of an upstream insertion (32). Using PCR, we found that the consensus sequences for the *CYP51A1* gene were identical for all isolates tested (Appendix B). Amplified fragment length polymorphism (AFLP) is a genotyping technique that has been used to study fungicide resistance in other plant pathogen populations (10,24). Using previously classified *V. inaequalis* isolates, polymorphic bands from electropherograms were converted to a binary format and

analyzed. Sequential elimination of alleles 92, 9 and 15 was significant for one set of isolates, but the statistical technique could not be used with the other set of isolates, as duplicated isolates failed to yield identical electropherograms (Appendix C). Bioassays are an inexpensive method to screen organisms for sensitivity to compounds and calculate the compounds' unknown concentration (6). We found that the mean colony growth of *Botryosphaeria dothidea* (indicator fungus) was significantly different among incubation times and myclobutanil concentrations, following *V. inaequalis* conidial exposure (Appendix D). We were unable to calculate R_f values.

Future Research

Although *V. inaequalis* populations in Virginia were found to have shifted baseline sensitivities to myclobutanil, the 2006 collection had more resistant isolates (N=16) than all other years combined (N=4). We suspect this may be partially due to sampling technique, as samples taken in 2006 consisted of older and newer lesions, whereas samples taken in 2007 through 2010 consisted only of newer lesions (due to shoot growth monitoring). Because the spray interval was extended to allow scab infection to occur, myclobutanil may have had more of a fungistatic effect on newer lesions (which would have only been exposed to a single fungicide application), thereby allowing sensitive isolates to remain in the population. An examination of sampling technique could help determine whether it is involved in the classification of isolates. Future experiments could include subdividing individual trees or orchards and sampling scabby leaves randomly in one portion and selecting leaves based on shoot growth in the other. It would be beneficial to conduct this trial in both research and commercial

orchards. Continued divergence in the frequency of resistant isolates between the two sampling techniques would require further investigation.

Our research found that isolate recovery was highest early in the field season and that *V. inaequalis* isolates collected then were more sensitive to myclobutanil than isolates collected later in the field season, regardless of cultivar, tree treatment or year. The observed shift in the frequency of SI-resistant isolates as the season progresses may be due to resistant isolates having a greater temperature tolerance. A previous study in Pennsylvania found that benomyl resistance in *V. inaequalis*, when developed under field conditions, did not negatively affect fitness in the absence of the fungicide (23). Future experiments could include measuring factors of parasitic fitness (colony growth, conidial production, etc.) of resistant and sensitive isolates after incubation at different temperatures (19°C, 25°C, 30°C and 35°C) in the presence of myclobutanil.

The breakdown of ontogenetic resistance may also be a factor. Mature apple leaves are generally not susceptible to scab, but lesions have been reported on the undersides of senescing leaves in late summer and autumn (26,34,38). Despite the implications this has for scab management (late season infections, contribution to primary inoculum, etc.), research on the breakdown of ontogenetic resistance is quite limited (20,26). Future experiments could include assaying *V. inaequalis* isolates obtained after August – focusing sample collection on leaves with lesions on the lower leaf surface – or inoculating leaves of various ages with conidial suspensions and measuring the plant's chemical and protein-based defense mechanisms (13). Greenhouse studies could be used to mitigate the competing microflora and leaf contaminants commonly associated with

late season field sampling, but ontogenetic resistance is slower to develop in greenhouse conditions.

Our research failed to find a potential SI resistance mechanism. There was no nucleotide variation within the *CYP51A1* gene of resistant or sensitive *V. inaequalis* isolates (Appendix B), but this finding agreed with a previous study from Michigan (32). However, over-expression of the *CYP51A1* gene was correlated with the presence of an upstream insertion in 82% of SI-resistant isolates from research orchards. Future experiments could include quantifying the copy number and expression level of the *CYP51A1* gene and relevant insertions in resistant and sensitive isolates from research and commercial orchards. Interestingly, the previous study failed to detect the insertion in 96% of resistant isolates from commercial orchards (32).

Although we identified three alleles, whose sequential elimination was a significant factor in SI resistance in *V. inaequalis*, we failed to segregate resistant and sensitive isolates using polymorphic bands (Appendix C). Since the technique does not provide specific information on gene function or which region of genomic DNA was amplified, future experiments could include using complementary DNA (cDNA) in place of genomic DNA in the PCR template to study gene expression (36) or sequencing identified alleles. However, studies with other plant pathogens also reported no association between AFLP banding patterns and fungicide resistance (10,14,24). This may be because AFLP primarily generates dominant markers (i.e. present or absent) from multiple loci, which for a large genome (*V. inaequalis*' is estimated to be 100 Mbp), might mean only a few hundred bands. For these reasons, we suggest that future experiments focus on investigating the genetic diversity of scab populations in either

diverse geographical areas (multi-country or multi-state) or on various hosts (such as crabapple and hawthorn) (15). Whichever approach is taken, consideration should be given to the highly subjective nature of marker scoring (17).

Our research found that the length of time *V. inaequalis* conidia were exposed to myclobutanil was a significant factor in the mean colony growth of the indicator fungus, *Botryosphaeria dothidea*. This may be due to metabolic activity of the conidia or natural breakdown of the chemical. Future experiments could include exposing *V. inaequalis* to C^{14}-labeled myclobutanil and monitoring the uptake and metabolism of the fungicide (33), evaluating efflux pump activity and expression in resistant and sensitive isolates in the presence of myclobutanil (9,16) or assessing the overall metabolic activity in the presence of damaging physical treatment (such as high temperature) (29).

Despite the long history of QoI use at the AHS AREC, *V. inaequalis* populations were sensitive to trifloxystrobin (mean EC_{50} value of 0.04 μg ml^{-1}). Commercial orchards that had received less QoI applications, however, had a greater frequency of QoI-resistant isolates. Future experiments may include evaluating both populations for the glycine to alanine mutation at position 143 of the *CYTB* protein (G143A) with either PCR (25) or real-time PCR (28). Since a single mutation can confer a high level of QoI resistance, *V. inaequalis* populations should be regularly monitored. We did not find a significant association between SI and QoI resistance, but this agreed with previous studies (11,18).

Surprisingly, some *V. inaequalis* isolates grew better at low levels of fungicide than on the unamended control. Variability in *V. inaequalis* cultures has been documented previously (30), but chemical hormesis – where low doses of a toxin stimulate a biological system (3) – has not been examined. A recent study with *Pythium*

aphanidermatum found that low doses of cyazofamid increased radial growth up to 13% (12). Future experiments may include exposing *V. inaequalis* isolates to sub-inhibitory concentrations of a fungicide and evaluating their tolerance of various environmental conditions (high temperature, low humidity, frequent drying, etc.). Caution must be taken with data interpretation, however, as the mechanism(s) involved in hormesis are unknown, and the concept remains contentious (27).

Further examination of potential fungicide resistance mechanisms would be greatly aided by sequencing the *V. inaequalis* genome and developing cDNA microarrays. To date, nine plant pathogenic fungi (37) and one group of related filamentous fungi (19) have been sequenced. Studies using those fungi and cDNA microarray technology have found that exposure of isolates to azole fungicides resulted in qualitative and quantitative differences in gene expression (1,7). Future experiments may assess *V. inaequalis* isolates the same way, and sequencing efforts are currently underway (31). Given the likelihood that SI resistance is pleiotropic in nature, and that QoI resistance is not always associated with the G143A mutation, sequencing information is essential to understanding the phenomena.

In the meantime, scab will continue to cause losses to Virginia's apple growers. Managing scab in the presence of fungicide resistance will require growers to rely more on broad-spectrum fungicides (such as the EBDCs) and combination products (such as fluopyram and trifloxystrobin; tradename Luna Sensation). For fruit intended for the domestic fresh market, growers may need to reexamine the cost-benefit ratio of supplementing their programs with additional cultural (such as scab-resistant cultivars) or mechanical (such as sanitation and pruning) control methods. Collaborative efforts

between plant pathologists, entomologists, and horticulturists are important in meeting the challenge of consistently producing uniform, commercial-quality fruit in the presence of a plethora of diseases, insect pests, and physiological disorders.

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APPENDIX A

Preliminary spatial distribution data from 2010

Distribution of SI resistance within the tree canopy in 2010

Protocol: Mature Stayman Winesap trees were selected from three-tree sets in a lower density orchard block (less than 150 trees A^{-1}) located at the Alson H. Smith, Jr., Agricultural Research and Extension Center (AHS AREC) in Winchester, Virginia. Myclobutanil (Rally 40W, Dow AgroSciences, Indianapolis, IN) was applied with an airblast sprayer at a concentrate rate of 5.0 oz 100 gal^{-1}. Mancozeb (Penncozeb 75DF, United Phosphorous, Inc., King of Prussia, PA) was tank-mixed and applied with myclobutanil at a rate of 3 lb 100 gal^{-1} A^{-1}. The non-treated trees were within the same rows as treated trees. Standard maintenance materials were applied to all trees in each test block, as needed (1). Ten scabby leaves were collected from three canopy heights (lower, middle and upper) and two zones (interior or exterior) within individual Stayman trees on 19 May, 12 June and 13 July 2009 and on 30 June 2010. Previously described methods of culturing monoconidial isolates of *V. inaequalis* and sensitivity tests based on percent growth suppression (PGS) were used (2). Isolates were incubated at 19°C and colony diameters were measured bi-directionally every week for 4 weeks. Sensitivity tests were conducted in triplicate. A total of 40 isolates were tested. Data were analyzed using PROC GLM in SAS (Windows, release 9.2, SAS Institute Inc., Cary, NC).

Results: The mean colony growth was significantly different ($P < 0.001$) among canopy heights (lower, middle or upper) and zones (interior or exterior) in 2009, but not in 2010 ($P = 0.144$ and $P = 0.286$, respectively).

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APPENDIX B

2010 spray deposition data

Distribution of manganese within the tree canopy in 2010

Protocol: Four mature Stayman Winesap trees that were not included in any other fungicide trials were selected from three-tree sets in a lower density orchard block (less than 150 trees A^{-1}) located at the AHS AREC. Twenty-five leaves were collected from each of seven locations (lower west, middle west, upper west, lower east, middle east, upper east or middle by the trunk) within each tree. Mancozeb (Manzate Pro-Stick 75WG, DuPont, Wilmington, DE) was applied to both sides of the tree with an airblast sprayer at a rate of 3 lb A^{-1} on 7 July 2010. After the residue had dried, 25 leaves were collected from the same seven locations. All leaves were dried at room temperature for several days and then mailed to A&L Eastern Laboratories in Richmond, VA, for processing. Data were averaged from the four single-tree replications and analyzed using PROC GLM in SAS (Windows, release 9.2, SAS Institute Inc., Cary, NC).

Results: Manganese concentrations before or after mancozeb application were not significantly different amongst the seven locations within the tree (Table B.1). However, foliar deposit in the middle west location (160.25 ppm) was significantly higher than that of the middle by the trunk (76.75 ppm). We concluded that spray deposition was generally homogeneous throughout the tree canopy. However, the unusually hot and dry environmental conditions (average June temperature was 87.9; average rainfall was 2.48 inches) prior to sampling and the late timing of the mancozeb application may have been contributing factors.

Table B.1. Distribution of mancozeb within the tree canopy in 2010.

	Manganese Concentration (ppm)		
Sampling Zone	**Before**[a]	**After**[b]	**Foliar Deposit**[c]
Lower West	57.50 a	203.50 a	146.00 ab
Middle West	50.25 a	210.50 a	160.25 a
Upper West	56.75 a	141.75 a	85.00 ab
Lower East	59.75 a	178.25 a	118.50 ab
Middle East	63.50 a	190.50 a	127.00 ab
Upper East	53.50 a	195.75 a	142.25 ab
Middle by trunk	61.50 a	138.25 a	76.75 b

[a] Background levels of manganese before mancozeb application. Mean separation by Waller-Duncan K-ratio t-test (p=0.05). Data averaged from four single-tree reps.

[b] Mancozeb (Manzate Pro-Stick 75WG) was applied with an airblast sprayer to both sides of the tree at a rate of 3.0 lbs A^{-1} on 7 July 2010.

[c] Foliar deposit equals the difference in manganese concentration prior to and following mancozeb application for that canopy location.

APPENDIX C

CYP51A1 gene

Venturia inaequalis isolates tested for point mutations within the *CYP51A1* gene

Background: Previous studies in yeast and filamentous fungi have demonstrated that mutations within the cytochrome P450 (*CYP51A1*) gene (1,2,5,7) or overexpression of the gene (3,4) confer SI resistance. The *CYP51A1* gene encodes C-14 α-demethylase, an enzyme involved in the synthesis of ergosterol – the target of DMIs. Mutations within the gene alter the amino acid composition of the enzyme, thereby decreasing its affinity to the DMIs. In contrast, overexpression of the gene increases production of the enzyme, which overwhelms the DMI target sites. Studies with *V. inaequalis* have not found nucleotide variation within the *CYP51A1* gene, but SI resistance in some field strains has been correlated with overexpression of the gene and the presence of an upstream insertion (6).

Protocol: Nine isolates of *V. inaequalis* (three resistant, three moderately resistant, and three sensitive to myclobutanil) were tested for point mutations within the *CYP51A1* gene. Agar plugs from each isolate were transferred to flasks containing 100 ml of ¼-strength potato dextrose (PD) broth and incubated at 25ºC for 4 weeks. The PD broth and mycelia mixture was poured through two layers of cheesecloth, and fungal masses were scraped together using wooden coffee stirrers. Isolates were transferred into uncapped cryovials and lyophilized overnight in a Freezone 2.5 Liter Benchtop Freeze Dry System (Labconco Corp., Kansas City, MO). Lyophilized tissue for each isolate was transferred into tubes containing 0.5 mm silica beads (BioSpec Products, Inc., Bartlesville, OK) and homogenized for one minute at 2500 rpm in a Mini-Beadbeater-96 (Model #3110BX;

BioSpec Products, Inc., Bartlesville, OK). Genomic DNA was extracted using the BioSprint 15 workstation and Plant DNA kit (Qiagen, Inc., Valencia, CA) following manufacturer's protocols, and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Pittsburgh, PA). Allele-specific amplification was conducted using the Schnabel and Jones PCR cycling parameters (6). A 404-bp fragment spanning codon 133 was amplified using primers 5'-GTTTCCCTTCTTCGGCAACAC-3' and 5'-TGAGAGCTTCGGTGGTGAGAC-3'. A 1% agarose gel containing 1 µl of each sample and 2 µl of 6X DNA loading buffer (Fisher Scientific, Pittsburgh, PA) was used to confirm the presence of the 404-bp fragment. The *CYP51A1* gene was amplified using wild-type primer 5'-CTCCGATCTTGTCTA-3' and mutant primer 5'-CTCCGATGTTGTCTT-3'. The PCR amplicons were bidirectionally sequenced at the Virginia Bioinformatics Institute. The consensus sequences were analyzed using the Lasergene software package (DNASTAR, Inc., Madison, WI).

Results: The 404-bp fragment was present for all nine isolates, confirming amplification of codon 133 (region in the *V. inaequalis CYP51A1* gene that corresponds to a point mutation correlated with SI resistance in *Uncinula necator*) (6). The consensus sequences for the gene were identical for all isolates tested, and no point mutations in codon 133 were observed. Sequence data for *V. inaequalis* isolates ViB06, ViA25 and ViC20 were deposited in GenBank, Accession Numbers JQ956422, JQ956423, and JQ956424, respectively. We concluded the fungicide resistance observed in these nine isolates was not due to point mutations within the *CYP51A1* gene. However, we did not examine overexpression or the possible presence of a 553-bp upstream insertion (6).

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APPENDIX D

Amplified fragment length polymorphisms

Venturia inaequalis isolates examined for variation using amplified fragment length polymorphisms

Background: Amplified fragment length polymorphism (AFLP) is a genotyping technique that can be readily adapted to DNA of any origin or complexity without prior sequence information (1,3). It has been used to study genetic diversity (4,8) and fungicide resistance in other plant pathogen populations (2,5).

Protocol: Eighty-two isolates of *V. inaequalis* were examined for variation using amplified fragment length polymorphisms (AFLPs). Isolates had previously been classified by SI resistance and were divided into two groups (set 1: N=43 with six resistant and sixteen sensitive; set 2: N=39 with five resistant and eighteen sensitive). Genomic DNA was extracted using the BioSprint 15 workstation and Plant DNA kit (Qiagen, Inc., Valencia, CA) following manufacturer's protocols, and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Pittsburgh, PA). Digestions, ligations, pre-amplifications and selective amplifications were conducted following laboratory protocols (6,7). Genomic DNA was digested with two restriction enzymes (*MSE*I and *Eco*RI), and the fragments were ligated to end-specific oligonucleotide adaptors. A pre-selective PCR amplification was performed using primers complementary to the adaptor sequences to reduce the number of amplified bands. A selective PCR amplification was performed using fluorescent-labeled primers (*EcoR1+CC* labeled with FAM and *EcoR1+AA* labeled with HEX) that contained two additional bases. The first PCR amplicon served as a template in the selective PCR

amplification. Hi-Di formamide and GeneScan 500Liz size standard (Applied Biosystems, Inc., Carlsbad, CA) were added to each sample prior to the run. AFLPs were analyzed on a Genetic Analyzer 3130xl (Applied Biosystems, Carlsbad, CA) and GeneMarker Version 1.6 software (SoftGenetics, LLC., State College, PA) was used to visualize electropherograms. Polymorphic bands (alleles) were scored from 100 to 500 base pairs at a peak detection threshold intensity greater than 250 and then converted to a binary format (0=no peak; 1=peak) and analyzed using PROC GLM SELECT in SAS (Windows, release 9.2, SAS Institute Inc., Cary, NC).

Results: DNA extraction, PCR amplification and AFLP generation were successful, and electropherograms were obtained for all 82 isolates of *V. inaequalis*. Sequential elimination of alleles 92, 9 and 15 was significant ($P < 0.001$, $P = 0.009$, and $P = 0.004$, respectively) for set 2 according to LASSO selection, which adds or deletes parameters based on a constrained form of ordinary least squares regression. However, this genotyping technique cannot identify a gene's function or expression level, and we did not sequence the bands corresponding to alleles 92, 9 and 15. LASSO selection failed to identify any alleles for set 1. Despite identical settings on the Genetic Analyzer 3130xl, the two AFLP data sets were not comparable, as isolates SI2 and SI20 (which were included in both runs) failed to yield identical electropherograms.

Acknowledgements: We thank Ciro Velasco-Cruz and Zaili Fang from the Laboratory of Interdisciplinary Statistical Analysis for SAS recommendations and technical support.

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APPENDIX E

Bioassay

Bioassay examining myclobutanil metabolism in *Venturia inaequalis* isolates with various fungicide sensitivities

Background: Plant pathologists have routinely used inexpensive bioassays to screen organisms for sensitivity to compounds (4,9). For those that are time- or labor- intensive to culture *in vitro*, another organism (referred to as an indicator organism) is selected based on its growth in response to known dilutions of a standard. The growth at each dilution is plotted against the log concentration, and the resulting standard curve is used to calculate the concentration of unknown samples. Previous studies have used *Colletotrichum acutatum*, *C. gloeosporioides*, *Monilinia fructicola* and *Botrytis cinerea* (1,5) as indicator fungi. Since metabolic degradation of pesticides is one method fungi use to decrease a chemical's toxicity (6), bioassays are often followed by thin layer chromatography (TLC) to separate and detect compounds and their metabolites (8). The purpose of this research was to examine myclobutanil metabolism in *V. inaequalis* isolates.

Protocol: The growth rate of five potential indicator organisms (two isolates of *Botryosphaeria dothidea*, two isolates of *Colletotrichum acutatum*, and one isolate of *Monilinia fructicola*; all obtained from infected apple fruit at the AHS AREC, Winchester, VA) were evaluated on potato dextrose agar amended with 0, 0.01, 0.1, 0.5, 1.0, 5.0 or 10.0 μg ml^{-1} myclobutanil (Dow AgroSciences, Indianapolis, IN) every day for three days. Indicator organism was selected based on the correlation coefficient (R^2 value) of growth rate versus the log myclobutanil concentration. Eleven isolates of *V.*

inaequalis (one resistant, four moderately resistant, three sensitive, and three not previously classified) were examined for myclobutanil metabolism using a bioassay and TLC. To obtain conidia for the bioassay, *V. inaequalis i*solates were grown for 14 days in 4% (w/v) malt extract broth in medicine bottles following previously published protocols (7,10). Conidia were harvested and suspensions were standardized to an optical density of 0.2 ± 0.02 (approximately 1 x 10^6 conidia ml^{-1}) with a Spectronic 20 (Bausch and Lomb, Rochester, NY) set to 525 nm (11). Screwtop tubes containing 3ml of 4% (w/v) malt extract broth, 1 ml conidial suspension and 1ml of myclobutanil (0, 0.05, 0.1, 0.5 or 1.0 μg ml^{-1}) were set up for each *V. inaequalis* isolate and incubated at 19ºC for 1, 4, 8, 24, 48 or 72 hours. Following incubation, 1ml aliquots of each sample were transferred to Petri dishes, and potato dextrose agar was added. Plates were inoculated with 2mm diameter agar plugs of the indicator organism. Plates were incubated at 19ºC and growth measurements recorded after 48 hours. Bioassay data were analyzed using PROC GLM in SAS (Windows, release 9.2, SAS Institute Inc., Cary, NC). Aliquots of all samples were also applied to TLC silica gel plates (EMD Chemicals, Darmstadt, Germany), and plates were developed using ethyl acetate (2) or 75:25 (v/v) hexane:acetone solvent (3). R_f values were calculated following TLC plate exposure to a UV light.

Results: *B. dothidea* isolate B was selected as the indicator organism (R^2 = 0.9425) after two days growth. The mean colony growth of the indicator organism on plates amended with aliquots from the *V. inaequalis* conidial suspensions was significantly different ($P <$ 0.001) among incubation times and myclobutanil concentrations (Figure E.1). Tukey's HSD test indicated significant differences between short (1 or 4 hours) and long (48 or 72 hours) incubation times. The myclobutanil concentration of unknown samples was

calculated using the equation $y = -0.4593x + 0.5677$ ($R^2 = 0.9374$); concentrations were generally lower than the *V. inaequalis* conidia were exposed to, suggesting breakdown of the fungicide. However, TLC plates failed to develop with either solvent (as compounds remained at the origin and did not move), so we were unable to determine if differences in the indicator fungus' growth were due to metabolic action on the fungicide by the *V. inaequalis* conidia.

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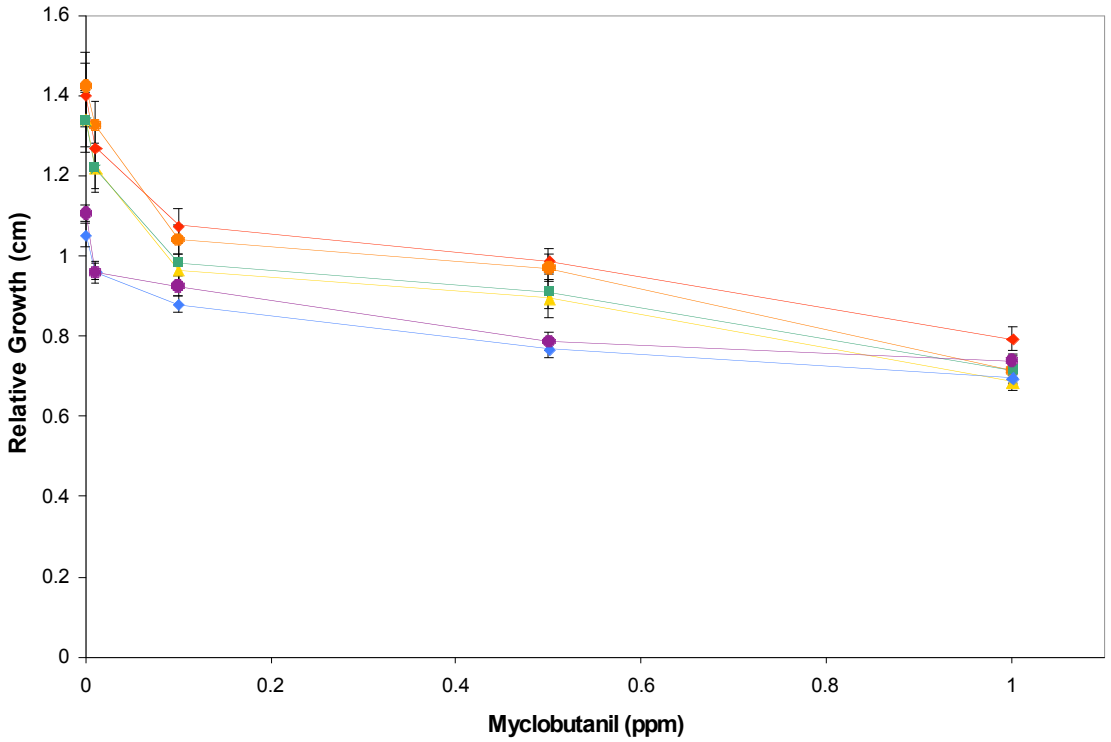
Figure E.1. Average growth rate of indicator fungus (*Botryosphaeria dothidea*) on potato dextrose agar amended with aliquots from conidial suspensions of *Venturia inaequalis* isolates following exposure to myclobutanil. *V. inaequalis* isolates (N=11) were incubated with various myclobutanil concentrations for 1 hour (red), 4 hours (orange), 8 hours (yellow), 24 hours (green), 48 hours (blue) or 72 hours (purple).



APPENDIX F

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