SUSTAINABLE USE OF SUCCINATE DEHYDROGENASE INHIBITORS FOR THE MANAGEMENT OF APPLE SCAB

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Apples are an important specialty crop in New York State, and NYS is the second largest producers of apple nationwide. Of economic importance is the impact of managing the fungal pathogen causing apple scab, *Venturia inaequalis.* If left unmanaged, this disease will render the fruit unmarketable due to the malformation and commercially unacceptable olive-green to brown lesions that develop on fruit. The disease can also have negative impacts on overall tree health by reducing photosynthesis when the severity of leaf infection is high. In the absence of durable host resistance, commercial management relies heavily on the use of fungicides to prevent and control apple scab infections. Misuse of fungicides targeting a single site within fungi has resulted in sequential development of resistance to many classes of fungicide, resulting in ineffective fungal disease management. The first goal of this research was to characterize the efficacy and target site of succinate dehydrogenase inhibitors (SDHIs), a class of fungicides in which new chemistries have been released. This work demonstrated high *in vitro* efficacy of SDHI fungicides and provided the framework for future phenotypic and genotypic screening for fungicide resistance. The second goal of this research was to improve our understanding of how to best use these fungicides to prevent resistance development. We investigated practices involving application at both

low and high doses as well as uses in different mixtures. We found that application at the higher doses often led to isolates with sensitivity shifted towards resistance. Additionally, this work reiterated the importance of mixing fungicide modes of action to prevent selection for resistance. Finally, the third goal of this research was to create a management plan that uses SDHIs with more sustainable biopesticide chemistries. We found potential for a program where SDHIs rotated with biopesticides could provide season-long management of apple scab. The work completed in this dissertation will help contribute to sustainable use of SDHI fungicides for the management of apple scab.

BIOGRAPHICAL SKETCH

Katrin Ayer was born and raised in Hartford County, Connecticut where she attended Farmington High School. It was through enjoyment of her science courses that she decided to further pursue biology when she attended Hobart and William Smith Colleges, in Geneva NY. Her summers after her sophomore and junior years were spent conducting research at the New York State Agricultural Station (now Cornell AgriTech) in the lab of Dr. Kerik Cox. It was those summers that she became acquainted with the field of plant pathology through studying lesion severity and spore production of *Venturia inaequalis* in one year, and fungi and bacteria associated with a rising pest of apples in the next. She graduated with a B.S. in Biology and minor in Hispanic Studies in 2016. Afterwards, she decided to pursue her Ph.D. in plant pathology at Cornell University under the advisement of Dr. Kerik Cox, where she continued her studies with the causal agent of apple scab, *Venturia inaequalis*.

Dedicated to my family.

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CHAPTER 1

INTRODUCTION

Apple production. Apples (*Malus ×domestica)* are an economically important specialty crop in the United States, with approximately 323,000 acres planted nationwide (USDA NAAS 2018). Globally, the United States is the second largest producer of apple, behind China (Food and Agriculture Organizations of the United Nations 2013 and a commercial value of utilized production at 3-3.5 billion dollars in the (USDA NASS 2018). Nationally, New York is second to Washington in production of apple, with an average annual total utilized production of 29.5 million bushels (NYAA 2018). The majority (53%) of NY apples are grown for the fresh market, while 47% are grown for processing, totaling approximately 50,000 acres of land across 1,000 farms (USDA NASS 2012). The major production regions in NY include the Champlain Valley, Hudson Valley, Finger Lakes, Niagara Frontier, and Central NY (USDA Agricultural Census 2007). New York has a large diversity of cultivars produced compared to other areas of the country, however, most of the acreage is planted to 'McIntosh', 'Empire', 'Red Delicious', 'Cortland', 'Golden Delicious', 'Rome', 'Idared', 'Crispin', 'Paula Red', and 'Gala'. (New York Apple Growers Association 2018). Further, many different apples have been developed in New York at Cornell AgriTech (Formerly the New York State Agricultural Experiment Station; NYSAES), including 'Cortland', 'Jonagold', 'Empire', 'SnapDragon', 'Ruby Frost', 'Liberty', 'Freedom' and more recently 'Cordera', 'Pink Luster' and 'Firecracker'.

In recent years, apple trellising systems and tree architecture have become modernized, replacing fewer, larger central leader trees (approximately 330 trees per ha) (Figure 1.1 A) with a higher density planting of smaller trees grafted to size-controlling rootstocks at anywhere from 1,200-5,400 trees per ha (Figure 1.1 B) (Robinson et al. 2013). This change in orchard design

provides many benefits including an increased yield per hectare, increased sunlight received by fruit, increased ability to incorporate mechanization, and reduced labor time and associated costs (Robinson et al. 2013; Lauri et al. 2008; Robinson 2004). Despite these advances in planting systems, there are still many challenges to sustainable apple production in the Northeastern US including cost and time of labor, safety of workers, climate change, consumer preference, fruit finish disorders, weed and insect presence, and disease pressure. Fortunately, trees trained to modern high-density systems should have smaller foliar canopies and may be less conducive for disease development (Tivoli et al. 2013). In the NY region, the production of apples can be challenged by several diseases, including but not limited to, fire blight, apple scab, powdery mildew, cedar apple rust, fly speck/sooty blotch, and bitter rot (Figure 1.2). Of these, apple scab, caused by the ascomycete fungus *Venturia inaequalis*, is one of the most economically important fungal diseases of apple.

Figure 1.1. Adaptation of modern tree architecture by apple grower in Walden, NY, transitioning from trees the left panel to those in the right panel. **A.** Older, larger central leader apple trees planted at approximately 330 trees/ha. **B.** Modern, smaller apple trees planted at approximately 1,700trees/ha suitable for increased orchard mechanization, and optimal yield.

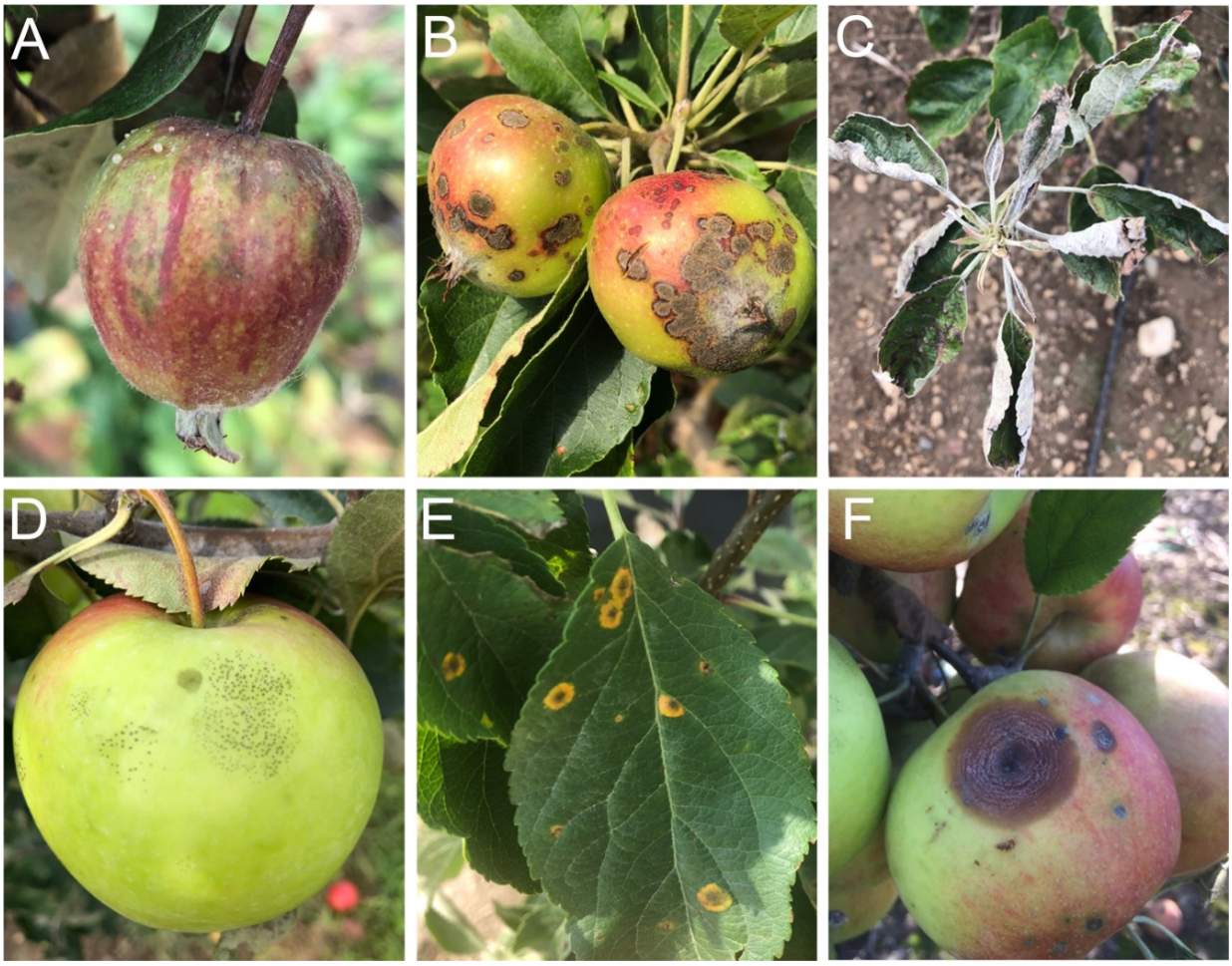

Figure 1.2. Major disease of apple including **A.** the causal agent of fire blight, *Erwinia amylovara*, oozing from immature fruit, **B.** brown, cracked apple scab lesions on fruit infected with *Venturia inaequalis*, **C.** signs of powdery mildew sporulating with *Podosphaera leucotricha* conidia on terminal shoots, **D.** Flyspeck and Sooty Blotch symptoms on fruit caused by a complex of diseases, **E.** Pycnia spore stage of *Gymnosporangium juniperivirginianae* causing cedar apple rust on upper side of leaves, and **F**. Bitter rot lesions sporulating on fruit caused by *Colletrotrichum sp.*

Biology of apple scab. Apple scab is one of the most economically important diseases of apple in the northeast United States due to the prevailing temperate climate of cool wet spring weather, which is conducive to the lifecycle of the pathogen *V. inaequalis* (Figure 1.2 B) (MacHardy 1996). *V. inaequalis* overwinters on leaf litter on the orchard floor, initially as a saprotroph, where it will then produce pseudeothecia filled with developing asci (a fruiting body

resulting from sexual reproduction in which meiospores are formed). During the spring, asci mature within pseudeothecia and ascospores are released during daytime rainfall (Aylor 1998; Gadoury et al. 2004). In the spring, maturation of asci, ejection, and infection by ascospores is highly dependent upon temperature, humidity, light, and leaf wetness (MacHardy and Gadoury 1989; Brook 1969). These ascospores can infect young tissues, including cluster leaves and cluster fruit. In the weeks following this primary infection, olive-green lesions will develop on fruit and leaves and will have a fuzzy appearance as they sporulate with asexual conidia (MacHardy 1996).

If primary ascospore infections are not adequately managed, secondary infection cycles, produced from splashed dispersed conidia, may occur during each period of precipitation as long as temperatures remain below 30°C. Fruit lesions formed early in the season can lead to fruit cracking and the introduction of secondary pathogens (MacHardy 1996). At the season's end, leaves will senesce and fall to the ground, with those infected with *V. inaequalis* contributing to the overwintering inoculum (Figure 1.3). During severe epidemics, apple scab infection of leaves can greatly reduce photosynthesis and lead to premature defoliation, while fruit infections will lead to misshapen and cosmetically unappealing fruit, rendering them unsuitable for the fresh market (Spotts and Ferree 1979; Rosenberger 2016). Apple scab infections may also be quiescent and further develop in cool wet storage condition, leading to pinpoint lesion that put fruit out of grade for the fresh market. While especially problematic in temperate climates, apple scab is endemic to production regions worldwide and therefore a variety of management techniques are incorporated, based on disease pressure and local weather patterns.

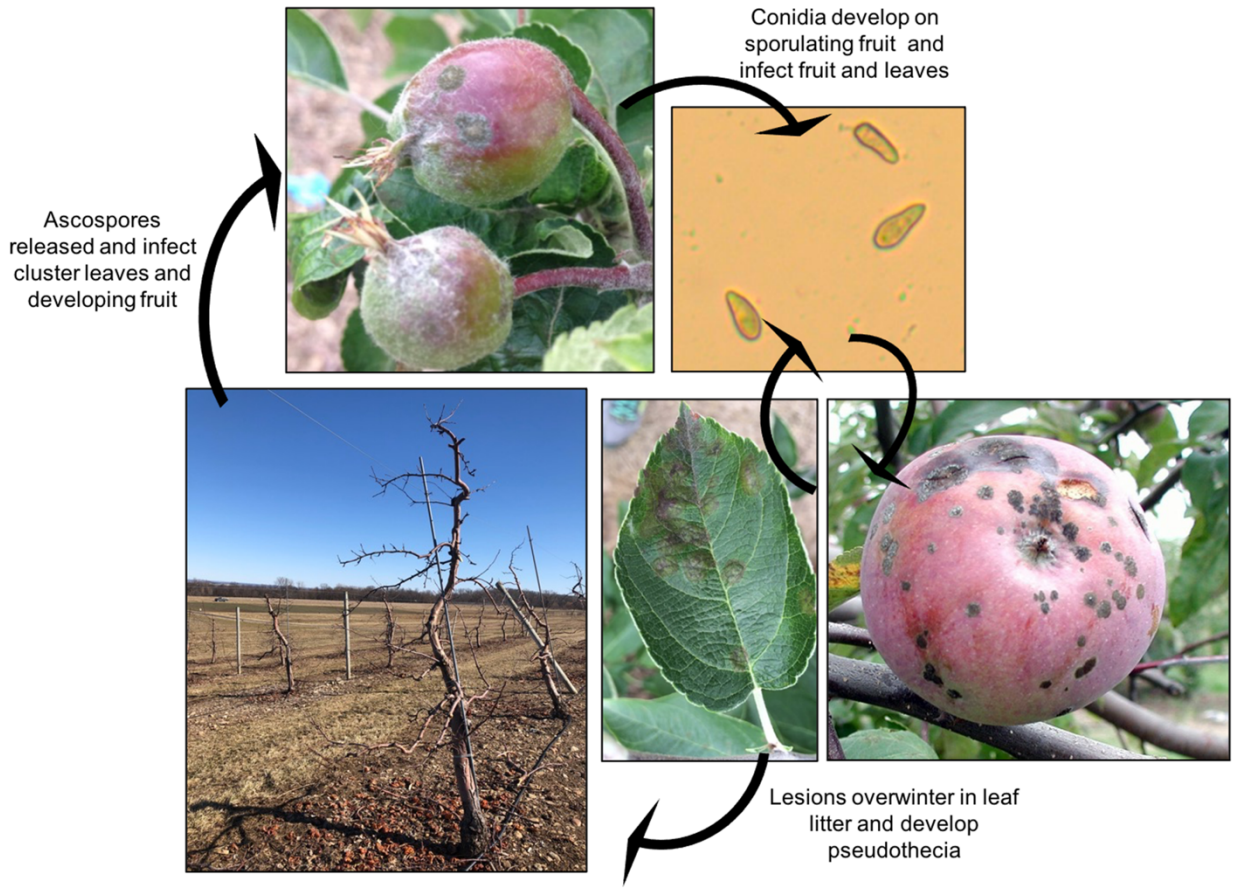

Figure 1.3. Simplified life cycle of *V. inaequalis.* Inoculum overwinters under the tree canopy where pseudothecia with ascospores form. The primary infections are caused by released ascospores during rain events on cluster fruit and leaves. Resulting lesions sporulate with conidia and cause secondary infections on fruit and leaves later in the season.

Apple scab management. Apple scab management is focused on controlling primary infections to reduce overall orchard inoculum and interrupt the disease cycle. If these primary ascospore infections are prevented, it will greatly reduce inoculum and the potential for secondary infections of fruit later in the season. (MacHardy et al. 2001). Cultural practices are recommended to reduce overwintering pseudothecia on leaf litter on the orchard floor. This can be accomplished by springtime applications of urea to the orchard floor or use of a flail mower to shred leaves under the canopy (Cox et al. 2018). Further, delayed-dormant applications of copper

may be used to reduce primary inoculum in the early season (Montag et al. 2006). Indeed, these methods can greatly reduce the risk of primary scab and ascospore release in the spring (Sutton et al. 2000; Gomez et al. 2007).

The use of resistant varieties is effective and there are 18 identified genes conferring qualitative resistance to *V. inaequalis* to date (Bus et al. 2011; Papp et al. 2020). For example, *Rvi6 (*VF gene), deriving from *Malus floribunda,* has been an effective source of qualitative resistance to *V. inaequalis* (Bus et al. 2011). However, breakdown of *Rvi6* has been reported in Europe (Parisi et al. 1993) and recently in New York (Papp et al. 2020) in a planting that did not receive chemical management. Further, apart from 'Honeycrisp', cultivars with disease resistance often lack consumer-name recognition due to taste, color, and storage preferences, and therefore are not commercially popular (Volk et al. 2015; Merwin et al. 1994).

In the absence of durable resistance in commercially acceptable cultivars, growers rely on chemical management throughout the growing season to prevent crop loss and to reduce inoculum pressure in addition to cultural practices. It is estimated that growers spend approximated $550 in fungicide applications per acre per year to manage apple scab and other fungal diseases (Cox 2015), and such management can require more than 10 applications per season. Fungicides are applied from silver tip (dormant applications) throughout the season to protect against both the primary and repeating secondary infection cycles of apple scab. Use of disease support systems (DSSs), such as the Network for Environment and Weather Applications (NEWA; newa.cornell.edu; Carroll and DeGaetano 2011), can help with timing of management decisions. Forecasting models such as NEWA can predict when an infection event will occur as well as the local weather conditions during those infection events. Such tools allow for fungicide applications to be made preventatively as opposed to curatively and potentially reduce need for

fungicide applications during low-risk weather.

Conventional fungicide programs consist of a combination of synthetic broad-spectrum contact fungicides such as captan and mancozeb, as well as single-site fungicides. Fungicides such as mancozeb and captan have multi-site modes of action and aren't at risk for resistance development. They are useful in rotation and in tank mixtures with single-site fungicides and providing high efficacy against apple scab (Brent and Hollomon 1995; Guillino et al. 2010). Unfortunately, the multi-site mode of action causes these fungicides to lack specificity, have greater off-target effects, and require larger use volumes in application. This has called into question sustainability of these products (Runkle et al. 2017; EPA 1999; EPA 2005).

Many biopesticides also have a multi-site mode of action, have been approved for organic status, are accepted for their environmental softness, and may be a useful tool in disease management. Their modes of action can be derived from antimicrobial metabolites, competition for nutrients and ecological niche on plant surfaces, and plant defense activators (Kohl et al. 2019; Caulier et al. 2019). However, biopesticides are not heavily utilized in many conventional management programs due to their observed lack of efficacy in field trials (Cromwell et al. 2008; Pscheidt et al. 2001; Pscheidt and Bassinette 2014; Rosenberger et al. 2000; Strickland and Cox 2020; Yoder et al. 2007; Yoder et al. 2014b). More effective organic-approved alternatives include inorganic copper, sulfur, and lime sulfur (calcium polysulfide) (Cooley et al. 2008).

By comparison, modern single-site fungicides target a single process within the fungus and hence, are more effective in lower quantities. Important classes of these single-site fungicides include demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), and succinate dehydrogenase inhibitors (SDHIs), belonging to groups 3, 11, and 7 respectively as defined by the Fungicide Resistance Action Committee (FRAC). These fungicides inhibit *V.*

inaequalis by targeting processes such as ergosterol biosynthesis (DMI) and cellular respiration (QoI and SDHI). While these fungicides have high activity against *V. inaequalis*, the frequency of applications and specificity for the target site applies a high selection pressure for the development of resistance (FRAC 2020).

Fungicide resistance and SDHI fungicides. Resistance development to single-site fungicides has created challenges for successful management of apple scab, as an increase in resistance leads to a decreased or complete lack of efficacy for the fungicide (Frederick et al. 2014; Lesniak et al. 2011; Villani and Cox 2014). Resistance can be qualitative, where a single mutation may confer full resistance, or quantitative, where numerous individual mutations result in smaller shifts in sensitivity but may have an additive effect. Mutations may involve alteration of the target site, changes in target site expression levels, breakdown of the fungicide, use of an alternative pathway, or presence/increase in efflux pumps/ABC transporters (Ma 2005; Sang et al. 2018). Development of resistance consequently leads to ineffective disease control in the field. Important resistant management practices include limiting the number of applications within a growing season, rotating between fungicides with different target sites, and avoiding consecutive applications of the same chemistries (Brent and Hollomon 1998). Recent registration of newly developed succinate dehydrogenase inhibitors (SDHI) has allowed for new opportunities in management against *V. inaequalis* with potential for rotation with other single-site fungicides where widespread resistance was imminent.

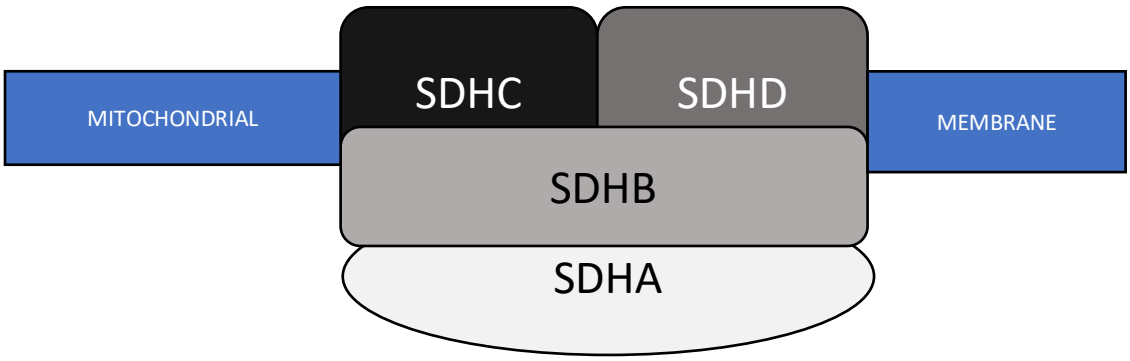

Figure 1.4. Depiction of the succinate dehydrogenase enzyme, the target site of succinate dehydrogenase inhibitors (SDHI). SDHI fungicides come in direct contact with the SDHB, SDHC, and SDHD proteins.

SDHI fungicides interfere with fungal cellular respiration through inhibition of complex II of the mitochondria (FRAC 2020). The target site consists of four subunits, *sdhA* (flavoprotein), *sdhB* (iron-sulfur subunit), *sdhC* (membrane-bound protein), and *sdhD* (membrane-bound protein) (Figure 1.4) (Avenot and Michailides 2010). Binding to the interface between the SDHB, SDHC, and SDHD subunit interferes with the cycling of succinate to fumarate (Sierotzki and Scalliet 2013; Avenot and Michailides 2010). First generation SDHI fungicides were primarily used to manage basidiomycetes with limited effectiveness against ascomycetes, however, the next generation SDHI fungicides have improved activity against ascomycetes, with fungicides including fluopyram, penthiopyrad, benzovindiflupyr, fluxapyroxad, pydiflumetofen, and inpyrfluxam (Xiong et al. 2015). These chemistries show great promise for control of apple scab, and a high level of efficacy has been reported in other fungal pathogen of fruit such as *Botrytis cinerea* and *Alternaria alternata*. Unfortunately, fungicide resistance has been reported in both of these pathogens (Fernandez-Ortuno et al. 2017; Malik et al. 2013; Avenot and Michailides 2009). To date, widespread resistance to SDHI

fungicides in *V. inaequalis* has yet to be reported. Therefore, further investigation to understand activity of these chemistries, risk of resistance development, mechanisms of resistance, and best management practices is necessary to ensure sustainable SDHI use and a continued high level of efficacy against *V. inaequalis* in important apple growing regions throughout New York and the US.

Dissertation Research Goal. The overarching goal of my research is to increase sustainable management of the apple scab pathogen, *V. inaequalis* through responsible use of SDHI fungicides, ensuring these fungicides are used effectively, applications are not selecting for resistance, and management plans are sustainable for continued future use in the NY apple industry. Specifically, this dissertation aims to answer the following broad questions: 1) How effective are SDHI fungicides against *V. inaequalis*; 2) How can we reduce the risk of practical resistance through altering application practices; and 3) Can biopesticides be used in congruence with the use of SDHI fungicides to limit the use of multi-site protectant fungicides?

Specific Research Objectives

Chapter 2: In this chapter, we characterized the efficacy of SDHI fungicides as well as the genetic composition of target genes (*Visdh* genes). We established baseline *in vitro* sensitivity to four different SDHI fungicides; fluxapyroxad, pydiflumetofen, inpyrfluxam, and benzovindiflupyr as well as their cross-sensitivity. This work gives us insight into how to best use these fungicides as well as a framework allowing for future phenotypic and genotypic resistance monitoring.

Chapter 3: After determining efficacy, in this chapter we aimed to identify practices that best slow selection for fungicide resistance development in order to ensure longevity of SDHI fungicide utility in management of apple scab. Specifically, in this chapter we looked at the effect of application dose and tank mixture on SDHI fungicide sensitivity over the course of four years. We worked to understand whether use of high doses or low doses exert more selective pressures as well as whether a single-site fungicide or multi-site fungicide is a better mixing partner with an SDHI fungicide for reducing selective pressure. Such work is imperative for future studies on the efficacy of SDHI fungicides.

Chapter 4: In this chapter, we look to integrate biopesticides in a management program with SDHI fungicides to reduce the heavy reliance on synthetic multi-site fungicides captan and mancozeb. This program utilizes horticultural planting systems as well as use of a DSS to best optimize a program with biopesticides to enhance the potential for disease management.

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CHAPTER 2

CHARACTERIZATION OF THE *VISDHC* AND *VISDHD* GENES IN *VENTURIA INAEQUALIS*, AND SENSITIVITY TO FLUXAPYROXAD, PYDIFLUMETOFEN, INPYRFLUXAM, AND BENZOVINDIFLUPYR

Abstract

Succinate dehydrogenase inhibitors (SDHI) are an important class of fungicides for management of apple scab, especially as resistance to other classes of fungicides has become prevalent in the northeastern United States. Considering the single-site mode of action, there is a high risk of resistance development to SDHI fungicides. Such risk mandates the need for proper monitoring of shifts in population sensitivity. This study aims to provide a means for phenotypic and genotypic characterization of SDHI fungicide resistance for *Venturia inaequalis*, the causal agent of apple scab. To complement the published sequence of *VisdhB*, target genes *VisdhC* and *VisdhD* were identified using sequences of homologous genes in other fungal organisms and a draft genome of *V. inaequalis*. Using mycelial growth and conidial germination assays, baseline sensitivities and cross sensitivities of *V. inaequalis* were determined for several SDHI fungicides. Mean baseline EC_{50} values for conidial germination of benzovindiflupyr, fluxapyroxad, pydiflumetofen, and inpyrfluxam were found to be 0.0021, 0.0284, 0.014, and 0.0137 μg ml^{-1}, respectively. Mean baseline EC_{50} values for mycelial growth of benzovindiflupyr, fluxapyroxad, pydiflumetofen, and inpyrfluxam were found to be 0.0575, 0.228, 0.062, and 0.0291 μg ml^{-1}, respectively. A significant and positive correlation in sensitivity was found between benzovindiflupyr, fluxapyroxad, pydiflumetofen, and inpyrfluxam as well as penthiopyrad and fluopyram, with the highest correlation between benzovindiflupyr and penthiopyrad for mycelial

inhibition of *V. inaequalis* ($r = 0.950$, $P<0.001$). For inhibition of conidial germination, the highest correlation was observed between penthiopyrad and fluopyram ($r = 0.775$, $P<0.001$). Furthermore, the sequences of the *VisdhC* and *VisdhD* genes were identified and characterized for baseline isolates of *V. inaequalis*. Residues of similar position to mutations found in other systems that confer resistance to SDHI fungicides were identified in baseline isolates, but no mutations were identified in baseline isolates or those previously exposed to SDHI fungicides. This study will serve as a reference for future monitoring of resistance to SDHI fungicides in *V. inaequalis* at both a phenotypic and genotypic level.

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Introduction

Apple scab, caused by the ascomycete fungus *Venturia inaequalis* (Cooke) G. Winter, is one of the most economically devastating diseases of apple (MacHardy 1996). Conventional management relies on multiple fungicide applications throughout the growing season due to the lack of commercially popular cultivars with host resistance (MacHardy 1996; Merwin et al. 1994). Increased use of single-site fungicides has resulted in subsequent selection for resistant isolates and a consequential loss of fungicide efficacy, as seen with wide-spread resistance to Quinone outside Inhibitors (QoI) (Frederick et al. 2014; Lesniak et al. 2011; Villani and Cox 2014). Recently, new chemistries in the class of succinate dehydrogenase inhibitors (SDHIs) have been registered for disease control in apples, and with these new products come new opportunities for effective control of apple scab. While SDHI fungicides were first commercialized in the 1960s, these original chemistries were not widely used in apple due to the narrow range of activity restricted to basidiomycetes (Xiong et al. 2015; Sierotzki and Scalliet 2013). Next generation SDHI fungicides have high efficacy on a broader spectrum extended to ascomycete fungi, including the apple scab pathogen, through targeting complex II of the mitochondria and interfering with cellular respiration (Hagerhall 1997; Sierotzki and Scalliet 2013; Villani et al. 2016).

The high intrinsic activity and specificity for their target presents a high risk of resistance development to SDHI fungicides (Avenot and Michailides 2010; Hu et al. 2015). Additionally, as other methods of chemical control become obsolete due to resistance development, there will be a heavier reliance on the use of the newer SDHI fungicides, limiting options for chemical rotation (Cox 2015; Chapman et al. 2011). This highlights the necessity of sustainable use of SDHI fungicides with emphasis on resistance monitoring to protect the durability of this class of

fungicides. While there are currently no known populations of *Venturia inaequalis* with resistance to SDHI fungicides, resistance to SDHIs has been noted in other fungal species, including *Botrytis cinerea* (Fernández-Ortuño et al. 2017), *Stagonosporopsis citrulli* (syn. *Didymella bryonia)* (Thomas et al. 2012), *Alternaria solani (*Mallik et al. 2013*) Sclerotinia sclerotiorum* (Wang et al. 2015), and *Alternaria alternata* (Avenot et al. 2009). These observations of resistance necessitate the availability of careful monitoring protocols for *V. inaequalis* to best understand resistance development and ensure permanency of SDHI fungicides in this system.

The *sdh* target gene is composed of four subunits; a flavoprotein (SDHA*)* an iron-sulfur unit (SDHB*)* and two membrane-bound units (SDHC and SDHD), but SDHI fungicides only come in direct binding contact with the SDHB, SDHC, and SDHD subunits, otherwise known as the ubiquinone binding pocket (Sierotzki and Scalliet 2013). Mutations conferring resistance can therefore occur in any of the three subunits where SDHIs directly bind. The most common of these mutations is typically found in the SDHB subunit, where a critical histidine residue is replaced by arginine, leucine, or tyrosine to confer resistance (Sierotzki and Scalliet 2013). At this time, only the *VisdhB* gene has been characterized for *V. inaequalis* (Villani et al. 2016). While several mutations associated with SDHI resistance have been identified in the SDHB subunit in other phytopathogenic fungi, this does not explain all incidences of resistance. The H134R mutation and the D123E mutation has been well documented for *Alternaria alternata* in the SDHC and SDHD subunit, respectively (Avenot et al. 2009). This illustrates the importance of elucidating the sequence of the *VisdhC* and *VisdhD* genes for resistance monitoring.

Following extended exposure to SDHI fungicides, shifts in sensitivity have been observed in other fungal pathogens, including *B. cinerea* (Hu et al. 2015) and *Monilinia*

fructicola (Amiri et al. 2010), illustrating the importance of monitoring phenotypic changes in sensitivity. To most accurately assess changes in sensitivity, it is first necessary to determine effective concentrations of SDHI fungicides at which 50% of growth is inhibited (EC_{50} values) for baseline isolates. Baseline sensitivities have previously been determined for the registered SDHI fungicides penthiopyrad, fluopyram, and benzovindiflupyr (Villani et al. 2016), leaving the baseline sensitivity of recently registered fluxapyroxad and the forthcoming inpyrfluxam and pydiflumetofen. Furthermore, due to the similarity in chemistries and mode of action, it is appropriate to address concerns of cross-sensitivity between all the SDHI fungicides.

The objectives of this study were to (i) clone the remaining target genes of SDHI fungicides: *VisdhC* and *VisdhD* (ii) determine the baseline sensitivity to four SDHI active ingredients: fluxapyroxad, pydiflumetofen, inpyrfluxam and benzovindiflupyr and (iii) determine cross-sensitivity between all six SDHI fungicides that are currently registered or in development for use on apple.

Materials and Methods

Isolate collection. Fifty baseline isolates with no previous exposure to modern fungicides were collected from apple trees in Romulus, Geneva, and Waterloo, NY from 2013-2017 to use for characterization of baseline sensitivity (Villani et al. 2015). To compare the baseline with a population exposed to SDHI fungicides, > 30 additional isolates were collected from a 20-year-old research orchard in Geneva, NY, consisting of 'Empire' and 'Cortland' apples on M.9/M.111 interstem rootstocks. During the growing season prior to collection, trees were subjected to four applications of Sercadis® (BASF, Research Triangle Park, NC) a formulated product containing fluxapyroxad. Sercadis® was applied at rate of 256.2 mL/ha dilute to run-off with a AA2 GunJet

handgun (TeeJet Technologies, Glendale Heights, IL) at 551 kPa. Fungicides were applied in coordination with infection periods at pink, bloom, first cover (10 days after petal fall), and second cover (approximately 24 days after petal fall) with multi-site protectants applied at green tip and petal fall. During June 2016 and 2017, a minimum of 15 leaves with apple scab lesions were randomly collected from the treatment replications. In addition, symptomatic leaves were collected from the baseline locations sampled from previously (Villani et al., 2015). From these leaves, isolated individual lesions arising from a single ascospore infection event (Köller et al. 2004; MacHardy and Gadoury 1989), were selected for monosporic isolation. Conidia of *V. inaequalis* were obtained, as previously described (Frederick et al. 2014; Villani et al. 2015), by first excising individual lesions with a 5mm diameter cork borer. Lesions were then placed in 1.2ml deionized water and shaken for 60 seconds to dislodge and suspend conidia in water at an approximate concentration ranging from 10^2 to 10^3 *V. inaequalis* conidia ml^{-1} (Villani et al. 2016). The leaf discs were removed and discarded from the tubes with a sterile toothpick. Conidial suspensions were stored at -20°C for up to five years prior to fungicide evaluations.

Identification of *VisdhC* and *VisdhD* genes. The draft genome sequence of *Venturia inaequalis* for isolate 3a-27-17, as described by Villani et al. (2016), was used to identify areas of high homology with closely related fungal organisms. Accessions from NCBI Genbank for the *sdhC* and *sdhD* genes from *Alternaria alternata* (accession number KJ426267 and KJ426275 respectively) and *Botryotinia fuckeliana* (accession number GQ253443 and GQ253441 respectively) were used to locate putative genes in *V. inaequalis*. A genome search in the assembly of isolate 3a-27-10 for homologous contiguous areas was completed with the local CLC Main Workbench 7 (version 7.8.1, Qiagen Bioinformatics, Redwood City) BLAST

function. Primers were then designed to target putative upstream and downstream sequences of *VisdhC* and *VisdhD* genes (Table 2.1).

To amplify putative *VisdhC* and *VisdhD* genes, DNA was extracted from baseline isolate 10-3-14, which had been cultured on potato dextrose agar (PDA) (Difco Laboratories Inc., Detroit, MI) and incubated at room temperature (20 to 23°C) for four weeks. To extract genomic DNA (gDNA), approximately 50 mg of mycelium was excised from the agar and ground in liquid nitrogen using a mortar and pestle. An Omega BioTek E.Z.N.A. Plant DNA Kit (Omega Bio-Tek, Norcross, GA) was used to isolate total gDNA in accordance with the manufacturer's instructions. To locate introns and coding regions, an RNA extraction was also completed for the same isolate. Briefly, isolate 10-3-14 was grown and tissue was lysed as described above. RNA was extracted using an Omega BioTek E.Z.N.A Plant RNA Kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's instructions. DNA contamination was removed using TURBO DNA-*free* Kit (Ambion by Life Technologies, Carlsbad, CA) following the manufacturer's instructions. Complementary DNA (cDNA) was generated from RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories Inc.) following the manufacturer's instructions.

Both extracted cDNA and gDNA were then amplified through polymerase chain reaction (PCR). Primer pairs VisdhC-106F/VisdhC+200R and VisdhC1F/VisdhC654R were used to amplify gDNA of the *VisdhC* gene while primer pairs and VisdhD-139F /VisdhD713R and VisdhD-139F / VisdhD695R were used to amplify the *VisdhD* gene. cDNA of *VisdhC* and *VisdhD* was amplified using primer pairsVisdhC1F/VisdhC654R and VisdhD1f/VisdhD713R, respectively. A PCR was completed at the following cycles using a T100 Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA); 2 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at specified annealing temperature (Table 2.1) and 45 seconds at 72°C, followed by a

final extension at 72°C for 7 minutes. For each primer pair, 25 µl reactions were prepared composed of the following: 12.5 µl 1x EmeraldAmp GT PCR Master Mix (Takara Bio/Clontech Laboratories, Inc., Mountain View, CA) 8 µl sterile distilled water, 1 µl of both reverse and forward primers (10 µM), and 2.5µl fungal cDNA or gDNA. PCR products were separated on a 1% agarose gel (Bio-Rad Laboratories Inc.) and stained with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA) in 1x Tris-acetate-EDTA buffer at 100 V for 1 hour. Gel images were taken using KODAK Gel Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY).

PCR products were purified using PCR Clean-up & Concentrator (Zymo Research, Irvine CA) and submitted for Sanger sequencing with both forward and reverse primers at Cornell University Sequencing facility in Ithaca, NY, using their Applied BioSystems Automated 3730xl DNA Analyzer. Additional primers VisdhC582R and VisdhD433R were used to sequence the purified PCR samples to confirm the position of the start codons for the *VisdhC* and *VisdhD* gene, respectively. All sequencing data was analyzed using CLC Main Workbench. Suspected residue sites for mutations conferring SDHI resistance were identified based on homology to mutations found in other pathosystems. The NCBI BLAST function was used to determine percent homology of *VisdhC* and *VisdhD* to those of other fungal species.

Determination of baseline sensitivity to next generation SDHI fungicides. To determine the concentration at which 50% of conidial germination and mycelial growth was inhibited by each of the SDHI fungicides (EC_{50} value), *in vitro* inhibition assays were completed. Technical grade fluxapyroxad (BASF Cooperation), pydiflumetofen (Syngenta Crop Protection, Greensboro, NC), and inpyrfluxam (Valent Technical Company, Dublin, CA) were dissolved in

acetone to attain the following final nine concentrations through serial dilutions once amended in media: 0 (control), 0.001, 0.01. 0.05. 0.1, 0.5, 1.0, 5.0, and 10 μg ml^{-1}. Baseline EC_{50} determination was also repeated for technical grade benzovindiflupyr (Syngenta Crop Protection) as an internal control to ensure these new isolates were also representative of isolates used in previous work done by Villani et al. (2016). Each concentration was amended into autoclaved potato dextrose agar (PDA) (Difco Laboratories) that had cooled to 55°C. PDA was determined to be the most suitable media for use with technical grade SDHI fungicides for *in vitro* sensitivity of *V. inaequalis* by Villani et al. (2016). All media were also amended with 50 μg ml^{-1} streptomycin sulfate and 50 μg ml^{-1} chloramphenicol to prevent high levels of bacterial contamination (PDA++).

To assess preventative activity of SDHI fungicides, we conducted a modified conidial germination assay (Villani et al, 2016). Conidial solutions of 100 μl collected from 50 baseline isolates were evenly distributed on PDA++ amended with each SDHI fungicide (fluxapyroxad, pydiflumetofen, inpyrfluxam, and benzovindiflupyr) at each concentration as well as a control plate (no fungicide added). Plates were incubated at 22°C for one week in 12 h light-dark intervals. After the incubation period, five germ tubes or micro-colonies from five individual conidia per plate were measured as replicates using SPOT Idea 3.1 with SPOT Imaging Basic software package (Diagnostic Instruments Inc., Sterling Heights, MI) attached to an Olympus SZX12 stereoscope (Olympus America Inc., Center Valley, PA). Conidial germination assays were performed for each fungicide, which was independently tested for the same 50 baseline isolates. All fungicides were assessed within the timeframe of three months in controlled laboratory conditions. In addition to EC_{50} values for inhibition of conidial germination, EC_{50} values for mycelial growth on the four SDHI fungicides were also determined to evaluate post-

infection activity. Fifty baseline isolates obtained from stored (2013-2015) or fresh (2016-2107) single-spored conidial suspensions (see above) were plated on PDA++ and incubated for three weeks at 22°C. Mycelium was transferred using a 3mm cork-borer in duplicate on each concentration for the four SDHI fungicides. After three weeks of incubation at 22°C, the radial diameter was measured for both plugs on each plate.

For inhibition of both conidial germination and mycelial growth, mean percent relative growth was determined. The effective concentration needed for 50% inhibition based on the non-amended control was calculated using a probit analysis with the Statistical Analysis System (SAS) Version 9.4 (Cary, NC) SAS as previously described (Kreis et al. 2016).

Determination of cross-sensitivity to SDHI fungicides. Given the similarity in target site of the SDHI fungicides, cross-sensitivity of *V. inaequalis* to the six SDHI fungicides was determined. Technical grade fluxapyroxad, pydiflumetofen, inpyrfluxam, penthiopyrad (DuPont Crop Protection), fluopyram (Sigma-Aldrich, St, Louis, MO), and benzovindiflupyr were dissolved in acetone to a concentration of 1x and 10x the baseline EC_{50} values for each fungicide (determined above). Cross-sensitivity was determined for both inhibition of mycelial growth and conidial germination. The discriminatory doses for penthiopyrad, and fluopyram used for analysis of cross-sensitivity were based on EC_{50} values determined in Villani et al, (2016). EC_{50} values used for penthiopyrad and fluopyram for conidial germination inhibition were 0.08, and 0.176 μg ml^{-1}, respectively, while EC_{50} values for mycelial growth inhibition were 0.8 and 2.02 μg ml^{-1}, respectively. The concentration of 10x the EC_{50} value was selected as a discriminatory dose for consistency between different SDHI fungicides. Due to little to no mycelial growth at a concentration of 10x the EC_{50} value, mycelial growth cross-sensitivity was repeated at 1x the

EC_{50} value.

Fifty isolates, consisting of either baseline or exposed phenotypes, were used to assess cross-sensitivity for the stage of conidial germination inhibition. Conidial suspensions (100 µl) from each isolate were evenly distributed on PDA++ amended with each of the six different fungicides as well as two non-fungicide amended control plates. After one week, germ tube length or micro-colony diameter were measured as described above. Cross-sensitivity was also determined for inhibition of mycelial growth. Fifty isolates of actively growing *V. inaequalis* cultures from above (four weeks old) were plated on PDA++ amended with each of the different fungicides, including two non-fungicide amended control plates with a cork borer 5 mm in diameter. After four weeks of incubation at 22°C, diameters of radial growth were measured for each isolate on each fungicide. Percent relative growth was calculated for each isolate on all six SDHI fungicides for both mycelial and conidial growth. Pearson Correlations were determined for all pairwise fungicide combinations using the Proc CORR procedure in SAS, Version 9.4.

Multiple Fungicide Resistance Analysis. We hypothesized that target-site mutations may be conferring the higher relative growth observed in isolates exposed to SDHI fungicides when compared to growth of baseline isolates. To determine if high relative growth values (> 50%) were associated with mutations in the target site of SDHI fungicides, DNA was extracted from mycelium of two isolates with the highest percent relative growth on SDHI fungicides as described above. Target genes; *VisdhB*, *VisdhC*, and *VisdhD* were amplified as described above and as in Villani et al. (2016) (Table 2.1). PCR products were purified and sequenced in the forward and reverse direction as described above. All returned sequences were analyzed for SNPs in CLC Main Workbench.

Given the potential for an absence of missense mutations in *Visdh* genes, we wished to determine whether high relative growth values for exposed isolates could be due to multi-fungicide resistance mechanisms, such as overexpression of multi-drug efflux pumps (Hahn 2014; Jurick et al. 2017). We hypothesized that if isolates with reduced sensitivity to SDHI fungicide also had high relative growth values when exposed to fungicides from different classes (e.g. FRAC groups), then multi-fungicide resistance mechanisms may be involved. To assess the possibility of multi-fungicide resistance in exposed populations, a subset of isolates from the cross-sensitivity experiments (three baseline isolates and nine exposed isolates with relative growth greater than 50% on a minimum of two SDHI fungicides tested) were subjected to relative growth assays on PDA++ amended with technical grade myclobutanil (FRAC group 3) (Sigma-Aldrich, St Louis, MO), dodine (FRAC group U12) (Sigma-Aldrich, St Louis, MO), and trifloxystrobin (FRAC group 11) (Sigma-Aldrich, St Louis, MO). Myclobutanil, dodine, and trifloxystrobin were dissolved in acetone to obtain discriminatory concentrations of 0.1, 0.2, and 0.02 μg ml^{-1}, respectively, as previously described (Koller et al. 2004; Villani et al. 2015; Frederick et al. 2014). Two 3 mm mycelial plugs growing on PDA were transferred to PDA ++ amended with each fungicide at the discriminatory dose as well as two non-fungicide amended control plates. Plates were incubated at 22°C for three weeks. Radial growth was measured on each plate, once per colony along the largest diameter, and percent relative growth was calculated for each fungicide as described above.

Results

Identification of *VisdhC* and *VisdhD* genes. The *VisdhC* and *VisdhD* genes were cloned from a baseline isolate 10-3-14 to identify potential mutations that may confer future resistance

to SDHI fungicides. *VisdhC* and *VisdhD* genes were successfully amplified and sequenced with the primers listed in Table 2.1 and deposited into GenBank under accession numbers MH484042 and MH484043, respectively.

Amplification of gDNA of the *V. inaequalis sdhC* gene with primer pairs VisdhC-106F/VisdhC+200R and VisdhC1F/VisdhC654R revealed fragments of 589 and 706 bp, respectively. To identify exons (coding region) and introns within *VisdhC*, amplification of cDNA was conducted with primer pair VisdhC1F/VisdhC654R and revealed a 558 bp fragment. Alignment of gDNA and cDNA *VisdhC* sequences yielded introns of 49 and 99 bp occurring at nucleotide positions +41 and +175, respectively. With introns, *VisdhC* was determined to be 712 bp in length. The translated *Vi*SDHC protein had 56% similarity to that of *Botrytis cinerea* and 57% similarity to *Monilinia fructicola*. Residue 144 (histidine) was noted (Supplementary Table S2.1) due to its proximity and similarity to common mutations found in other phytopathogens that confer resistance to SDHI fungicides, where a histidine residue is replaced with arginine (e.g. H134R) (Avenot et al. 2009).

Amplification of gDNA of the *V. inaequalis sdhD* gene with primer pairs VisdhD-139F/VisdhD713R and VisdhD-139F/VisdhD695R, revealed fragment size of 851 and 835 bp, respectively. Coding regions were amplified with primer pair VisdhD1f/VisdhD713R and revealed a fragment size of 600 bp. The *VisdhD* gene was 713bp (with introns) and allowed us to identify two introns of 59 and 54bp occurring at positions +233 and +310. Residue 130 (aspartic acid) was noted (Supplementary Table S2.1) due to its proximity and similarity to common mutations found in other systems that confer resistance to SDHI fungicides, where an aspartic acid is replaced by an arginine. This correlates with the D133R mutation in *A. alternata* (Avenot et al. 2009) and the D132R mutation in *Sclerotinia sclerotiorum* (Avenot and Michailides 2010).

The translated *Vi*SDHD protein had 57% homology to that of *Botrytis cinerea*.

Table 2.1. Primers used in identification of *VisdhC* and *VisdhD* genes

Primer Name	Amplification Target	Sequence (5'-3')	Annealing Temperature (°C)
VisdhC-106F[a]	VisdhC upstream (Forward)	TTGGCTTTGACTATTCGG	49
VisdhC+200R	VisdhC downstream (Reverse)	ATTGAGGTGTTTGAGATGAC	49
VisdhC1F	VisdhC coding region (Forward)	ATGATGTCCAACAGAGCTCTCCA	50
visdhC654R	VisdhC coding region (Reverse)	TCAAATATACATGTGATCGGC	50
VisdhC582R	VisdhC start (Reverse)	GGAATGTGAATGGTAGAG	47[b]
VisdhD1F	VisdhD coding region (Forward)	ATGGCCTCAATTGCTCA	52
VisdhD713R	VisdhD coding region (Reverse)	TTAGCTCGCGAACCTGCCGA	52
VisdhD-139F	VisdhD upstream (Forward)	TGATCATGGGACCTAAG	50
VisdhD695R	VisdhD middle (Reverse)	CCGAATTTATCCAACGATT	50
VisdhD433R	VisdhD start (Reverse)	AAGCGCTCGAATGTCCAGTG	58[b]

[a]Primer names are based on location of 5' end
[b]Indicates primer not used for PCR but for clarification in sequencing, therefore annealing temperature not used

Supplemental Table 2.1. Protein translation of target genes *VisdhC* and *VisdhD* from baseline isolate of V. *inaequalis*, with critical residues highlighted based on mutations in other systems.

Gene	Protein translation	Documented mutation
VisdhC	MMSNRALQITARRVAAQKPTTAFARFASPAAVATNTHFL HRRQVATQHVSVDNNDILVAQRKLRPVSPHLGIYKPQITW IPSMFNRITGAILSGGFYLFGIGYLVAPAFGWHLESAVLAA SFATWPIAAKVLAKMSLALPFTF**H**SFNGLRHLMWDMTKG ITNAQVARSGWFVVGLSFVSAFYLAVGY	**HI34R** (*Alternaria alternata*) (Avenot et al. 2009)
VisdhD	MASIAHSTMLRQAFRAAPTKQISSRTASTLISSPLRTARPA VQQPLRSAFVQDSIPTSSRVAAFHATGSKAILPPLPQAVTG DVNTPARVPEPSPSHGSYHWTFERLISAGIVPLTMAPFIGG SLNPLL**D**GVFCAALLAHSHIGWDAMITDYFPGWRVPKVR AALNWTLRIATVMVGVGLYEFETSTWQSLDKFGRFAS	**D133R** (*Alternaria alternata)* (Fan et al. 2015) **D123E** (*Alternaria alternata)* (Avenot et al. 2009)

Baseline sensitivity to fluxapyroxad, pydiflumetofen, inpyrfluxam, and benzovindiflupyr. Baseline sensitivity values for *V. inaequalis* were determined for four SDHI fungicides: fluxapyroxad, pydiflumetofen, inpyrfluxam, and benzovindiflupyr. Baseline sensitivities were determined for mycelial growth and conidial germination using growth inhibition assays. Discrepancies in isolate numbers between different fungicide or growth stage sensitivity assessment were the result of high contamination levels of yeast and other fungi. For inhibition of conidial germination, the mean EC_{50} value for fluxapyroxad was 0.0284 μg ml^{-1} (n=30) with a range from 0.00159 to 0.160 μg ml^{-1} (median = 0.0167 μg ml^{-1}) (Fig. 2.1a). The mean EC_{50} value for pydiflumetofen was 0.014 μg ml^{-1} (n=47) with a range from 0.00043 to 0.0106 μg ml^{-1} (median = 0.00281μg ml^{-1}) (Fig. 2.1b). For inpyrfluxam, the mean EC_{50} value was 0.0137 μg ml^{-1} (n=41) with a range from 0.00058 to 0.114 μg ml^{-1} (median = 0.008μg ml^{-1}) (Fig. 2.1c). Lastly, to ensure this set of baseline isolates performed similarly to those used by Villani et al. (2016), the mean EC_{50} values for mycelial growth inhibition were again determined for

benzovindiflupyr. The mean EC_{50} value for benzovindiflupyr was 0.00213 μg ml^{-1} (n=47), with a range of 0.00163 to 0.00337 μg ml^{-1}.

Compared with the EC_{50} values for conidial germination, EC_{50} values for mycelial growth inhibition were always greater for each of the SDHI fungicides. The mean EC_{50} value for fluxapyroxad was 0.228 μg ml^{-1} (n=22), with a range from 0.0236 to 0.65 μg ml^{-1} (median = 0.184 μg ml^{-1}). There was a hundredfold increase from the min to the max EC_{50} (Fig. 2.1d). The mean EC_{50} value for pydiflumetofen was 0.062 μg ml^{-1} (n=47) with a range from 0.000142 to 1.0317 μg ml^{-1} (median = 0.076 μg ml-1) (Fig. 2.1e). For inpyrfluxam, the mean EC_{50} value was 0.0291 μg ml^{-1} (n=32) with a range from 0.00239 to 0.128 μg ml^{-1} (median = 0.0181 μg ml^{-1}) (Fig. 2.1f). Mean EC_{50} values for benzovindiflupyr were 0.0575 μg ml^{-1} (n=47), with a range of 0.000532 to 0.920 μg ml^{-1}.

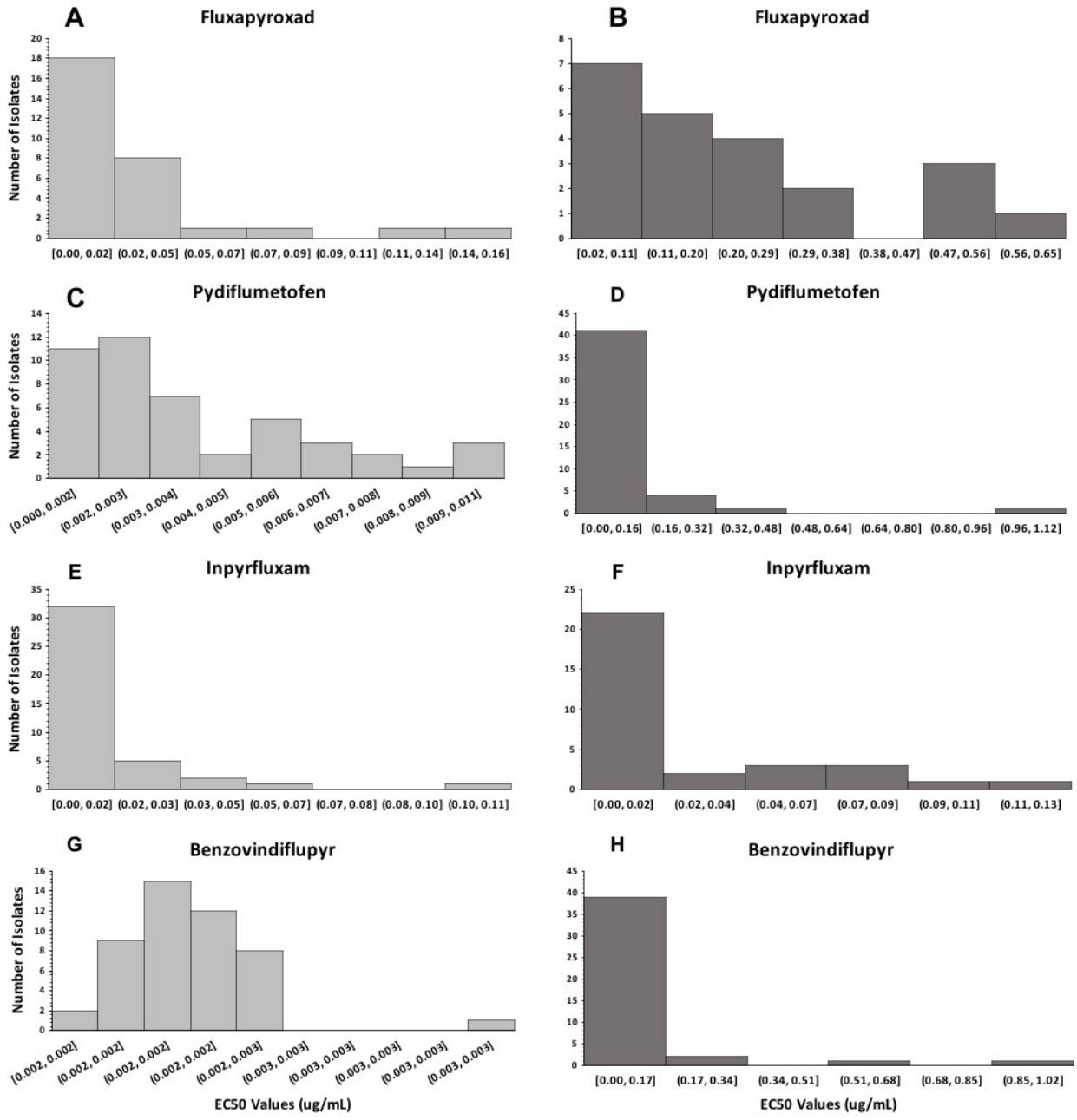

Figure 2.1. Distribution of values of effective concentrations by which growth was inhibited by 50% for baseline isolates of *V. inaequalis* for the SDHI fungicides fluxapyroxad (**A&B)**, pydiflumetofen (**C&D)**, inpyrfluxam (**E&F)**, and benzovindiflupyr (**G&H)** Graphs in light grey **(A, C, E, & G)** indicate EC_{50} values for conidial germination inhibition, while graphs in dark grey **(B, D, F, & H)** indicate EC_{50} values for mycelial inhibition.

Cross-sensitivities to SDHI fungicides. Due to the similarity in mode of action between all SDHI fungicides, cross-sensitivity was also examined to determine correlations in isolate sensitivity to different SDHI fungicides. The correlation coefficients *(r)* from each pairwise fungicide combination are printed below (Table 2.2). For inhibition of conidial germination, cross-sensitivity were overall lower, with the highest correlation seen between fluopyram and penthiopyrad (r= 0.775, P<0.0001). High correlations also existed between pydiflumetofen and fluopyram (r= 0.741, P<0.0001), inpyrfluxam and benzovindiflupyr (r= 0.715, P<0.0001), as well as pydiflumetofen and penthiopyrad (r= 0.707, P<0.0001) (Table 2.2). Among all SDHI fungicides, there was a significant (P<0.05), positive relationship between all active ingredients with varying degrees of strength (Table 2.2). For inhibition of mycelial growth of *V. inaequalis*, the highest correlations were between benzovindiflupyr and penthiopyrad (r= 0.950, P<0.0001), penthiopyrad and pydiflumetofen (r= 0.907, P<0.0001), fluxapyroxad and benzovindiflupyr (r= 0.902, P<0.0001), and fluopyram and penthiopyrad (r= 0.905, P<0.0001) (Table 2.2). However, varying levels of significant (P<0.05) positive correlations were observed between all six fungicides.

Table 2.2. Pairwise correlations of fungicide sensitivities for each of the six SDHI active ingredients.

	Fluopyram	**Penthiopyrad**	**Pydiflumetofen**	**Benzovindiflupyr**	**Inpyrfluxam**	**Fluxapyroxad**
Fluopyram		0.775[a]	0.741	0.689	0.680	0.613
Penthiopyrad	0.905		0.709	0.589	0.677	0.600
Pydiflumetofen	0.865	0.907		0.682	0.596	0.565
Benzovindiflupyr	0.863	0.950	0.871		0.715	0.599
Inpyrfluxam	0.861	0.708	0.670	0.884		0.558
Fluxapyroxad	0.846	0.783	0.820	0.907	0.884	

[a] Values above grey cells indicate correlation coefficients *(r)* for cross-sensitivity based on conidial germination for each fungicide combination, while numbers below grey cells are correlation coefficients for cross-sensitivity as determined by mycelial growth. All values are statistically significant ($P<0.0001$).

Interestingly, when observing differences in relative growth between baseline and exposed isolates, there was a very dramatic difference in growth patterns. In baseline isolates, growth was more inhibited, with mean relative growth for baseline isolates on SDHI fungicides ranging from 1.1 to 11.96% when compared to PDA control, depending on the SDHI fungicide. Conversely, mean relative growth for exposed isolates on SDHI fungicides ranged from 31.32 to 75.15%, depending on the SDHI active ingredient (Fig. 2.3). Overall, baseline isolates were much more sensitive to all six SDHI fungicides than the exposed isolates.

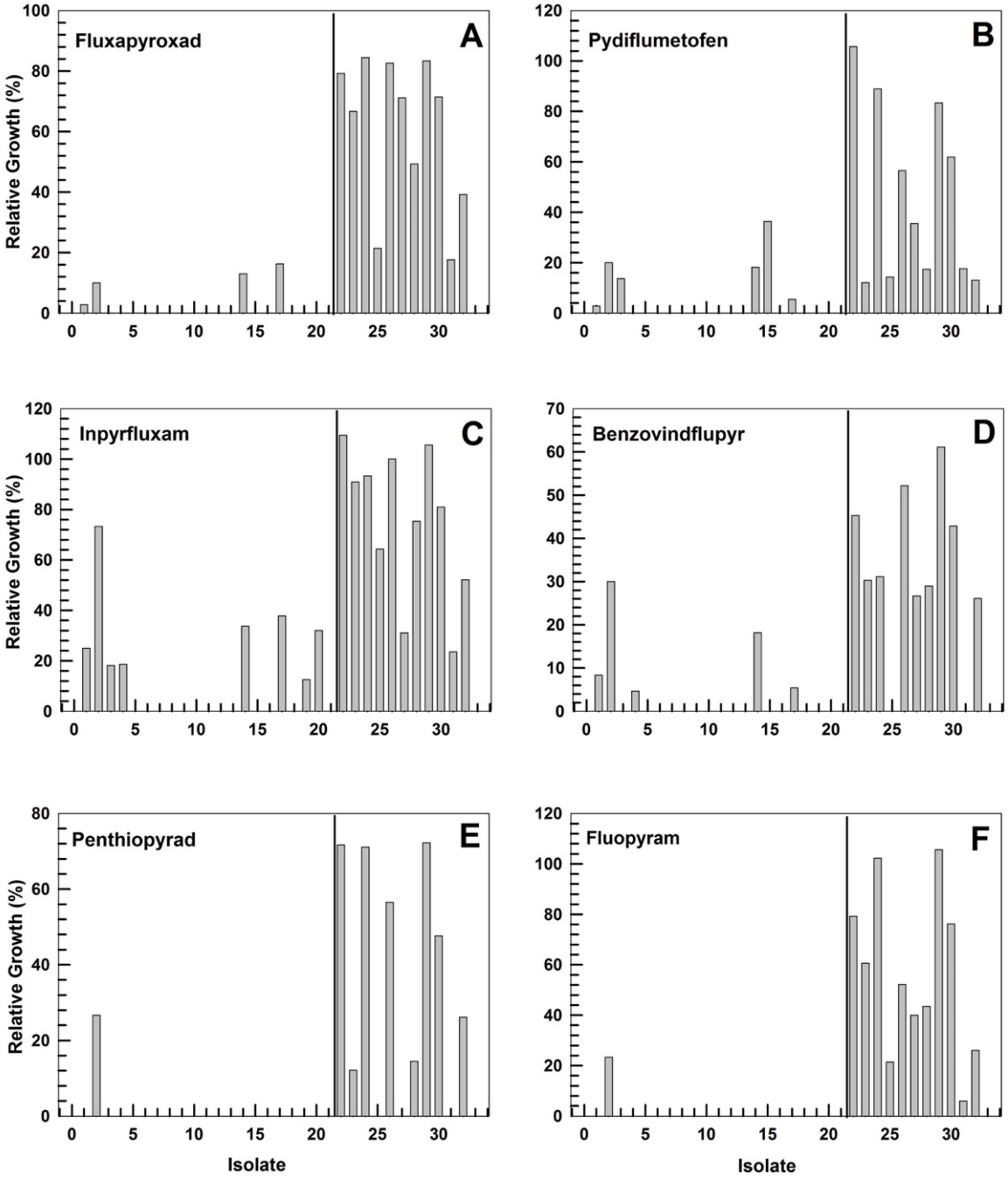

Figure 2.2. Percent mycelium relative growth values of baseline and exposed isolates on PDA++ medium amended with **A.** fluxapyroxad (0.228 µg/ml), **B.** pydiflumetofen (0.062 µg/ml), **C.** inpyrfluxam (0.0291 µg/ml), **D.** benzovindiflupyr (0.057 µg/ml), **E.** penthiopyrad (0.086 µg/ml), and **F.** fluopyram (2.02 µg/ml). Values represent the mean of two colonies for each isolate. Baseline isolates are to the left of the black line (1-21) and exposed isolates are to the right (22-32).

Presence of multiple fungicide resistance. No mutations in the protein sequence of the target site (*sdh* genes) were found in any of the exposed isolates with high relative growth as observed in the mycelial growth inhibition assays. Due to the absence of missense mutations in the *sdh* genes, we hypothesized that the observed insensitivity resulted from a multiple fungicide resistance mechanism that could therefore extend to different classes of fungicides. Indeed, isolates with high percent relative growth on SDHI fungicides also had high percent relative growth on media amended with myclobutanil, dodine, and trifloxystrobin, while all baseline isolates were much more sensitive to these fungicides. The mean relative growth for baseline isolates on myclobutanil, dodine, and trifloxystrobin were 11.56, 7.85, and 1.15%, respectively. Conversely, mean relative growth for exposed isolates on myclobutanil, dodine, and trifloxystrobin were 100.38, 57.71, and 63.33%, respectively, when compared to the PDA control (Fig 2.3).

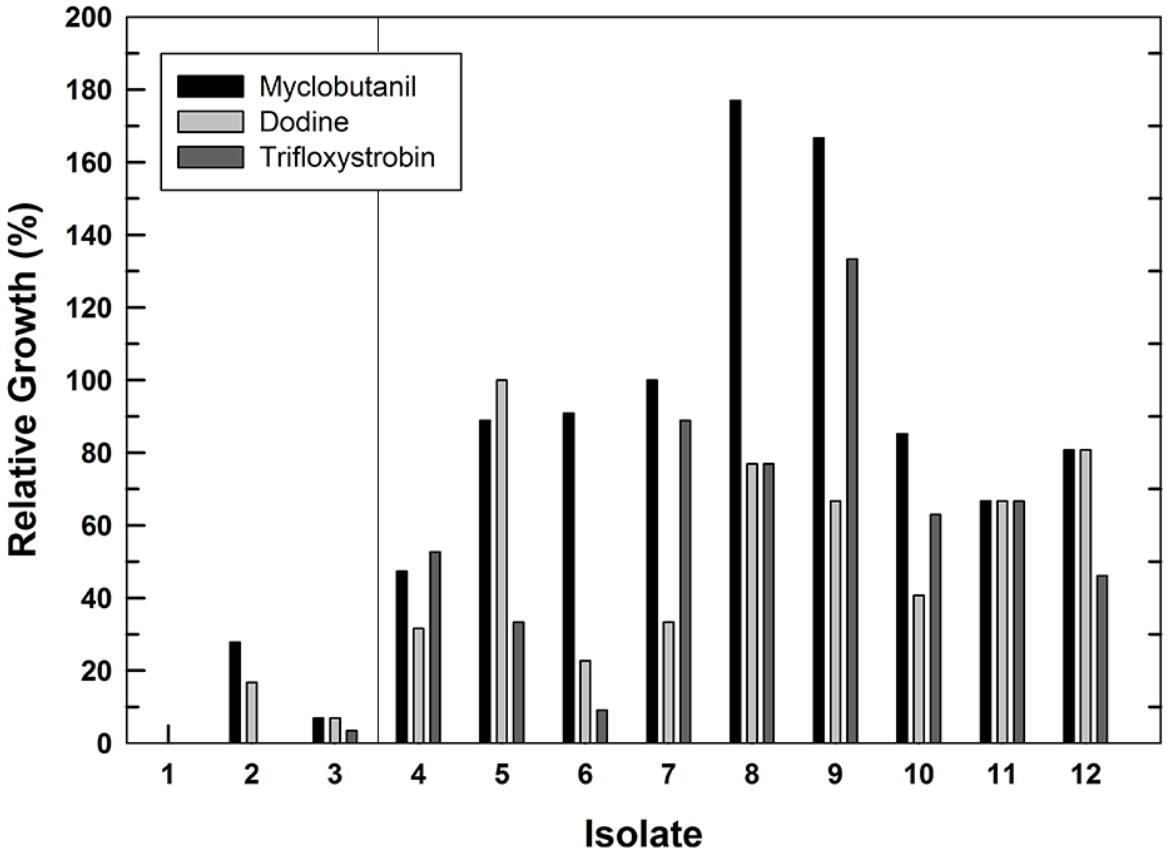

Figure 2.3. Percent mycelium relative growth values of baseline and exposed isolates on PDA medium amended with myclobutanil (0.1 µg/ml), dodine (0.2 µg/ml), or trifloxystrobin (0.02 µg /ml). Values represent the mean of two colonies for each isolate. Isolates to the left of the line (1-3) are baseline, while those to the right of the line (4-12) are exposed isolates.

Discussion

Increased reliance on SDHI fungicides for management of apple scab will require precise resistance monitoring for populations of *V. inaequalis*. In order to establish a comprehensive SDHI resistance monitoring program, phenotypic and genotypic characterization of several novel SDHI fungicides was completed for *V. inaequalis*. The *VisdhC* and *VisdhD* genes were characterized for a baseline isolate of *V. inaequalis* to determine potential mutation sites that

might confer resistance to SDHI fungicides. Furthermore, baseline sensitivities of *V. inaequalis* mycelium and conidial germination for several SDHI fungicides were determined. *V. inaequalis* baseline sensitivities for fluopyram, penthiopyrad, and benzovindiflupyr have been previously determined (Villani et al. 2016), but baseline sensitivities had yet to be determined to fluxapyroxad, pydiflumetofen, and inpyrfluxam. Lastly, cross-sensitivities for all six SDHI fungicides were determined using both baseline and exposed isolates of *V. inaequalis*.

In this study, the *VisdhC* and *VisdhD* genes were successfully cloned and characterized, and, along with the *VisdhB* gene sequence (Villani et al. 2016), will complete the characterization of the SDHI binding pocket for *V. inaequalis*. The low percent identity observed between the *VisdhC* and *VisdhD* genes and other fungal organisms is not surprising due to the known lack of conservation of the SDH subunits (Cecchini 2003). This lack of conservation underscores the importance of understanding resistance on a genotypic level, as different mutations have been identified occurring in the *sdhB*, *sdhC*, and/or *sdhD* subunit, as found in *A. alternata* (Avenot et al. 2009, 2008). Different SDHI fungicides might be able to differentially control resistant isolates depending on the mutation present. Certainly, some mutations confer full resistance to SDHI fungicides, while others only confer partial resistance (Sierotzki and Scalliet 2013). For example, in *B. cinerea,* the N230I mutation in the SDHB subunit may confer moderate resistance to boscalid, but low level of resistance to bixafen and carboxin (Veloukas et al. 2013). By comparison, the H272R mutation in the same subunit confers moderate resistance to boscalid, fluopyram, fluxapyroxad, but a lower level of resistance to SDHI active ingredients bixafen and carboxin (Veloukas et al. 2013). While we identified no isolates with mutations that resulted in a change in the protein sequence for any of the genes, the *Visdh* gene sequences will allow for development of primers targeting specific mutation sites identified based on homology

to other fungal species to aid in future genotypic monitoring when growers experience product failures.

Baseline sensitivity was also determined for SDHI fungicides fluxapyroxad, pydiflumetofen, and inpyrfluxam in addition to previously determined EC_{50} values for fluopyram, penthiopyrad, and benzovindiflupyr. Sensitivity was determined for both conidial germination and mycelial growth due to activity of these fungicides against both life stages (Villani et al. 2016). Of all SDHI fungicides evaluated in this study, benzovindiflupyr had the highest intrinsic activity against conidial germination of *V. inaequalis* followed by inpyrfluxam, pydiflumetofen and then fluxapyroxad. For mycelial growth inhibition, inpyrfluxam had the highest activity against *V. inaequalis*, followed by benzovindiflupyr, pydiflumetofen and then fluxapyroxad. Mean EC_{50} values for fluxapyroxad were eight times greater for mycelial growth than conidial germination. Pydiflumetofen had the most consistent EC_{50} value between the two stages of growth (mycelial and conidial). Due to high efficacy against both mycelium and conidia observed, it can be expected that these SDHI fungicides will also have good protectant and curative activity against *V. inaequalis* in the field, with higher efficacy when used as a protectant as opposed to when applied post-infection.

The baseline EC_{50} values of the SDHI fungicides evaluated in the current study fall within the range of EC_{50} values analyzed by Villani et al. (2016). While little has been published on some of these SDHI fungicides in other systems, fluxapyroxad and pydiflumetofen EC_{50} values for *V. inaequalis* are comparable to what was found in *A. alternata* (Avenot et al. 2013; Olaya et al. 2016). Similar to the high level of intrinsic activity we observed, Olaya et al. (2016) also observed that pydiflumetofen had the highest activity against *A. solani* when compared to several other SDHI fungicides. This is the second time benzovindiflupyr EC_{50} values have been

evaluated for *V. inaequalis*. The range observed here overlapped with the EC_{50} value for benzovindiflupyr, previously determined by Villani et al. (2016), which demonstrates the consistency between the two sets of isolates used. To our knowledge, this is the first study looking at the efficacy of inpyrfluxam against any fungal phytopathogen. We found that this fungicide has a high level of *in vitro* activity and potential for application for the management of apple scab due to level of activity comparable with SDHI fungicides registered for use against apple scab.

Differences in the level of intrinsic activity for SDHI fungicides could be explained by differences in affinity for the SDHI target site in *V. inaequalis*. Indeed, the level of homology between the *VisdhC* and *VisdhD* genes and those of the other fungal pathogens of perennial fruit trees (i.e. *M. fructicola, A. alternata,* and *B. cinerea)* is quite low. Hence, the intrinsic activity of the SDHI fungicides presented here may be different in other ascomycete pathogens as the fungicides bind to the SDH binding pocket a little differently. Alternatively, the level of intrinsic activity of these SDHI fungicides may be similar for other ascomycetes, but it could be that mutations have a differential effect on the development of insensitivity. While pydiflumetofen and inpyrfluxam are SDHI fungicides currently being developed and evaluated for use in commercial apple production, fluxapyroxad, fluopyram, penthiopyrad, and benzovindiflupyr are all currently registered for use on apple in New York at the time of these experiments. Commercial products containing these active ingredients, such as products like Aprovia™ or Sercadis®, should have high efficacy in the field based on high levels of activity measured *in vitro*.

Benzovindiflupyr, penthiopyrad, fluxapyroxad, and inpyrfluxam all belong to the chemical group pyrazole 4–carboximide, while pydiflumetofen belongs to the chemical group N-

methoxy-(phenyl-ethyl)-pyrazole-carboxamides), and fluopyram belongs to the chemical group pyridinyl-ethyl-benzamide (FRAC 2018). Despite the array of chemical groups that are represented within the class of SDHI fungicides, they all have the same target site, which may be problematic for cross-resistance. At this time, resistance to SDHI fungicides has not yet been reported in *V. inaequalis,* and therefore, cross-resistance remains unknown. However, due to the variation in sensitivities to differing chemistries of SDHI fungicides, we determined cross-sensitivity for a collection of baseline and exposed isolates for all six of these active ingredients.

Significant ($P < 0.0001$) and positive correlations were observed between all SDHIs, with varying degrees of strength of correlation depending on the two fungicides in comparison. The occurrence of cross-sensitivity is not surprising due to similar mode of action between all SDHIs. Benzovindiflupyr, penthiopyrad, fluxapyroxad, and inpyrfluxam are from the same SDHI chemical group, while both fluopyram and pydiflumetofen are from two differing chemical groups. Because of chemistry similarity, we would expect that SDHIs belonging to the same chemical group would have a higher level of cross-sensitivity. Surprisingly, this was not always the case. While high correlations were observed between benzovindiflupyr and penthiopyrad (r= 0.950) for mycelial inhibition, two chemistries belonging to the group of pyrazole-4-carboxamides, fluxapyroxad and penthiopyrad also belonged to the same group, but exhibited a much lower correlation (r= 0.783). Similar trends were observed between mycelial and conidial stages between inpyrfluxam and pydiflumetofen. We observed a low correlation for mycelial inhibition (r= 0.670) between these two fungicides, which is also mirrored for conidial inhibition (r= 0.599). Interestingly, a relatively low correlation was seen between benzovindiflupyr and penthiopyrad (r= 0.589) for conidial inhibition, regardless of the aforementioned high correlation for mycelial inhibition. Despite being in different chemical groups, penthiopyrad and fluopyram

were highly correlated for both mycelial and conidial inhibition (r= 0.905 and 0.775 respectively). This same pattern between penthiopyrad and fluopyram was observed by Villani et al. (2016).

The observed cross-sensitivity between specific pairs of SDHI fungicides may be indicative of potential cross-resistance we might observe when resistance develops to certain SDHI fungicides in populations of *V. inaequalis*. Hence, careful monitoring of SDHI control failures in commercial apple orchard will be needed to identify populations shifting toward resistance. While these indications of cross-sensitivity present some concern, it should be noted that cross-sensitivity is not always predictive of cross-resistance. Veloukas et al. (2013) found that fluopyram and boscalid provided different levels of control against varying mutations found in *B. cinerea*. This is supported by Mallik et al. (2013) who found that fluopyram controlled boscalid-resistant isolates of *A. solani*. Such an observation may be slightly misleading given that boscalid and fluopyram are in different SDHI chemical groups. Evaluating cross-resistance once SDHI resistance develops in field populations will bring further insights by illustrating how trends in cross-sensitivity between SDHI fungicides is related to trends in cross-resistance. While correlation does not imply causation, knowledge of correlative cross-sensitivity in *V. inaequalis* further emphasizes the importance of chemical rotation even within the class of SDHI fungicides despite the variation in chemical composition that exists.

While it is expected that there would be no cross-resistance between the SDHIs and other fungicide classes such as DMIs and QoIs due to differences in target site (FRAC 2018), Koller and Wilcox (2001) suggest that populations of *V. inaequalis* with resistance to one fungicide are more likely to subsequently develop resistance to unrelated fungicide chemistries. Such genetic plasticity could explain why the exposed isolates had reduced levels of sensitivity to SDHI

fungicides when compared to the baseline isolates. Furthermore, when the subset of baseline and exposed isolates was tested for sensitivity to several fungicide class, similar levels of insensitivity were observed for all fungicide classes, supporting the idea there may be some form of multiple fungicide resistance playing a role in isolate response. In *B. cinerea,* a mutation in the transcription factor, *Mrr1*, is associated with reduced sensitivity to multiple fungicides caused by overexpression of efflux pumps, denoted as MDR1 (multidrug resistance 1) and MDR2 (Hahn 2014; Jurick et al. 2017). To confirm that a similar phenomenon would be happening in *V. inaequalis*, further genomic investigation directed at mutations in *ViMrr1* would be required. In *V. inaequalis,* multiple fungicide resistance has not been associated with any one genetic determinant or fitness costs (Chapman et al. 2011), suggesting sensitive isolates may not be able to outcompete resistant isolates, which could pose new management challenges for modern fungicide use.-

Continual monitoring of SDHI sensitivity is vital for the preservation of this important fungicide tool for managing apple scab. With the addition of baseline sequences of the *VisdhC* and *VisdhD* gene as well as baseline sensitivities for all registered SDHI fungicides on apple, it will now be possible to pursue resistance monitoring on both a genotypic and phenotypic level. Knowledge of baseline sensitivities will benefit phenotypic monitoring by looking for shifts in sensitivity away from baseline, and reference sequences with annotations of where common mutation sites are located, will allow for a complete assessment of the *sdh* subunit forming the ubiquinone binding pocket. Overall, SDHI fungicides have good activity against both mycelial and conidial growth stages of *Venturia inaequalis*. Cross-sensitivity between SDHI fungicides occurs to certain degree depending on the chemical group. However, it is uncertain if the same correlation in cross-sensitivity will be observed once complete resistance arises in field

populations. Interestingly, isolates with previous exposure to fungicides seem to have reduced sensitivity to SDHIs, as well as other classes of fungicides suggesting that multiple fungicide resistance may play a role in future product failures. Hence, continued monitoring of SDHI resistance and multiple fungicide resistance will be imperative in populations of *V. inaequalis* in the years to come.

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CHAPTER 3

THE EFFECT OF SUCCINATE DEHYDROGENASE INHIBITOR FUNGICIDE DOSE AND MIXTURE ON DEVELOPMENT OF RESISTANCE IN *VENTURIA INAEQUALIS**

Abstract

Understanding how fungicide application practices affect selection for fungicide resistance is imperative for continued sustainable agriculture. Here, we examined the effect of field applications of the succinate dehydrogenase inhibitor (SDHI) fluxapyroxad at different doses and mixtures on the SDHI sensitivity of *Venturia inaequalis*, the apple scab pathogen. Fungicide applications were part of selection programs involving different doses (high or low) and mixtures (with a second single-site fungicide or a multisite fungicide). These programs were tested in two apple orchards over 4 years to determine potential cumulative selection effects on resistance. Each year after program applications, apple scab lesions were collected, and relative growth assays were conducted to understand shifts in fluxapyroxad sensitivity. After 4 years, there was a trend toward a reduction in sensitivity to fluxapyroxad for most selection programs in comparison to that in the non-selective-pressure control. In most years, the selection program plots treated with low-dose fluxapyroxad applications resulted in a larger number of isolates with reduced sensitivity, supporting the use of higher doses for disease management. Few significant differences ($P < 0.05$) in fungicide sensitivity were observed between isolates collected from plots where fungicide mixtures were applied compared to that in untreated plots, supporting the use of multiple modes of action in field applications. In all, appropriate doses and mixtures may contribute to increased longevity of SDHI fungicides used on perennial crops like apples.

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Introduction

Apple scab, caused by the ascomycete fungus *Venturia inaequalis* (Cooke) G. Winter, is one of the most economically significant diseases of apples produced in cool and wet climates (MacHardy 1996). Use of conventional, single-site mode-of-action fungicides is one of the most effective ways to manage apple scab (Cox 2015); however, continued reliance on fungicides from within a single mode of action can select for the development of fungicide resistance over time. Resistance to single-site fungicides has occurred multiple times in commercial populations of *Venturia inaequalis* in the United States as seen with quinone outside inhibitors (QoI) (Lesniak et al. 2011, Fredrick et al. 2014), demethylation inhibitors (DMI) (Villani et al. 2016a), and dodine (Koller et al. 1999). This history of resistance development reinforces the importance of fungicide resistance management with the newest fungicides in the class of succinate dehydrogenase inhibitors (SDHI). These fungicides are presently being marketed for apple scab management (Agnello et al. 2019), and except for the pyridinecarboxamide boscalid, SDHI fungicides are highly effective against *V. inaequalis* (Ayer et al. 2019, Villani et al. 2016b, Sundin and Outwater 2017). At the time of the study, there have been no published reports of resistance to SDHI fungicides in *V. inaequalis.* Hence, there is an opportunity to better understand the development of SDHI fungicide resistance in this pathosystem before resistance is widespread, which could lead to increased opportunities for management, decreased fungicide costs to growers, and increased practical longevity for this class of fungicides.

As previously described, in order for a resistant population of fungal species to become established within a field, an individual needs to acquire a rare, advantageous mutation conferring resistance (Hobbelen et al. 2014). This individual must then be able to survive and reproduce exclusive of any associated fitness costs. Persistence of the individual is then

dependent on selection pressure exerted by the fungicide applied, where it then might become established within the field (Hobbelen et al. 2014). An improved understanding on how the progression of resistance development can be slowed through alteration of fungicide application practices is of paramount to the development of sustainable management strategies. Alterations can be made to various aspects of fungicide applications, including dose, mixture, interval, and frequency. Here, we provide reasoning to further investigate effects of fungicide dose and mixture.

Fungicide labels may present a range in quantity that can legally be applied to the crop of interest, allowing growers to make a choice in their application dose (Brent and Hollomon 1995). It is generally believed that applying a lower dose of a fungicide will reduce disease control, resulting in a higher pathogen population in the field. Due to the larger population size left in the field, it is thought that sensitive isolates will be able to outcompete any resistant isolates, preventing establishment (Fig. 3.1B) (Fry and Milgroom 1990, van den Bosch et al. 2011). Conversely, application of a higher fungicide dose will eliminate a larger portion of the pathogen population from the field, affording better disease control and lowering the probability that an advantageous mutation emerges post-application from an existing sensitive isolate (Fig. 1C) (van den Bosch et al. 2011, Milgroom 1990). Unfortunately, leaving sensitive isolates in the fields may not be appropriate for crops destined for the fresh market, such as fruit and vegetables. Due to greater level of disease control achieved, high-dose applications may result in a more profitable crop, although it may seem more expensive initially. In summary, application of a low dose may slow establishment, while high-dose applications may slow emergence of resistance (Hobbelen et al. 2014).

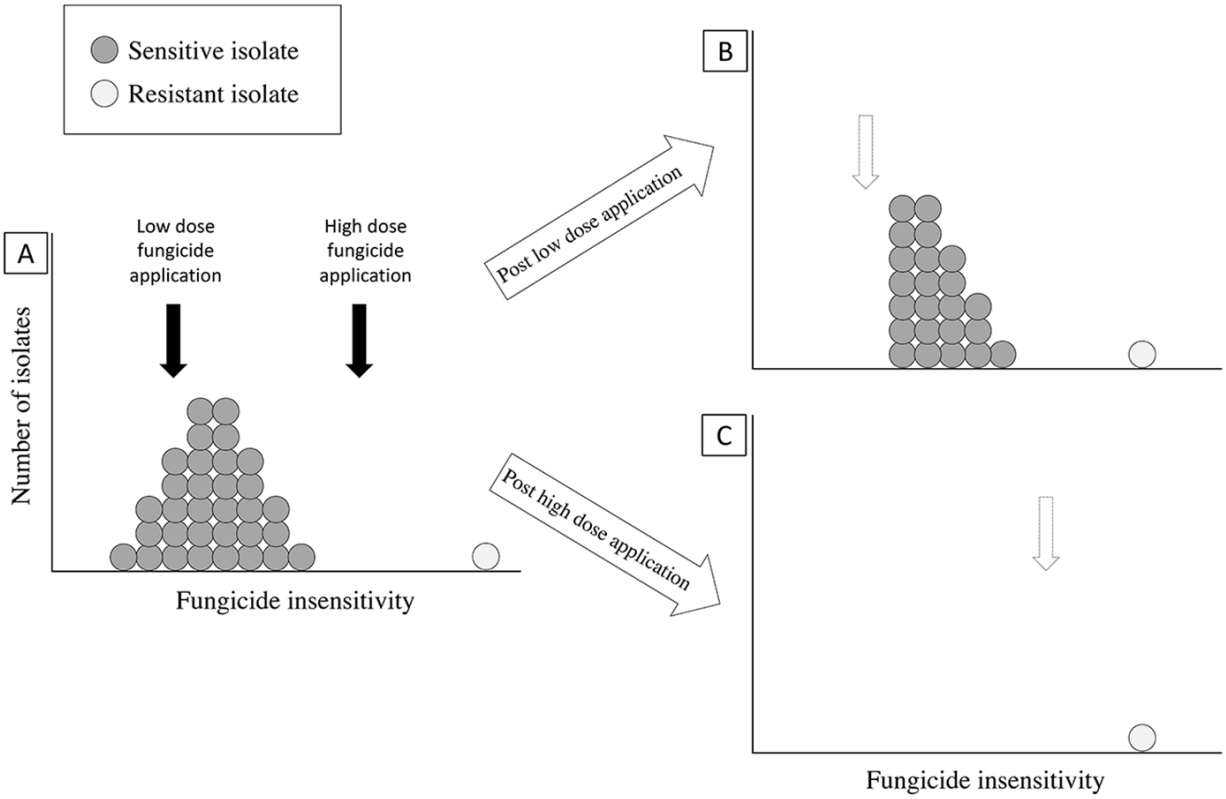

Figure 3.1. Predictions of fungicide resistance selection and establishment post application of a high or low fungicide dose. **A.** Hypothetical pathogen population pre-existing in the field **B.** Resulting population phenotypes after selection at the end of the season with a low dose application. The sensitive members are thought to slow establishment of resistance through competition. However, there is a higher probability of resistance emerging again because the remaining population size is larger. **C.** Resulting population phenotype after selection with a high dose application. A highly resistant isolate remaining at the end of the season is thought to have a low probability of success at causing subsequent disease or overwintering (low inoculum potential). There are also fewer isolates left behind to acquire additional advantageous mutations. However, if the resistant isolate successfully overwinters, a resistant population could become established.

Only limited and slightly conflicting experimental data exist in support of whether applications of high or low doses truly slow selection for fungicide resistance in the field. While some studies have shown support for use of low doses to slow establishment of resistance (Hobbelen et al. 2014; van den Bosch et al. 2011; Metcalfe et al. 2000; Mikaberidze et al. 2017), it is commonly recommended to apply the high dose (Beckerman et al. 2013), and studies have

shown that use of sublethal fungicide doses can be problematic for fungicide resistance (Amaradasa and Everhart 2016). Other studies have found that the fungicide dose applied does not influence resistance development (Burnett and Zziwa 1997; Pijls and Shaw 1997). However, to our knowledge, none of these experiments examined continuous selection on the same pathogen populations in the field over multiple years. Rather, literature in this field is dominated by extensive modeling work (Hobbelen et al. 2014; van den Bosch 2011; Mikaberidze et al. 2017) that is limited mainly to cereal cropping systems.

To complicate the decision on application dose, many commercialized fungicides exist as premixes or are often combined in tank mixes with a second fungicide. This is an accepted practice to reduce selection pressure against an individual single-site fungicide, as the probability of acquiring multiple advantageous mutations to two different fungicides is lower than that of acquiring a single mutation (Brent and Hollomon 1995). To the best of our understanding, there have not been controlled experiments evaluating whether combining a single-site fungicide with a second single-site fungicide or with a multisite fungicide is more effective in reducing resistance development. Mixtures that contain a second single-site fungicide may improve disease control but may have a higher risk of resistance. Mixed single-site fungicide application may also lead to an unnecessary expenditure of one of the few use allowances per growing season according to the label. On the other hand, application with a broad-spectrum multisite fungicide has a low risk of resistance development but often necessitates the application of higher quantities and may potentially have more off-target effects (Brent and Hollomon 1995; Beckerman et al. 2013) than single-site fungicides. Furthermore, there may be future use restrictions on broad-spectrum multisite fungicides as part of a move toward environmental sustainability.

Due to the lack of direct field experimentation on how fungicide dose and mixture affect resistance development in perennial specialty crop systems over multiple years, we wished to examine these aspects in the apple scab pathosystem. Specifically, we wished to evaluate these concepts for the SDHI fungicides in relationship to resistance selection in *V. inaequalis*. By using the perennial specialty crop system of apple and a fungal pathogen that overwinters directly under trees with short range dispersal (MacHardy 1996; Frederick et al. 2015), a year-to-year study could be conducted in which pathogen populations can be tracked over time. Furthermore, as apple is a crop that necessitates repeat applications, stronger selection pressure is placed on fungal organisms in an apple orchard within a single year, making it a more appropriate pathosystem to examine in terms of rapid fungicide resistance development.

We aimed to experimentally evaluate four different selection programs over four growing seasons, addressing the following questions: (i) how does the dose of SDHI fungicide application affect the selection of resistance, and (ii) how does the fungicide mix partner affect the selection for SDHI fungicide resistance in *V. inaequalis*? We further wished to evaluate the efficacy of these programs in the field throughout the 4 years to understand effects on practical resistance in addition to in vitro fungicide sensitivity of population members.

Materials and Methods

Fungicide resistance selection programs. This work was conducted in two research orchards at two different locations in Geneva, NY, during four consecutive growing seasons from April 2016 to July 2019. Orchard 1 consisted of 20-year-old ‘Empire’ and ‘Jonagold’ apple cultivars on M.9/M.11 interstem rootstocks. Orchard 2 consisted of 9-year-old ‘Jersey Mac’ apples on B.9 rootstocks. Both orchards were arranged in 94.2 m^2 program plots (with a

minimum of 100 m or 10,000 m^2 between plots) to prevent cross-movement of inoculum between selection program plots. Both orchards are isolated from other populations and sources of *V. inaequalis* by more than 5 km. The *V. inaequalis* populations of both orchards have been well documented in numerous studies on fungicide resistance (Frederick et al. 2014; Frederick et al. 2015; Villani et al. 2015). These two orchards have high disease pressure from year to year and therefore field trials rely on carryover overwintering inoculum in the spring and have been conventionally managed for apple scab and powdery mildew, as well as summer diseases flyspeck/sooty blotch, black rot, and white rot, throughout the years. Both orchards have a history of practical resistance to QoI and DMI fungicides (Frederick et al. 2014; Villani et al. 2015; Cox et al. 2013) but little to no application of SDHI fungicides prior to this study.

Four different fungicide resistance selection programs using the SHDI fungicide fluxapyroxad were implemented in two orchards over 4 years, abiding by product use restrictions according to the label. Selection program plots were arranged in a randomized block design with four replicate plots at each orchard. The selection programs included the following: treatment 1, no selection pressure (untreated control); treatment 2, a low-dose fluxapyroxad (single-site) program (Sercadis, 77.015 g active ingredient [A.I.]/ha; BASF, Research Triangle Park, NC); treatment 3, a high-dose fluxapyroxad program (Sercadis, 154.06 g A.I./ha); treatment 4, a program of fluxapyroxad and pyraclostrobin (Merivon, premixed, 73.21 g A.I./ha fluxapyroxad and 73.21 g A.I./ha pyraclostrobin; BASF, Research Triangle Park, NC) representing two single-site fungicides; and treatment 5, a program of fluxapyroxad (Sercadis, 77.015 g A.I./ha) and mancozeb (Koverall 75, 1,891.43 g A.I./ha; FMC, Philadelphia, PA) representing a single-site fungicide mixed with a multisite fungicide (Table 3.1). Here, we are assigning the name “low dose” to the application rate of 77.015 g A.I./ha of Sercadis and assigning the name “high dose”

to the application rate of 154.06 g A.I./ha of Sercadis. Replicated selection program plots were maintained for 4 years to increase the selection pressure over time. Fungicide applications were made at 933 liters/ha using a handgun (689.5 kPa) at approximately 7- to 10-day intervals in accordance with infection periods, approximately at the phenological stages of pink, bloom, first cover, and second cover, as defined by Meier (2001). Only four applications of fluxapyroxad per season were made as not to exceed the maximum allowed number of applications to comply with the product labels for Sercadis (BASF) and Merivon (BASF). Applications of mancozeb (Koverall 75; FMC) and sulfur (Microthiol Disperss 10 lbs; United Phosphorus, Inc., King of Prussia, PA) were made at the phenological stages of green tip and petal fall to comply with Sercadis (BASF) and Merivon (BASF) product label instructions, whereby no more than two consecutive applications of an SDHI fungicide are made within a growing season. A final application of mancozeb was included in each program at the end of the season to limit the development of apple scab.

Trt	Fungicide(s)	Commercial product(s)	Application dose	Resistant management practice tested
1	Untreated control	-	-	No selection pressure
2	Fluxapyroxad (26.55%)	Sercadis	77.015 g A.I./ha	Single-site applied at a low dose
3	Fluxapyroxad (26.55%)	Sercadis	154.6 g A.I./ha	Single-site applied at a high dose
4	Fluxapyroxad (21.26%) & pyraclostrobin (21.26%)	Merivon	73.21 g A.I./ha & 73.21 g A.I./ha	Single-site applied with a second single-site
5	Fluxapyroxad (26.55%) & mancozeb (80%)	Sercadis & Koverall	77.015 g A.I./ha & 1.891.43 g A.I./ha	Single-site applied with a multi-site

Table 3.1. Fungicides, commercial products, and doses used for each for each fungicide selection treatment program. Selection programs were applied at the apple phenological stages of pink, bloom, 1st cover, and 2nd cover.

Fluxapyroxad sensitivity determination following selection programs. Approximately 21 days after the last of four SDHI applications were made within the growing season, >15 leaves with sporulating lesions of apple scab were collected from each of the four replicate trees, for a total of >60 isolations per selection program for each orchard. Individual isolated leaf lesions representing single ascospore infections (Koller et al. 2004, MacHardy and Gadoury 1989) were excised and subjected to a relative growth assay as described previously (Ayer et al. 2019; Villani et al. 2016b). Sensitivity to fluxapyroxad was determined using a discriminatory dose of 0.25 µg/ml−1 based off previously determined baseline 50% effective concentration (EC_{50}) values of fluxapyroxad for *V. inaequalis* (Ayer et al. 2019). Potato dextrose agar (PDA)

(Difco Laboratories, Inc., Detroit, MI) was amended with the discriminatory dose of technical-grade fluxapyroxad dissolved in acetone, in addition to two antibiotics, streptomycin sulfate and chloramphenicol, at 50 $\mu g/ml^{-1}$ to avoid bacterial contamination (PDA++), along with a control plate of PDA++ without fluxapyroxad. At the end of a 1-week incubation period at room temperature, conidial germination was measured and percent relative growth (%RG) was calculated for each isolate in comparison to growth on a control PDA++ plate, as previously described (Villani et al. 2016b).

To examine differences in the population distribution of fluxapyroxad sensitivity phenotypes between selection programs, a nonparametric Kolmogorov-Smirnov (K-S) two-sample test was performed in Statistical Analysis System (SAS version 9.4; Cary, NC) as described in previous studies (Frederick et al. 2014, Frederick et al. 2015, Villani et al. 2015). Distribution of isolate phenotypes were compared for all fungicide selection programs within years as well as for the same program between years. Data were visualized with a distribution graph presented as a modified histogram with bins of 0, 50, 100, 150, and 200 %RG using SigmaPlot 11.0 (SyStat Software, Inc., San Jose, CA).

Impact of selection programs on the incidence of apple scab. To determine the impact of fungicide selection programs on the development of apple scab symptoms and practical fungicide resistance, the incidence of apple scab symptoms was assessed on terminal leaves in both orchards approximately 21 days after the last application. The incidence of apple scab symptoms on terminal leaves was determined by counting the number of terminal leaves with apple scab lesions out of eight fully expanded leaves from the distal end of the shoot. For each of four replicate plots, 10 shoots were assessed. The effect of selection programs on the incidence

of apple scab was determined by generalized linear mixed models using the PROC GLIMMIX procedure of SAS. All percentage data were subjected to arcsine square root transformation prior to analysis.

In each of the 4 years of experimentation, weather data and infection period predictions were collected to better understand disease pressure and apple scab development within each orchard. Temperature, relative humidity, and hourly leaf wetness data, as well as predicted apple scab infection periods, were collected from the Network for Environment and Weather Applications (NEWA, http://newa.cornell.edu/; Ithaca, NY) using the weather station (RainWise, Inc., Trenton, ME) present onsite at the research orchards in Geneva, NY. Total leaf wetness hours and total number of predicted infection periods were determined from April to June, the typical period of primary apple scab infection in Geneva, for all 4 years. To help explain relationships between disease incidence and %RG, a Pearson correlation analysis (SAS v9.4; PROC CORR) was completed for selection programs receiving fluxapyroxad for all 4 years. Values from the non-selective-pressure control (no fluxapyroxad applications) were excluded for this analysis as incidence would be unusually high in the untreated control.

Sequencing of the *VisdhB* gene to determine qualitative resistance. In order to determine if any isolates possessed qualitative resistance to SDHI fungicides, the *VisdhB* gene was sequenced as previously described (Villani et al. 2016b). Each year, a subset of isolates with >50 %RG on 0.25 μg/ml^{-1} fluxapyroxad-amended medium were sequenced to determine if a reduced sensitivity phenotype was caused by a SNP in the target gene. In short, vegetative mycelial growth was grown on PDA++ at room temperature, DNA was extracted using the E.Z.N.A. plant DNA kit (Omega Bio-Tek, Norcross, GA), a PCR was completed using

previously described *VisdhB* primers, sequences were purified, and samples were submitted to Cornell University's sequencing facility in Ithaca, NY, for Sanger sequencing on their Applied BioSystems automated 3730xl DNA Analyzer. Returned sequences were then analyzed in CLC Main Workbench 20 (version 20.1.1) (Qiagen Bioinformatics, Redwood City, CA) (Villani et al. 2016b).

Results

Isolates were successfully cultured from leaf lesions collected from all fungicide selection programs (Table 3.1) in all years. Differences in isolate number between programs and years reflect success of conidial isolation and overall disease incidence, while differences in isolate number between orchards reflect the age and size of evaluated trees (Table 3.2). Out of all the isolates with a percent relative growth (%RG) of >50 that were sequenced for detection of single-nucleotide polymorphisms (SNPs) in the *VisdhB* gene (n = 20), none had a coding frame mutation in the *VisdhB* gene, and all sequences were identical to that in GenBank (accession number KR139837).

There were few differences in sensitivity for isolates collected from the non-selective-pressure program (untreated control; treatment 1) from year to year. In 2017, distributions of fluxapyroxad RG values of isolates from the non-selective-pressure (untreated control) plots were statistically different from 2016 control sensitivities in both orchards 1 and 2 (Kolmogorov-Smirnov analysis test statistic [Ksa] = 2.012 and 2.132, respectively; $P < 0.05$). However, we noted that hours of leaf wetness (464 h) and number of infection events (15 infection events) were the highest in 2017 compared to data in 2016, 2018, and 2019 (Table 3.3). There were no significant differences in the distributions of fluxapyroxad RG values of isolates from the non-

selective-pressure (untreated control; treatment 1) plots between 2018 and 2016 for both orchard 1 (Ksa = 1.078; P = 0.196) and orchard 2 (Ksa = 1.173; P = 0.1278). Likewise, at the end of the experiment (which began in 2016 and ended in 2019), there were no significant differences in the distributions of fluxapyroxad RG values of isolates from the non-selective-pressure (untreated control; treatment 1) plots for both orchard 1 (Ksa = 1.194; P = 0.116) and orchard 2 (Ksa = 1.126; P = 0.158).

Table 3.2. Number of *V. inaequalis* isolates successfully retrieved and tested across all years, and treatments between **A.** Orchard 1 and **B.** Orchard 2.

A.	**Control**	**Low dose fluxapyroxad**	**High dose fluxapyroxad**	**Pyraclostrobin & fluxapyroxad**	**Mancozeb & fluxapyroxad**
2016	33	50	30	36	34
2017	76	78	55	148	62
2018	78	65	79	78	80
2019	83	102	105	162	107
B.	**Control**	**Low dose fluxapyroxad**	**High dose fluxapyroxad**	**Pyraclostrobin & fluxapyroxad**	**Mancozeb & fluxapyroxad**
2016	35	35	35	34	35
2017	32	40	39	39	30
2018	40	35	38	35	39
2019	29	48	31	14	41

Table 3.3. Table summarizing cumulative hours of leaf wetness and number of apple scab infection periods from April-June in 2016, 2017, 2018, and 2019 according to NEWA model predictions (NEWA.cornell.edu).

	2016	**2017**	**2018**	**2019**
Hours of leaf wetness	244	464	342	449
Number of infection periods	7	15	11	14

Table 3.4 Fluxapyroxad sensitivity distribution comparisons of isolates obtained from selection treatments in 2016-2019 for Orchard 1 and Orchard 2. Values displayed are the Ksa test statistic from the Kolmogorov–Smirnov test. Asterisk indicates $P < 0.05$.

		Orchard 1				Orchard 2			
		Control	**Low dose fluxapyroxad**	**High dose fluxapyroxad**	**Fluxapyroxad and pyraclostrobin**	**Control**	**Low dose fluxapyroxad**	**High dose fluxapyroxad**	**Fluxapyroxad and pyraclostrobin**
2016	Low dose fluxapyroxad	1.373*				1.554*			
	High dose fluxapyroxad	1.081	0.549			1.195	1.076		
	Fluxapyroxad and pyraclostrobin	1.310	0.940	0.652		0.988	1.389*	2.038*	
	Mancozeb and fluxapyroxad	0.894	0.704	0.548	0.738	0.837	1.315	0.837	1.113
2017	Low dose fluxapyroxad	1.997*				0.553			
	High dose fluxapyroxad	0.888	1.538*			1.925*	1.963*		
	Fluxapyroxad and pyraclostrobin	1.207	1.740*	0.496		1.102	1.27	2.038*	
	Mancozeb and fluxapyroxad	1.014	1.677*	0.432	0.777	0.935	1.277	1.278	0.834
2018	Low dose fluxapyroxad	1.069				3.641*			
	High dose fluxapyroxad	1.542*	1.265			2.004*	2.141*		
	Fluxapyroxad and pyraclostrobin	1.922*	1.053	1.224		2.901*	1.434*	1.101	
	Mancozeb and fluxapyroxad	1.877*	1.353	1.163	0.582	2.974*	1.784*	1.069	0.840
2019	Low dose fluxapyroxad	1.988*				2.218*			
	High dose fluxapyroxad	1.514*	1.249			1.473*	1.294		
	Fluxapyroxad and pyraclostrobin	0.949	2.014*	1.534*		0.689	1.528*	1.216	
	Mancozeb and fluxapyroxad	3.115*	1.726*	2.394*	2.986*	1.33	1.24	0.381	1.116

Fluxapyroxad sensitivity following different dose selection programs. In 2016, after one season of fungicide applications, there were no significant differences in %RG between isolates collected from the high-dose (treatment 3) and low-dose (treatment 2) fluxapyroxad selection program plots across both orchard blocks (Table 3.4). For both orchards 1 and 2, only the distributions of %RG of isolates from plots treated with the low-dose fluxapyroxad selection programs significantly differed from those of the non-selective-pressure control plots (Ksa = 1.373 and 1.554, respectively; $P < 0.05$) (Table 3.4 and Fig. 3.2A and B) with isolates from the low-dose selection program having a shift toward a reduction in sensitivity.

In 2017, after two seasons of selection pressure from the SDHI fungicide programs, we did begin to observe greater differences between isolates collected from the different selection programs. In orchard 1, the distribution of RG values of isolates recovered from the low-dose fluxapyroxad selection program plots had a greater shift toward resistance (Ksa = 1.538; $P < 0.05$) (Table 3.4). In orchard 2, the distribution of RG values of isolates collected from the high- and low-dose fluxapyroxad selection program plots were significantly different (Ksa = 1.963; $P < 0.05$) (Table 3.4), with isolates recovered from the high-dose selection program plots displaying a greater shift toward resistance (Fig. 3.2 C and D).

After the third season of SDHI fungicide applications, in 2018, we continued to observe differences between isolates collected from the different dose selection programs. In orchard 2, the distribution of fluxapyroxad RG values for isolates collected from the high- and low-dose fluxapyroxad selection program plots were significantly different (Ksa = 2.141; $P < 0.05$) (Table 3.4), with isolates collected from the low-dose selection program plots having a greater shift toward resistance (Fig. 3.2F). In orchard 1, however, there was no significant difference in the distribution of fluxapyroxad RG values of isolates collected from the high- and low-dose

selection programs (Ksa = 1.265; $P > 0.05$) (Fig. 3.2E, Table 3.4).

In 2019, after the fourth and final season of the selection programs, the observed trends were similar to those in 2018, but were less striking. In orchard 1, there were still no statistically significant differences in fungicide sensitivity between isolates collected from selection program plots treated with low and high doses of fluxapyroxad, although both programs were statistically significantly different from the non-selective-pressure program (untreated control) (Ksa = 1.988 and 1.514, respectively; $P < 0.05$) (Table 3.4 and Fig. 3.2G), with isolates collected from the two fluxapyroxad plots having a greater shift toward resistance. In orchard 2, the largest shift in sensitivity was seen in the isolates collected from plots treated with the low-dose applications of fluxapyroxad compared to those from the untreated control (Ksa = 2.218; $P < 0.05$) (Table 3.4 and Fig. 3.2H).

Fluxapyroxad sensitivity following different mixture section programs. There were no statistically significant differences in fungicide sensitivity between isolates collected from the two mixture selection program plots, fluxapyroxad mixed with pyraclostrobin (treatment 4) and fluxapyroxad and mancozeb (treatment 5), in all 4 years and in both orchards, with the exception of orchard 1 in 2019 (Ksa = 2.986; $P < 0.05$) (Fig. 3.3 and Table 3.4), where isolates collected from plots treated with fluxapyroxad and mancozeb displayed a shift toward resistance.

Fluxapyroxad sensitivity following fluxapyroxad applied alone or in mixture. In 2016 for orchard 2, there was a significant difference between the distribution of sensitivities of isolates collected from selection program plots treated with fluxapyroxad alone compared to those treated with fluxapyroxad mixed with pyraclostrobin (Ksa = 1.389; $P < 0.05$) (Table 3.4),

with isolates collected from the low-dose fluxapyroxad selection program having a shift toward resistance. However, no other statistically significant differences were observed. In 2017 in orchard 1, isolates collected from plots treated with low-dose applications of fluxapyroxad alone had a statistically significant shift toward resistance compared to both selection programs consisting of mixtures of fluxapyroxad with pyraclostrobin or mancozeb (Ksa = 1.740 and 1.677, respectively; $P < 0.05$) (Table 3.4). In 2018 for orchard 2, there was a significant difference between the distribution of sensitivities of isolates collected from selection program plots treated with fluxapyroxad alone (at the low dose) and from both fungicide mixture selection programs (Ksa = 1.4343 and 1.784, respectively; $P < 0.05$), where again the isolates collected from plots treated with fluxapyroxad alone were more greatly shifted toward resistance (Table 4). In 2019 in orchards 1 and 2, there was a significant difference between the distribution of sensitivities of isolates collected from selection program plots treated with fluxapyroxad alone (treatment 2) and from those treated with fluxapyroxad mixed with pyraclostrobin (Ksa = 2.014 and 1.528, respectively; $P < 0.05$) (Table 3.4), where isolates collected from plots treated with fluxapyroxad alone displayed a shift toward a reduction in sensitivity.

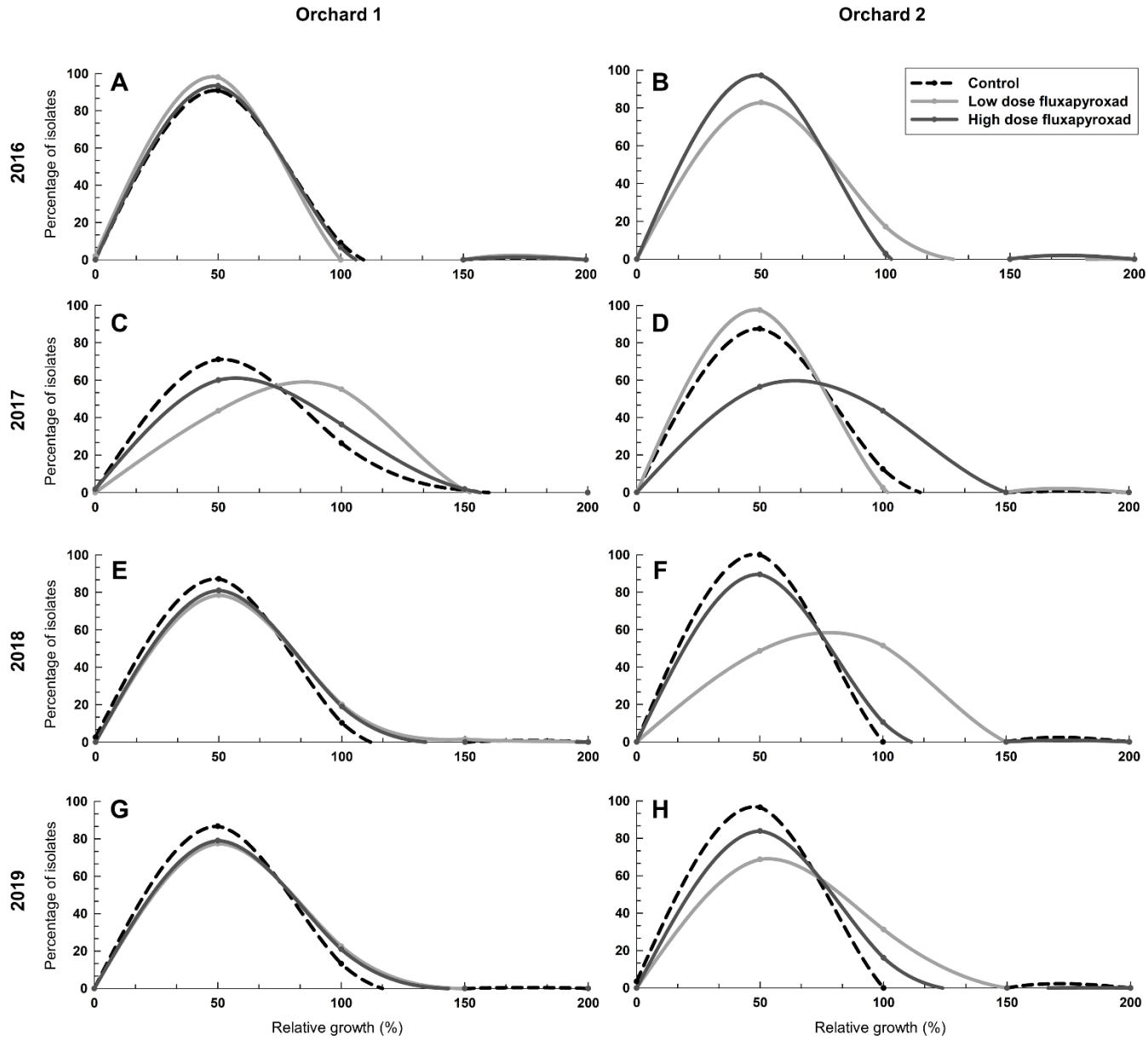

Figure 3.2. Distribution of *V. inaequalis* fluxapyroxad sensitivity from 2016-2019 (down columns) in Orchard 1 and 2 (across rows) between isolates collected from plots treated with a low dose of fluxapyroxad (77.015 g A.I./Ha.) and high dose fluxapyroxad (154.6 g A.I./Ha). Fungicide sensitivity is expressed as the relative growth of single conidial colonies on media amended with 0.25ug/ml dose of fluxapyroxad. Values are the mean and standard error of five isolated single conidial colonies for >60 clonal conidial isolates.

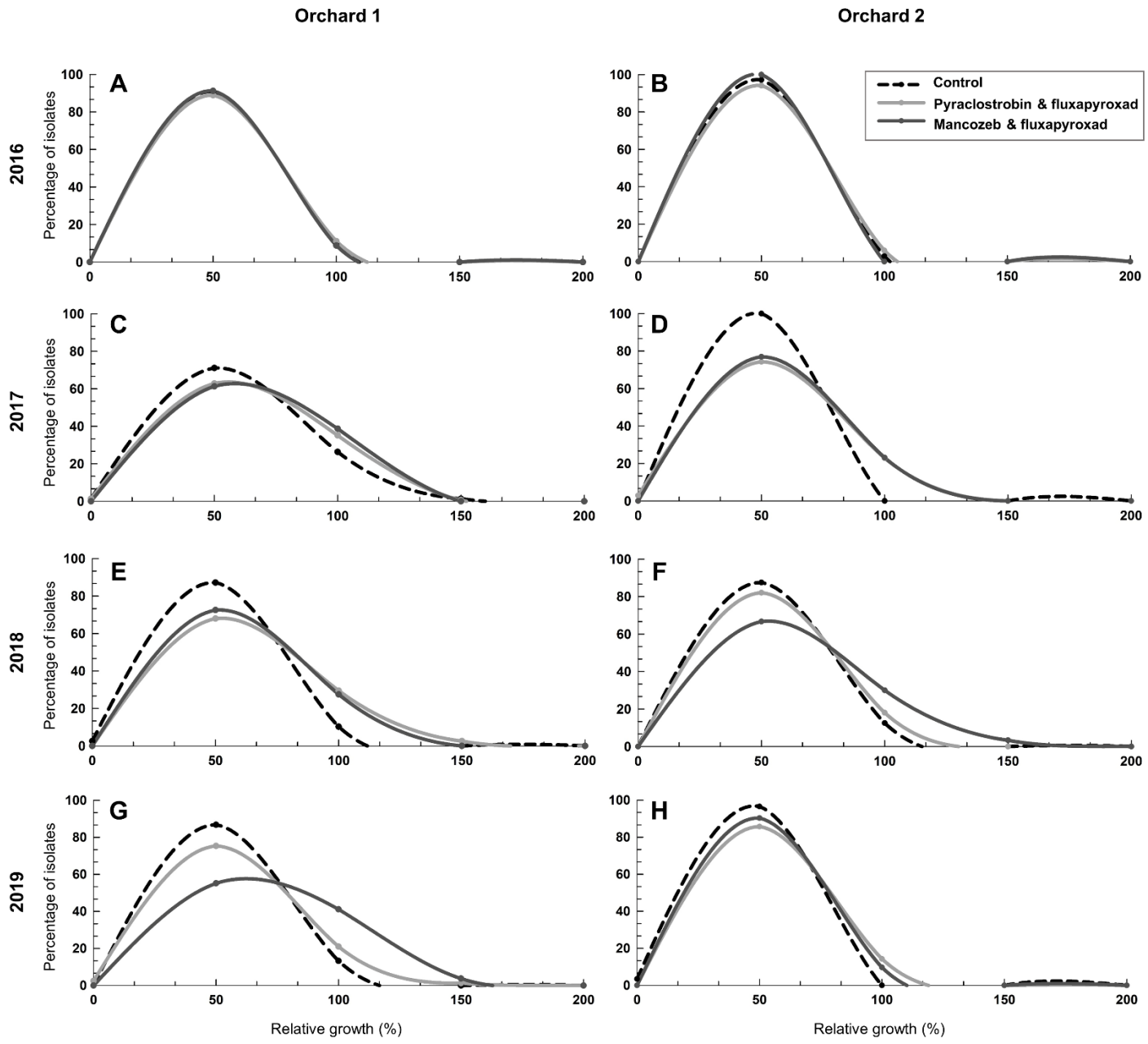

Figure 3.3. Distribution of *V. inaequalis* fluxapyroxad sensitivity from 2016-2019 (down columns) in Orchard 1 and 2 (across rows) between isolates collected from plots treated with fluxapyroxad mixed with pyraclostrobin or mancozeb. Fungicide sensitivity is expressed as the relative growth of single conidial colonies on media amended with 0.25ug/ml dose of fluxapyroxad. Values are the mean and standard error of five isolated single conidial colonies for >60 clonal conidial isolates.

Impact of selection programs on the development of apple scab. Both orchards had high levels of apple scab pressure, with incidence of apple scab symptoms on terminal leaves ranging between approximately 30 to 60% in the non-selective-pressure program (untreated control) each year (Fig. 3.4). There was no observable loss of efficacy for fluxapyroxad products after 4 years, as the percentage of apple scab control was comparable between selection programs in 2016 and 2019 (Fig. 3.4). For example, in 2016, percent control ranged from approximately 55 to 75% in orchard 1 and 50 to 90% in orchard 2 across all programs, with the low dose of fluxapyroxad affording the lowest degree of control (55% and 47% in orchards 1 and 2, respectively), the high dose affording the most control in orchard 1 (76%), and both of the mixture selection programs equally affording the most control in orchard 2 (88%). Similarly, by the end of the experiment in 2019, percent control ranged from approximately 45 to 70% in orchard 1 and 60 to 90% in orchard 2 (Fig. 3.4) across all programs, with the low dose of fluxapyroxad affording 50% and 90% control in orchards 1 and 2, respectively, and the high-dose fluxapyroxad applications affording the least control (45% and 60% in orchards 1 and 2, respectively), the program of fluxapyroxad with mancozeb affording the most control in orchard 1 (72%), and the high dose of fluxapyroxad affording the most control in orchard 2 (88%). Interestingly, a reduced level of control was observed for programs in 2017, when the weather was more conducive for disease development. For example, percent control was as low as 38% in orchard 1 for the selection program of isolates treated with the high dose of fluxapyroxad and 28% in orchard 2 for the selection program of fluxapyroxad with pyraclostrobin. The highest percent control was observed in the selection program of fluxapyroxad and pyraclostrobin in orchard 1 (50%), and in orchard 2 it was observed in the selection program of the high dose of fluxapyroxad (68%) (Fig. 3.4). The exceptionally large numbers of leaf wetness hours and

infection periods in 2017 and corresponding infections warranted an examination of the relationship between relative growth and disease incidence. Unfortunately, we found no relationship between relative growth and disease incidence in 2017. We did find a slight, yet positive, relationship between disease incidence in the field and mean percent relative growth across all years, but it was nonsignificant ($R^2 = 0.11$, $P = 0.063$).

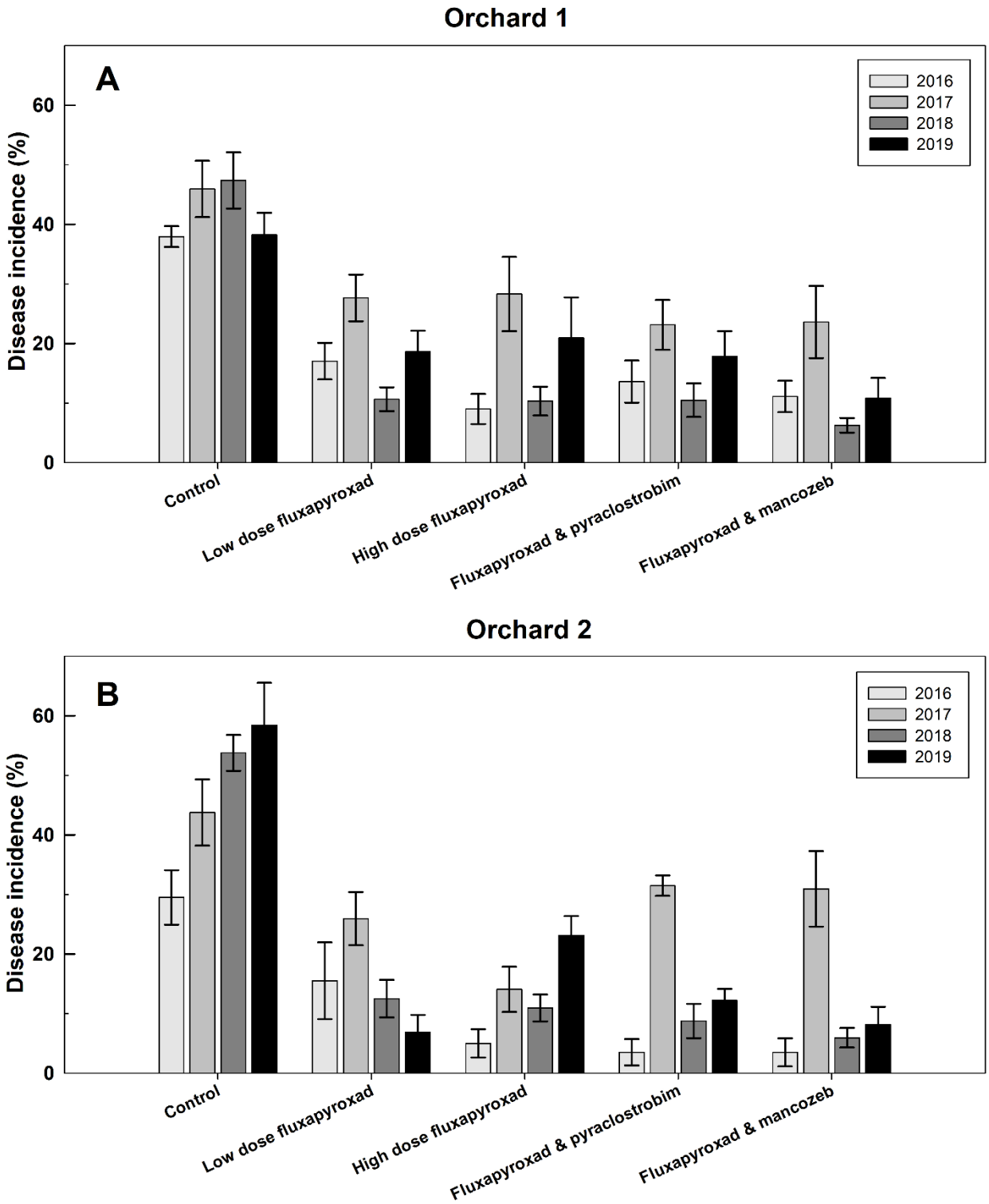

Figure 3.4. Development of apple scab symptoms on terminal leaves following fluxapyroxad selection programs in 2016 through 2019 in **A.** Orchard 1 and **B.** Orchard 2. The incidence of apple scab symptoms on terminal leaves is expressed as the mean and standard error of eight distal terminal leaves from 10 shoots from each of four replicate plots.

Discussion

There is little information on the impacts of accepted fungicide resistance management practices on field populations of pathogens of perennial specialty crops. It is well supported that rotation of fungicide mode of actions is an important resistance management strategy (Brent and Hollomon 1995). However, there is a clear problematic pattern of resistance development to important single-site fungicides across pathosystems of perennial fruit crops (Lesniak et al. 2011; Frederick et al. 2014; Villani et al. 2015, Fernandez-Ortuno et al. 2017, Mallik et al. 2013), demanding increased knowledge of practices that slow resistance development while providing acceptable disease control. Understanding the influence of both fungicide application dose and tank mixture on fungicide resistance development could help provide vital information to growers that could improve the longevity of single-site fungicides in their field.

This work aimed to understand the development of fungicide resistance as a result of fungicide selection programs in the form of different fungicide doses and tank mixtures over the course of 4 years. These results present evidence that with increased use of the single-site SDHI fungicide fluxapyroxad, there is a shift toward resistance. Population distributions from both orchards and plots receiving selection programs showed a trend of a shift toward resistance after 4 years. Perhaps the most striking shift in data is the differences in relative growth seen between 2016 and 2017. Indeed, this contrast can partially be explained by differences in weather conditions, as extended periods of leaf wetness are conducive to the development of apple scab (MacHardy 1996). An increase in the development of apple scab resulting from a favorable environment likely increased the overall population size in that year. A large population size in 2017 may have led to a greater probability of conidium having an advantageous mutation leading to a reduction in sensitivity. With the opposite, a suppression of population size, there is a

reduced chance of acquiring advantageous mutations (Milgroom 1990). Therefore, while seemingly intuitive, our results support the idea that managing population size may be one of the best strategies for reducing resistance development.

In 2017, lower %RG was observed overall in isolates from plots treated with a mixture of fluxapyroxad with a second fungicide. This reiterates the importance of incorporating mixtures as part of resistance management practices. However, a reduced level of disease control in 2017 is most likely due to high inoculum rather than fungicide failure. As noted by the change in population distributions between 2017 and 2018, resistance phenotypes can be lost if there is a bottleneck in overwintering inoculum, limiting phenotypic diversity (Nei et al. 1975). Indeed, by 2019, control of apple scab by fluxapyroxad programs was comparable to that observed in 2016. At the same time, some loss of fungicide sensitivity was maintained in 2018 and 2019, potentially due to increased selection with continued SDHI applications. Furthermore, the large range in percent disease control may be due to various levels of fungicide coverage. With adequate season-long management that limits overwintering population size through incorporation of cultural practices and comprehensive chemical control, it is possible that a grower could eradicate these high-relative-growth isolates from their orchard. Alternatively, isolates with resistance or reduced sensitivity may be lost if a fitness cost is associated with resistance, as this has been found in some fungi with SDHI resistance (Veloukas et al. 2014), but not all (Fan et al. 2015). However, in this example, no isolates were recovered with qualitative or complete resistance resulting from a mutation in the *VisdhB* gene.

Although the mechanisms for the observed reduction in sensitivity remains to be elucidated, other studies have noted similar reductions in the form of a quantitative or multigenic resistance response in fruit pathosystems, including that of apple scab. A two-phase resistance

response to QoI fungicides has been previously observed in *V. inaequalis*, whereby a quantitative resistance response was first observed before qualitative (monogenic) resistance conferred by target site mutations (Koller et al. 2004). Other studies in *V. inaequalis* have found that isolates with resistance to a fungicide with one mode of action were more likely to be resistant to a fungicide with a completely different mode of action, potentially resulting from multidrug efflux pumps (Koller and Wilcox 2001). Similarly, in a study examining *Botrytis cinerea* of blackberry, Cosseboom et al. (2020) found isolates with multi-fungicide resistance to be competitive and persist in the planting even in the absence of selective pressure (Cosseboom et al. 2020). An increased understanding of the mechanism(s) responsible for reduced sensitivity in the present study would provide a better understanding of fungicide resistance selection. However, despite being documented at the turn of the century, quantitative and multi-fungicide mechanisms of resistance have remained elusive to date in fruit pathosystems (Koller et al. 2004; Koller and Wilcox 2001; Cosseboom et al. 2020).

It is not surprising that fungicide selection programs did not display the exact same trends in both orchards each year, as the development of an advantageous mutation that confers resistance in a population member is both rare and random (Koller et al. 2004; Koller and Wilcox 2001; Cosseboom et al. 2020; Lucas et al. 2015). Our results support the idea that there is a higher probability of isolates with resistance or reduced sensitivity emerging from populations exposed to low doses of fungicides. In this study, isolates with reduced sensitivity were most frequently recovered from plots exposed to low doses of fluxapyroxad. However, this does not indicate that this will always be the case, nor does it suggest that isolates with reduced sensitivity cannot be recovered from plots treated with a high dose of fluxapyroxad or from plots treated with a mixture of fungicides. Careful management decisions are most important during years

where there is high disease pressure resulting from weather conditions conducive to infection. In such years, low-dose applications need to be made with caution to ensure adequate control of populations to reduce the number of population members subject to subsequent fungicide selective pressures. Low doses may select for reductions in sensitivity over time more rapidly than high-dose applications because carryover inoculum may be higher. Furthermore, tank mixtures should be incorporated to slow the development of resistance to SDHI fungicides. This study suggests that the exact mixing partner (a second single-site fungicide or a multisite fungicide) may not matter if there are multiple active ingredients and there is no resistance to the fungicide used as the mix partner.

Our work represents a field study that experimentally addresses the question how fungicide sensitivity is affected by application dose or tank mixture after multiple applications over several seasons in a perennial cropping system. However, similar studies have been completed to understand the role of fungicide dose on fungicide sensitivity. Amaradasa and Everhart (2016) have shown that exposure to sublethal fungicide doses increase mutation accumulation in fungi. Such a phenomenon may have an impact on fungicide resistance development when low doses are applied. This idea is further supported by Steva, who found that low-dose applications, indeed, select for isolates with reduced sensitivity more quickly than high-dose applications (Steva 1994). In contrast, modeling work for fungicide resistance in wheat has provided support that applications of high doses of fungicide exert higher selection pressure for resistance (van den Bosch et al. 2011). Conversely, other studies in *Monilinia fructicola* found that repeated fungicide applications at half of the labeled rate had no effect on genetic changes over two years (Dolwing et al. 2016). These discrepancies may be explained by differences in crop and chemical management requirements for the pathosystems, as well as by

differences between fungicide classes examined.

These questions extend beyond fungicide resistance, as similar studies have been investigated for herbicide and insecticide resistance development. In those fields, the consensus tends to support the idea that lower-dose applications allow for partial polygenic mutations conferring resistance to be acquired more rapidly (Beckerman et al. 2015, Neve and Powles 2005, Tabashnik et al. 2013). While the aforementioned work is in diploid systems, this is still in line with what is commonly accepted in the medical field in regard to antibiotic resistance development, where the goal of antibiotic administration is to eliminate the greatest quantity of bacteria possible (Cunha 1988, Roberts et al. 2008). Therefore, use of higher doses is commonly recommended in the medical field as well. However, controversy still surrounds this topic. Day and Read suggest that the use of the lowest effective dose or the highest tolerable dose are both effective strategies for minimizing resistance development in antimicrobial (Day and Read 2016). Other studies agree that it may not be as simple as a concept of "high versus low dose," and further investigation is necessary to reexamine commonly accepted practices (Kouyos et al. 2014). Indeed, availability of empirical evidence is limited in the medical field and therefore experimentation within agriculture may allow for cross-disciplinary comparisons.

In conclusion, this study aimed to help identify safe, effective, and sustainable fungicide use practices for apple growers in temperate climates worldwide. Further investigation is needed to better define the interplay between dose and population size on resistance development. To better understand the exact connection with fungicide resistance, controlled lab experimentation should also be conducted to eliminate external weather variables that impact field experiments. Overall, our results suggest that repeated application of a single-site SDHI fungicide at a low dose over several years may increase the chances of the emergence of resistance compared to

those when a high dose is applied. If a low dose is applied, the probability of resistance selection will most likely be lowered if mixed with either a second single-site fungicide or a multisite fungicide. Because no differences were present between applying fluxapyroxad with a second single-site fungicide or a multisite fungicide, orchard history should be considered (e.g., history of fungicide resistance, frequency of protectant applications) to determine the most appropriate fungicide mixing practices for the location. In addition, limiting the number of uses of SDHI fungicides by rotation with other fungicide classes is imperative, as increased use over 4 years in this study was shown to cause shifts toward resistance. Fungicide sensitivity testing is more important than ever to ensure appropriate use of fungicides. With intentional fungicide use, careful selection of doses and mix partners should be made to conserve fungicide sensitivity and contribute to more sustainable orchard management practices.

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CHAPTER 4

OPTIMIZING THE INTEGRATION OF A BIOPESTICIDE (*BACILLUS SUBTILIS* QST 713) WITH A SINGLE-SITE FUNGICIDE (BENZOVINDIFLUPYR) TO REDUCE RELIANCE ON SYNTHETIC MULTI-SITE FUNGICIDES (CAPTAN AND MANCOZEB) FOR MANAGEMENT OF APPLE SCAB*

Abstract

Apple scab is one of the most economically important diseases of apple in temperate production regions. In the absence of durable host resistance in commercially preferred cultivars, considerable applications of fungicides are needed to manage this disease. With the sequential development of resistance to nearly all classes of single-site fungicides in the apple scab pathogen *Venturia inaequalis*, synthetic multi-site fungicides, such as mancozeb and captan, often comprise the core of chemical management programs for apple scab. While these fungicides have demonstrable benefits for both disease and fungicide resistance management, the sustainability movement within agriculture aims to reduce reliance on such fungicides due to their broader environmental impacts. In this study, we establish a framework to enhance the feasibility of chemical management programs that do not rely on use of synthetic multi-site protectant fungicides to manage apple scab. Specifically, we wish to evaluate chemical programs that integrate the biopesticide, *Bacillus subtilis* QST 713 (Serenade Opti), in rotation with benzovindiflupyr (Aprovia), a single-site fungicide belonging to the class of succinate dehydrogenase inhibitors (SDHI), to circumvent the need for applications of synthetic multi-site fungicides. During implementation of these programs, disease incidence data were taken at biweekly intervals. Irrespective of the seasonal challenges presented in the two years of this study, when *Bacillus subtilis* QST 713 was used in place of captan and mancozeb mixtures, we

did not observe any significant differences ($P > 0.05$) in development of apple scab symptoms between any of the management programs for the vertical axis or super spindle orchards in either year. This potential for substituting synthetic multi-site fungicides with biopesticides is best realized when the programs are used with a decision support system in a super-spindle planting system, where trees have reduced canopy densities. This two-year study shows the potential to achieve adequate disease control using the integration of SDHI fungicides and biological controls without the use of synthetic multi-site fungicides.

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*Ayer, K.M., Strickland, D.A, Choi, M., and Cox, K. D. 2021. Optimizing the integration of a biopesticide (*Bacillus subtilis* QST 713) with a single-site fungicide (benzovindiflupyr) to reduce reliance on synthetic multi-site fungicides (captan and mancozeb) for management of apple scab. Plant Dis. *Accepted*.

Introduction

Apple scab is one of the most economically important diseases of apple in temperate production regions worldwide. The causal agent of apple scab, *Venturia inaequalis* (Cooke) G. Wint., is an ascomycete fungus that causes olive-colored scabby lesions on both leaves and fruit of apple, resulting in decreased yield of marketable fruit (Jones and Aldwinkle 1900; MacHardy 1996). *V. inaequalis* favors cool and wet environments, making it problematic in more temperate climates where season long management, including the use of chemical control, is necessary (MacHardy 1996). In the absence of durable host resistance in commercially preferred cultivars, more than 10 fungicide applications are needed to manage this disease within a single growing season (Agnello et al. 2019). Moreover, due to the history of sequential resistance shortly following introduction of single-site fungicides (Cox 2015), apple scab management programs in the eastern United States have begun to heavily rely on multi-site protectants from bud break (green tip) to early summer cover applications (Agnello et al. 2019; Beckerman et al. 2015a; Beckerman et al. 2015b; Jurick II and Cox 2017; Villani et al. 2019).

Unlike single-site fungicides, multi-site fungicides affect numerous different cellular processes in fungi and potentially other organisms, allowing them to be effective against many species of fungi, but at the expense of greater environmental impact. At the same time, multi-site fungicides pose a low risk for resistance development and have become important tools for resistance management when implemented in fungicide rotations and mixtures (Brent and Hollomon 1995; Guillino et al. 2010). Mancozeb and captan, (FRAC; Fungicide Resistance Action Committee group M03 and M04, respectively), are some of the most widely used multi-site fungicides due to their low cost, multi-site mode of action, and efficacy against pathogens affecting a broad range of crops (Runkle et al. 2017). However, despite utility in disease

management, environmental concerns exist surrounding the lack of target specificity of the mode of action for both mancozeb and captan. Certainly, mancozeb has been shown to have negative impacts on development and reproductive ability in mammals (Runkle et al. 2017), implicated as an environmental pollutant (Walia et al. 2014), and both captan and mancozeb are listed as probable human carcinogens (EPA 1999; EPA 2005). For these reasons, restrictions and limitations are in discussion for these chemistries in different countries, making it imperative to understand how disease management could continue without captan and mancozeb (Beckerman et al. 2015b).

Since the 1950s, single-site fungicides have been an integral part of apple scab management programs, but unfortunately, nearly all of these chemistries have fallen out of use due to sequential widespread resistance development shortly after introduction, with the benzimidazoles (Quello et al. 2010), dodine (Koller et al. 1999) demethylation inhibitors (Villani et al. 2016b), and quinone outside inhibitors (Frederick et al. 2014; Lesniak et al. 2011) being prime examples. Fortunately, the succinate dehydrogenase inhibitors (SDHI; FRAC group 7; FRAC 2020), have remained an integral part of chemical management programs due to their high level of efficacy and lack of widespread resistance in populations of *V. inaequalis* (Ayer et al. 2019a; Rosenberger et al. 2013; Sundin and Outwater 2016; Villani et al. 2016a;). In this regard, we believe SDHI fungicides to be an optimal choice in a program designed to reduce inputs of multi-site protectant fungicides. However, SDHIs are still classified as a medium-high risk of resistance development by the Fungicide Resistance Action Committee (FRAC), and it is essential to use these fungicides in a management program that incorporates different modes of action (FRAC 2020). Presently, the fungicides mancozeb and captan are used to fill this role of resistance management in commercial apple production (Agnello et al. 2019; Brent and

Hollomon 1995).

Biopesticides are gaining popularity as an environmentally sustainable option for disease management. When compared to synthetic fungicides or evaluated alone, biopesticides have often been shown to have reduced efficacy against apple scab (Cromwell et al. 2008; Pscheidt et al. 2001; Pscheidt and Bassinette 2014; Rosenberger et al. 2000; Strickland and Cox 2020; Yoder et al. 2007; Yoder et al 2014b). In recent years, formulations of biopesticides have become more refined, and numerous fruit tree trials have been conducted to discern their best use to maximize product efficacy in apple (Cox et al. 2017a; Cox et al 2017b). Biopesticides may have different modes of action, including through induced host resistance, direct competition for resources, parasitism, or the production of antimicrobial compounds (Kohl et al. 2019; Caulier et al. 2019). One of the most thoroughly studied organisms used in the development of biopesticides include the gram-positive bacteria *Bacillus subtilis* and *Bacillus amyloliquefaciens* (FRAC Group BM02) (Priest et al. 1987). The secreted antimicrobial metabolites of these bacterial species serve as the active ingredients in many biopesticide formulated products, allowing these products to be applied with antibiotics and copper without concern of reduced efficacy (Shafi et al. 2017; Cawoy et al. 2011). Indeed, biopesticides derived from these two species are some of the more promising active ingredients for managing several diseases of perennial fruit crops (Cox et al. 2017b, Lalancette et al. 2017; Cox and Villani 2015) and therefore were of interest for this study.

The concerns over multi-site protectant fungicides and the opportunities with single-site fungicides and biopesticides highlight the necessity for more sustainable chemical management programs for apple scab that would be more commercially appealing to marketers, regulators, and consumers. Therefore, we thought to explore the feasibility of management programs that do not rely on the use of synthetic multi-site protectants to manage both apple scab as well as

selection for fungicide resistance. Specifically, we examined the feasibility of programs that replaced synthetic multi-site fungicides with rotations between the biological control, *B. subtilis* QST 713, and succinate dehydrogenase inhibitors (SDHIs), to which there are no reports of resistance in *V. inaequalis*.

To increase potential success of replacing multi-site protectants with biopesticides, decision support systems (DSS) could be used to aid the effective and timely deployment of applications. DSSs based on apple tree phenology and development of pathogens in regard to weather conditions including leaf wetness, percent spore release, temperature, and rain fall, could be used to predict disease risk as well as intensity and length of infections (Carroll and DeGaetano 2011). These tools could also help make preventative fungicide applications as opposed to curative applications, which is important in reducing selection for fungicide resistance (Brent and Hollomon 1995). By making predictions of disease risk from forecast weather using host and pathogen biofixes, DSSs can determine whether a fungicide application may be warranted in the near future. In this regard, integrating a DSS in a disease management program may result in fewer applications of fungicides, with applications being more effective due to precision timing to risk periods. Therefore, we hypothesize that by using the apple scab component of the NEWA (Network for Environment and Weather Application; NEWA.cornell.edu; Carroll and DeGaetano 2011) DSS, applications of the highly effective SDHI fungicide could be timed during periods of high disease risk while biopesticides could be used to maintain fungicide coverage during periods of low disease risk. Further, should climate change predictions lead to periods of increased drought in temperate production regions, like New York (Sweet et al. 2017), there may be reduced need for multi-site synthetic fungicides and more opportunities for biopesticides.

Aside from precision application timing, orchard planting systems can have a substantial impact on disease development and spread. Toward the turn of the century, apple orchards in the eastern US were trained to the vertical axis system, where trees were planted at 1200 trees per ha (Robinson et al. 2013). Trees trained in this system and spacing typically have dense canopies, which in turn allow for increased humidity, reduced drying times of fruit and foliage, and can be more difficult to achieve adequate fungicide coverage. Indeed, many programs evaluating fungicides and biopesticides in apples used such planting systems (Bradshaw et al. 2016; Yoder et al. 2014a; Peter and Lehman 2019). In recent years, transitions towards smaller trees trained to a trellised super spindle system at 2,500-5,400 trees per ha allow for increased yield per hectare, increased mechanization, and reduced labor time and associated costs (Robinson et al. 2013). Such trees have smaller, thinner canopies, and would allow for reduced humidity, drying time of fruit and foliage, and better fungicide coverage, which would decrease the environmental factors favoring apple scab infection (Tivoli et al. 2013). Therefore, these super-spindle plantings present an additional opportunity to implement biopesticides in a management program to reduce reliance on multi-site protectant fungicides.

Altogether, the goal of this study was to provide a foundational framework to optimize the feasibility of integrating *Bacillus*-based biopesticides in a management program with highly effective single-site SDHI fungicides to reduce reliance on synthetic multi-site protectant fungicides. We hypothesized that by combining the advantages of using a modern super spindle planting system with appropriately timed applications using a DSS, both apple scab development and selection for fungicide resistance could be effectively managed using a program of rotating SDHIs with *Bacillus*-based biopesticides compared to programs alternating with multi-site protectant fungicides.

Materials and Methods

Experimental orchards. Management programs were applied in both an older, traditional vertical axis orchard and younger, modern super-spindle orchard in Geneva, NY. The vertical axis orchard consisted of 13-year-old 'Galas' grafted to B.9 rootstocks planted at approximately 1,200 trees to the Ha, while the super-spindle orchard consisted of fourth leaf (three-year-old) 'Gala' on G.935 rootstocks with a 3-wire training system, planted at approximately 3,000 trees to the Ha. Both plantings were within the same plot of land, approximately 600 m apart with no barriers, and therefore shared all local weather patterns. The experiment was conducted in 2019 and repeated in 2020 in both orchards. In each of the two orchards (vertical axis and super spindle), a weather sensor (HOBO remote monitoring system RX3000; Onset, Bourne, MA) was affixed to one tree of both orchards to record daily temperature and relative humidity, important variables for successful infection of *V. inaequalis*. For each location and year, temperature, average daily relative humidity, and total days with mean % relative humidity at or above 90% (an indicator of leaf wetness) was determined to assess differences in canopy microclimate.

Management programs. To assess the feasibility of using *Bacillus*-based biopesticides as a means of reducing reliance on multi-site protectant fungicides, we evaluated several management plans which included: 1) an untreated control serving as a negative control, 2) a commercial standard management program serving as a positive control, which consisted of applications of a tank mixture of multi-site protectant fungicides mancozeb (Manzate Max 5.46 L/Ha; UPL, King of Prussia, PA) and captan (Captec 4L 4.54 L/Ha; Arysta life Science, Cary,

NC) rotated with benzovindiflupyr (Aprovia 365.38mL/Ha; Syngenta, Greensboro, NC) on a calendar schedule, 3) a similar commercial standard management program rotating the mixture of mancozeb and captan with benzovindiflupyr, but with benzovindiflupyr application timing and use based on the apple scab component of the disease support system (DSS) NEWA (http://newa.cornell.edu/), 4) a program consisting of applications where the biological *B. subtilis* QST 713 (Serenade Opti 1.401 kg/Ha; Bayer Crop Science, Research Triangle Park, NC) was rotated with benzovindiflupyr on a calendar schedule and, 5) a program rotating applications of *B. subtilis* with benzovindiflupyr, with application timing and fungicide use based on the DSS (Table 4.1). We chose benzovindiflupyr as our model SDHI fungicide due to its low EC_{50} values against *V. inaequalis in vitro* (Villani et al. 2016a, Ayer et al. 2019a) and high levels of efficacy against apple scab in field studies (Phillion and Joubert 2015; Cox et al. 2017b). Plots for all management programs (treatments) were arranged in a randomized complete block design within both plantings with four single-tree replicates in the vertical axis orchard and four-tree panel replicates in the super spindle planting.

Table 4.1. Treatment programs applied to both the super spindle and vertical axis orchards in 2019 and 2020, including fungicide, product, application rate, and application timing. All programs were applied in both the vertical axis and super spindle orchards.

Treatment	Program	Active ingredients	Application timing
1	Untreated Control (no fungicides)	-	-
2	Manzate Max (5.46 L/Ha) + Captec (4.54 L/Ha) rotated biweekly with Aprovia (365.38 ml/Ha)	Mancozeb, Captan & Benzovindiflupyr	Calendar (every 7-10 days)
3	Manzate Max (5.46 L/Ha) + Captec (4.54 L/Ha) rotated with Aprovia (365.38 mL/Ha) during severe infection periods	Mancozeb, Captan & Benzovindiflupyr	Decision support system
4	Serenade Opti (1.401 kg/Ha) rotated biweekly with Aprovia (365.38 mL/Ha)	*Bacillus subtilis* QST 713 & Benzovindiflupyr	Calendar (every 7-10 days)
5	Serenade Opti (1.401 kg/Ha) rotated with Aprovia (365.38 mL/Ha) during severe infection periods	*Bacillus subtilis* QST 713 & Benzovindiflupyr	Decision support system

While not a commercially feasible option for disease management, or part of our formal experimental design evaluating full season programs, we added two additional programs in 2020 to better understand the individual impacts of the biopesticide and SDHI fungicide used in the programs described above. These ancillary programs included: A) single applications of *B. subtilis*, timed using the NEWA DSS, and B) single applications of benzovindiflupyr timed using the NEWA DSS in a manner identical to treatment 5, but with no applications made when *B. subtilis* was applied in treatment 5 (Supplementary Table S4.1).

Supplemental Table 4.1. Ancillary treatments added in 2020 compared to untreated control to examine individual impacts of the biopesticide and SDHI fungicide used in the programs on mean AUDPC.

Ancillary treatment	Program	Fungicide & Timing	Total Applications	Mean AUDPC			
				Vertical Axis Fruit	Vertical Axis Terminal	Super Spindle Fruit	Super Spindle Terminal
1	Untreated Control	-	0	44.58	26.88	9.38	31.79
A	Serenade Opti (1.401 kg/Ha)	*Bacillus subtilis* - DSS	8	3.33	11.09	0.00	5.23
B	Aprovia (365.38 mL/Ha)	Benzovindiflupyr - DSS	3	3.33	2.97	0.00	0

All management programs began on the same day at the phenological stage of tight cluster, on 25 April in 2019 and 2 May in 2020, and continued until the 8th application at fourth cover, at the end of June, depending on program and year. Prior to each program, applications of Kocide 3000 (6lbs/A; Certis USA, Colombia, MD) were applied to the entire orchard at the phenological stages of green tip and half-inch green to manage overwintering inoculum of apple scab and fire blight as part of standard commercial management practices (Agnello et al. 2019). With the exception of the ancillary control program in 2020 (trt. B), consisting of applications of benzovindiflupyr (Aprovia) alone, the United States Federal Insecticide, Fungicide, and Rodenticide Act (FIFIRA) Section 3 label was followed for application use guidelines for Aprovia (benzovindiflupyr), in that no more than two consecutive applications of the commercialized product would be applied and that the program would not exceed a total of four applications throughout the season. To prevent interplot interference between treatment plots due to drift, a Solo 425 piston backpack sprayer (Solo, Newport News, VA) was used in the super

spindle planting and a Solo 475-B gas-powered mist blower (Solo, Newport News, VA) in the vertical axis planting, where row and plot spacing was wider. Regardless of the sprayer used, fungicides were applied dilute to runoff, and all formulated products used were applied at the highest labeled commercial rate (Table 4.1) (Villani et al. 2015; Ayer et al. 2020).

For the calendar-based programs, applications were made on a 7-10 day schedule until petal fall, where intervals were extended to 10-14 days, which is standard practice for cover applications in apples (Ayer et al. 2019b; Strickland et al. 2020). Program rotational schedules were such that benzovindiflupyr was applied after two consecutive applications of either a mixture of captan and mancozeb (trt. 2) or *B. subtilis* (trt. 4). In treatment 3 and 5, benzovindiflupyr application timings were chosen based on apple scab infection periods as predicted by the NEWA DSS (NEWA.cornell.edu) with an ascospore release threshold of at least 15% and more than 35 h leaf wetness. For these treatments, if no infection was predicted for a given week, *B. subtilis* (trt. 5) or mancozeb mixed with captan (trt. 3) was applied to maintain fungicide coverage in between infection events or before smaller infection events.

Apple scab assessments. In each orchard and year, assessments of apple scab began once the first lesions appeared in the untreated plots (negative control) within a single orchard and were rated a total of five to seven times every 10-20 days to monitor disease progress over the season. In 2020, a sixth assessment of apple scab incidence on terminal leaves was made due to late season rainfall. In both orchards, the incidence of apple scab on terminal leaves and fruit was measured at each assessment date. The incidence of apple scab symptoms on terminal leaves was determined by counting the number of terminal leaves with apple scab lesions out of eight fully expanded leaves from the distal end of the shoot. Similarly, the incidence of apple scab

symptoms on fruit was expressed as the number of fruit with apple scab lesions out of the total fruit in a cluster. For each of the four replicate plots, 20 shoots or fruit clusters were assessed, unless there was insufficient fruit or numbers of shoots. Following the final assessment, incidence data for both terminal leaves and fruit, expressed as a percentage, was used to calculate the area under the disease progress curve (AUDPC) for the means of each replicate plot (Madden et al. 2007). In each orchard and year, the effect of management program on the AUDPC for apple scab of terminal leaves and fruit was determined by generalized linear mixed models using the PROC GLIMMIX procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). For models with significant fixed effects, differences between treatments were determined using the LSMEANS procedure in SAS 9.4 at the a = 0.05 level of significance with an adjustment for Tukey's HSD to control for family-wise error. For each orchard and year, the effect of management program on percent disease incidence and terminal leaves and fruit was evaluated by generalized linear mixed models using the PROC GLIMMIX procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). All percentage data were subjected to arcsine square-root transformation prior to analysis. For models with significant fixed effects, differences between treatments were determined using the LSMEANS procedure in SAS 9.4 at the a = 0.05 level of significance with an adjustment for Tukey's HSD to control for family-wise error.

SDHI fungicide sensitivity assessment. To allay concerns of selection for fungicide resistance following application of benzovindiflupyr and to assess potential fungicide resistance management, assessments of SDHI sensitivity were made for each management program in each orchard and year. Approximately 10 days following the final SDHI fungicide application, approximately five leaves with sporulating lesions of apple scab were collected from each of the

replicate plots for a total of 15-30 isolations from each management program and orchard. Individual isolated leaf lesions representing single ascospore infections (MacHardy and Gadoury 1989) were excised and subjected to a relative growth assay using a discriminatory dose of 0.02 μg ml^{-1} benzovindiflupyr based on 10x the baseline EC_{50} values for *V. inaequalis* as described previously (Ayer et al. 2109a; Villani et al. 2016a). In short, conidia were extracted from lesions as previously described (Ayer et al. 2109a; Villani et al. 2016a) suspended in dH_2O and spread onto plates of potato dextrose agar (PDA; Difco Laboratories, Inc., Detroit, MI) with the discriminatory dose of technical grade benzovindiflupyr (Syngenta; Greensboro, NC) in addition to two antibiotics; streptomycin sulfate and chloramphenicol at 50 μg ml^{-1} to avoid bacterial contamination (PDA++). At the same time, conidia from each isolated lesion were also spread on a control plate of PDA++ without benzovindiflupyr. After one week of growth at room temperature, conidial germination and secondary hyphal growth was measured using a SPOT Idea digital camera and SPOT Imaging Basic Software (Diagnostic Instruments Inc., Sterling Heights, MI) attached to an Olympus SZX12 stereoscope (Olympus America Inc., Center Valley, PA). Percent relative growth (%RG) on benzovindiflupyr amended media was calculated for each isolate in comparison to growth on the control PDA++ plate as previously described (Villani et al. 2016a; Ayer et al. 2019) and mean %RG was calculated for each treatment.

Results

Impact of seasonal weather on the orchard sites and management programs. In 2019, seasonal weather conditions were highly conducive for apple scab development while seasonal weather conditions in 2020 were not. Specifically, in May through June, there were 20.40 and 6.96 cm of rainfall, and 311 and 136 h leaf wetness in 2019 and 2020, respectively

(Table 4.2). In respect to seasonal weather trends, differences in the microclimate relative humidity between canopies of the trees in vertical axis and super spindle were also observed. In 2019, the mean percent relative humidity for canopies in the vertical axis planting was 77.25% from 1 April to 31 August, while mean relative humidity was 75.36% for canopies in the super spindle planting. Further, canopies in the vertical axis planting had 21 days where the mean daily relative humidity was over 90%, while there were 18 days in the super spindle orchard. In 2020, where there was only 6.96 cm of rain from May to June 2020, there were no appreciable differences in mean percent relative humidity or in days where the mean daily relative humidity was over 90% between the canopies of trees in the vertical axis and super spindle plantings (Table 4.2).

Table 4.2. Weather data displaying A) relative humidity between super spindle and vertical axis orchards from 1 April to 31 August in 2019 and 23 June to 31 August in 2020 and B) overall rainfall and leaf wetness in 2019 and 2020 from 1 May to 30 June.

A.	**Orchard**	**Mean %RH**	**Days over 90% mean RH**
2019			
	vertical axis	77.25%	21 d
	super spindle	75.36%	18 d
2020			
	vertical axis	73.96%	2 d
	super spindle	73.46%	2 d
B.	**Year**	**Rainfall**	**Leaf Wetness**
	2019	20.40 cm	311 h
	2020	6.96 cm	136 h

Table 4. 3. Cumulative application number of each active ingredient by program by the end of the season in 2019 and 2020.

Treatment		Program	*Bacillus subtilis*	Captan and Mancozeb	Benzovindiflupyr
2019					
	1	Untreated control	-	-	-
	2	Mancozeb, captan & benzovindiflupyr - calendar	-	6	2
	3	Mancozeb, captan & benzovindiflupyr -DSS	-	4	4
	4	*B. subtilis,* benzovindiflupyr-calendar	6	-	2
	5	*B. subtilis,* benzovindiflupyr-DSS	4	-	4
2020					
	1	Untreated control	-	-	-
	2	Mancozeb, captan & benzovindiflupyr - calendar	-	6	2
	3	Mancozeb, captan & benzovindiflupyr -DSS	-	5	3
	4	*B. subtilis,* benzovindiflupyr-calendar	6	-	2
	5	*B. subtilis,* benzovindiflupyr-DSS	5	-	3

At the end of the season, each treatment program received a total of eight fungicide applications, but the number of applications of each fungicide applied was determined by the application conditions that defined specific programs (Table 4.3). Programs where fungicides were applied on a calendar schedule received six applications of either the captan and mancozeb mixture (Captec 4L and Manzate Max) or *B. subtilis* (Serenade Opti) with two total applications of benzovindiflupyr (Aprovia) in both years. Programs with fungicide timing based on apple scab infection events as predicted by the NEWA DSS differed in the number applications of captan/mancozeb or *B. subtilis*, and the rotation partner of benzovindiflupyr between the two years (Table 4.3). In 2019, the NEWA DSS predicted four infection periods with more than 15%

ascospore release or 35 hours of leaf wetness. By comparison, programs with fungicide timing based on the NEWA DSS prediction only reached this threshold three times in 2020 (Table 4.3).

Impact of season weather and management of programs on the development of apple scab. In both years and orchards, disease assessments began the first week of June (3 June 2019 and 2 June 2020) with the onset of symptom development in the untreated plots (negative control) and continued through fruit maturation once incidence leveled out. While the lesions initially emerged at the same time, the development of apple scab in each orchard site differed between year with respect to seasonal weather, highlighted in the untreated plots. For example, in 2019, the mean AUDPC of untreated plots in the vertical axis orchard on fruit was 5190.83 (100% disease incidence), while untreated plots in the super spindle orchard untreated plots had a lower mean AUDPC of 4123.1 (79.38% incidence) (Figure 4.1; Table 4.4). In regard to apple scab symptoms on terminal leaves, the mean AUDPC for untreated plots in the vertical axis and super spindle orchard was AUDPC 3320.81 (28.13% incidence) and 4011.69 (48.13% incidence), respectively (Figure 4.2; Table 4.4). In 2020, where there was considerably less rainfall (6.96 cm) than 2019 (20.40 cm), the development of apple scab symptoms on fruit and terminal leaves was lower than that in 2019, but similar between the two plantings. For example, the mean AUDPC for apple scab symptoms on fruit in the untreated plots was only 44.58 (3.85% incidence) and 9.38 (1.43% incidence) on fruit in the vertical axis and super spindle orchards, respectively (Figure 4.3; Table 4.4). On terminal leaves, mean AUDPC was 26.88 and 31.79 in the vertical axis and super spindle orchards, respectively, representing 1% disease incidence at the end of the season in both orchards in 2020 (Figure 4.4; Table 4.4).

Table 4.4. Disease incidence of programs at the end of the season.

Trt	Program	Disease Incidence			
		Vertical Axis Fruit	Vertical Axis Terminal	Super Spindle Fruit	Super Spindle Terminal
2019					
1	Untreated control	100.00 a[y]	28.13 a	79.38 a	48.13 a
2	Mancozeb, captan & benzovindiflupyr - calendar	27.08 b	5.45 c	2.50 b	0.63 c
3	Mancozeb, captan & benzovindiflupyr - DSS	12.14 c	10.82 bc	0.00 b	2.97 bc
4	*B. subtilis*, benzovindiflupyr - calendar	25.21 b	13.76 b	15.56 b	6.88 b
5	*B. subtilis*, benzovindiflupyr - DSS	14.17 bc	5.94 c	2.78 b	7.08 bc
2020					
1	Untreated control	3.85 a	1.09 a	1.43 a	1.02 a
2	Mancozeb, captan & benzovindiflupyr - calendar	0.00 b	0.00 a	0.00 a	0.31 ab
3	Mancozeb, captan & benzovindiflupyr - DSS	0.00 b	0.00 a	0.00 a	0.00 b
4	*B. subtilis*, benzovindiflupyr - calendar	0.00 b	0.16 a	0.00 a	0.00 b
5	*B. subtilis*, benzovindiflupyr - DSS	0.00 b	0.00 a	0.00 a	0.16 ab

[y] Values within columns for a given year followed by a different letter are significantly different ($P < 0.05$) according to the LSMEANS procedure in SAS 9.4 with an adjustment for Tukey's HSD to control for family-wise error.

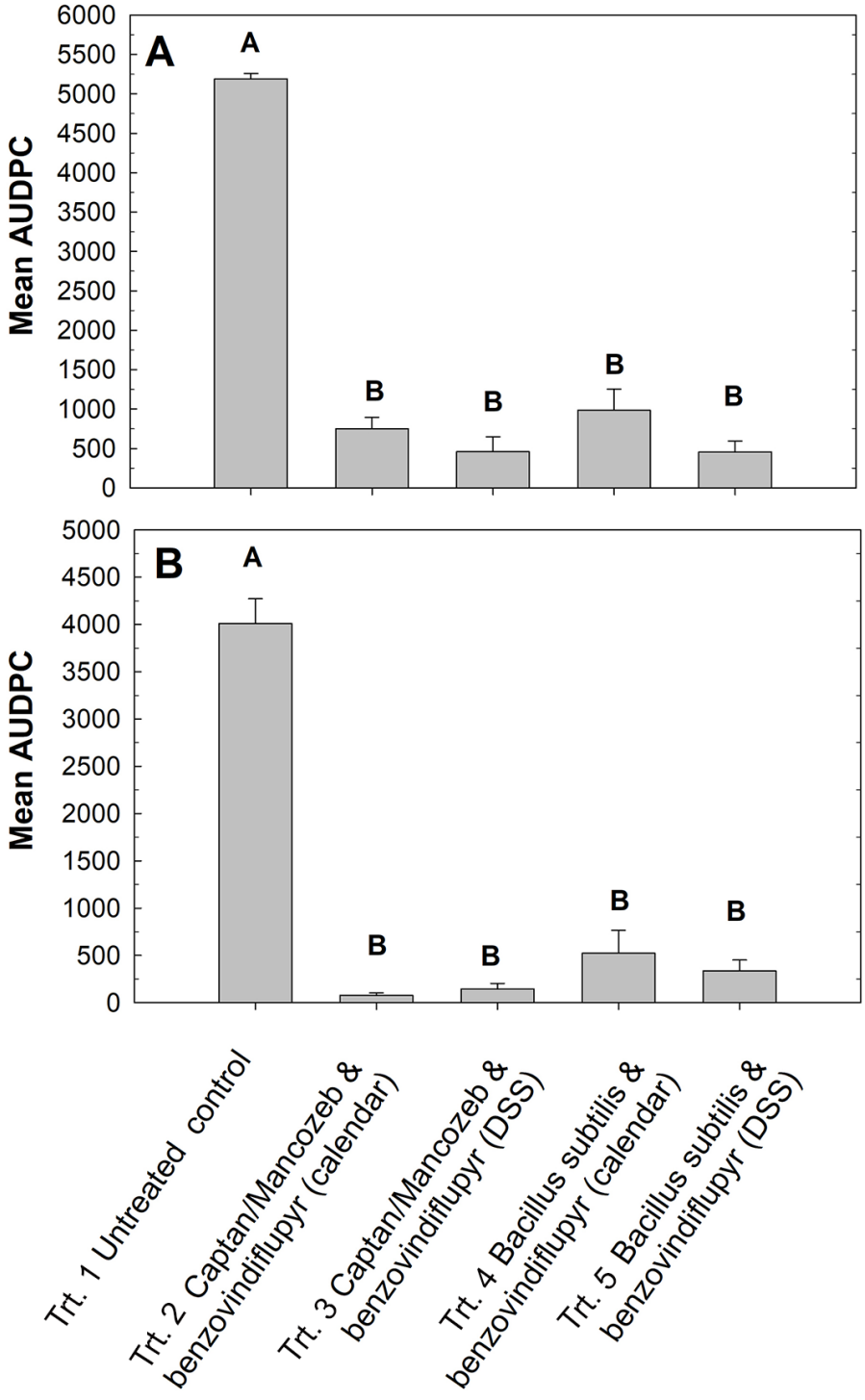

Figure 4.1. Mean area under the disease progress curve (AUDPC) for incidence of apple scab on fruit for the (A) vertical axis planting in 2019 and (B) super spindle planting in 2019. Within each graph, different letters above bars indicate significant differences between means based on Tukey HSD test ($P < 0.05$).

In both orchards in 2019, all management programs with fungicide applications (trts. 2-5) had lower mean AUDPCs for apple scab symptoms on fruit and terminal leaves than the untreated control plots ($P < 0.05$) (Figure 4.1 + 4.2). These programs were all equally effective for managing apple scab in that there were no significant differences between programs in mean AUDPC for apple scab symptoms on leaves or fruit ($P > 0.05$) (Figure 4.1 + 4.2). In 2019, end of season incidence of apple scab symptoms on fruit and leaves in management programs plots with fungicide applications was always below 30% and 20% in the vertical axis and super spindle orchards, respectively (Table 4.4).

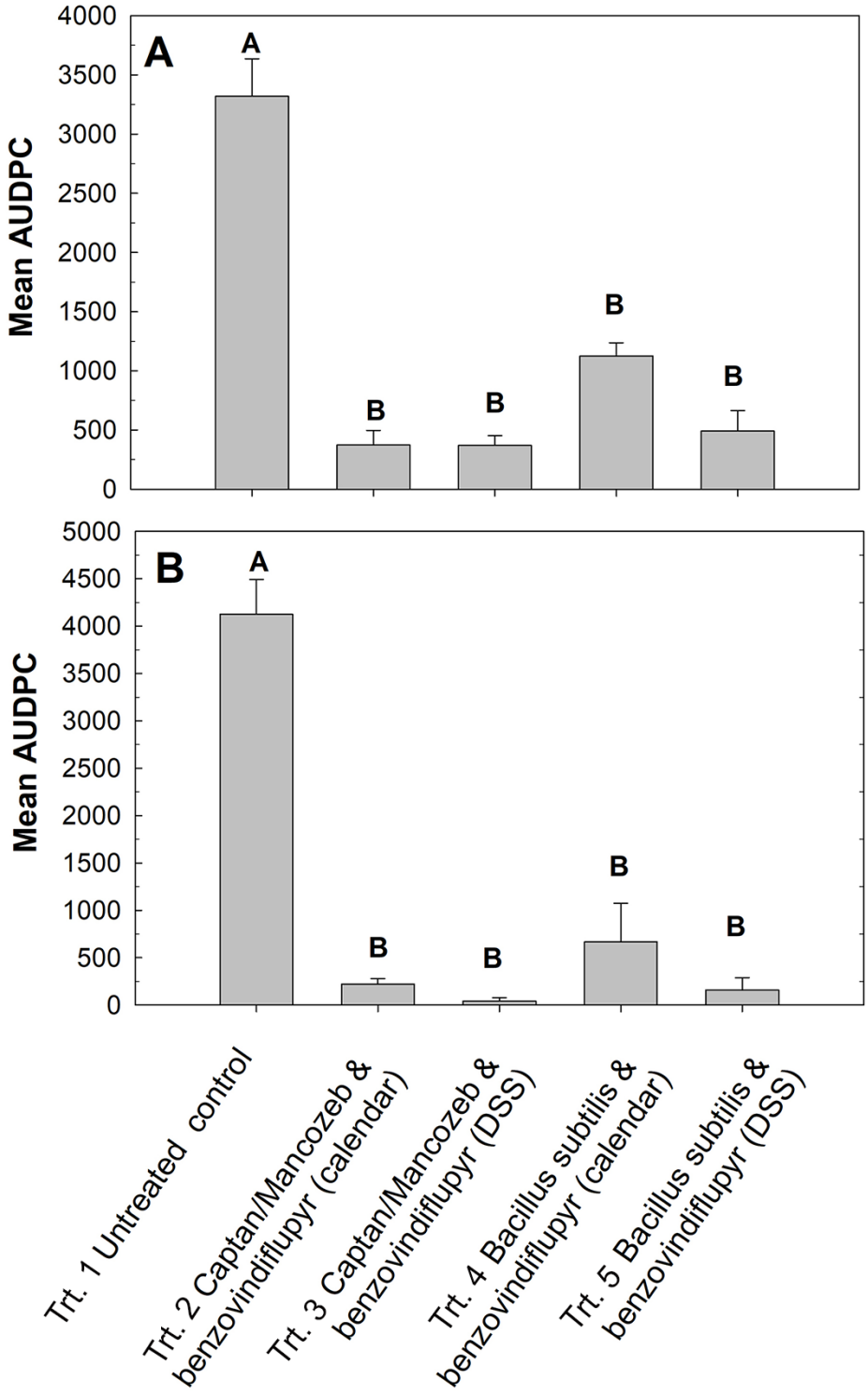

Figure 4.2. Mean area under the disease progress curve for incidence of apple scab on terminal leaves for the (A) vertical axis planting in 2019 and (B) super spindle planting in 2019. Within each graph, different letters above bars indicate significant differences between means based on Tukey HSD test ($P < 0.05$).

In 2020, management programs with fungicide applications (trts. 2-5) had complete (100%) disease control (0% incidence) on fruit, and only the untreated plots in both orchards developed apple scab on fruit (vertical axis 3.85% incidence, super spindle 1.43% incidence) (Table 4.4). However, there were no significant differences ($P > 0.05$) in mean AUDPC between any of these programs for the development of apple scab symptoms on fruit (Figure 4.3). The plots from the two ancillary single fungicide programs (trts. A + B), developed low levels of apple scab in the vertical axis orchard (< 0.5% incidence) and no apple scab in the super spindle orchard, but this was not significantly different from any of the programs or the untreated control in either orchard ($P > 0.05$) (Supplementary Table S4.1).

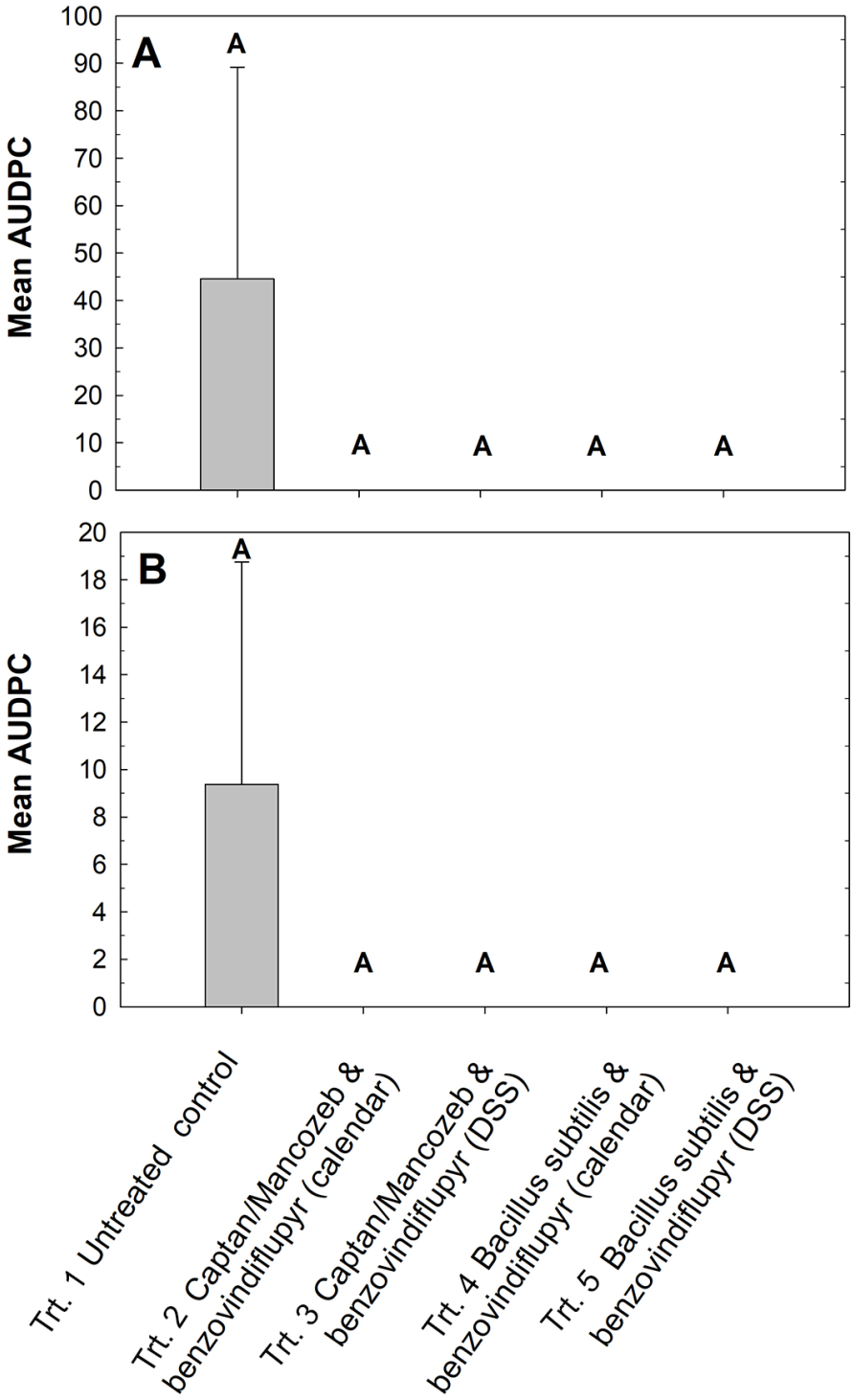

Figure 4.3. Mean area under the disease progress curve for incidence of apple scab on fruit for the (A) the vertical axis planting in 2020 and (B) the super spindle planting in 2020. Within each graph, different letters above bars indicate significant differences between means based on Tukey HSD test ($P < 0.05$).

There were similar levels of symptom development on terminal leaves in 2020 between the two orchards, and there were some significant differences ($P > 0.05$) among programs in the vertical axis planting regarding AUDPC (Figure 4.4). In the vertical axis orchard, only the AUDPC of management programs with fungicide timing based on the NEWA DSS (trts. 3 + 5) were statistically different from the untreated control (Figure 4.4). Both programs afforded complete control throughout the entirety of the season (mean AUDPC= 0, 0% disease incidence). In the super spindle orchard, all management programs with fungicide applications (trts. 2-5) and the two ancillary programs (trts. A + B) were significantly different from the untreated control ($P < 0.05$; Figure 4.4; Supplementary Table S4.1).

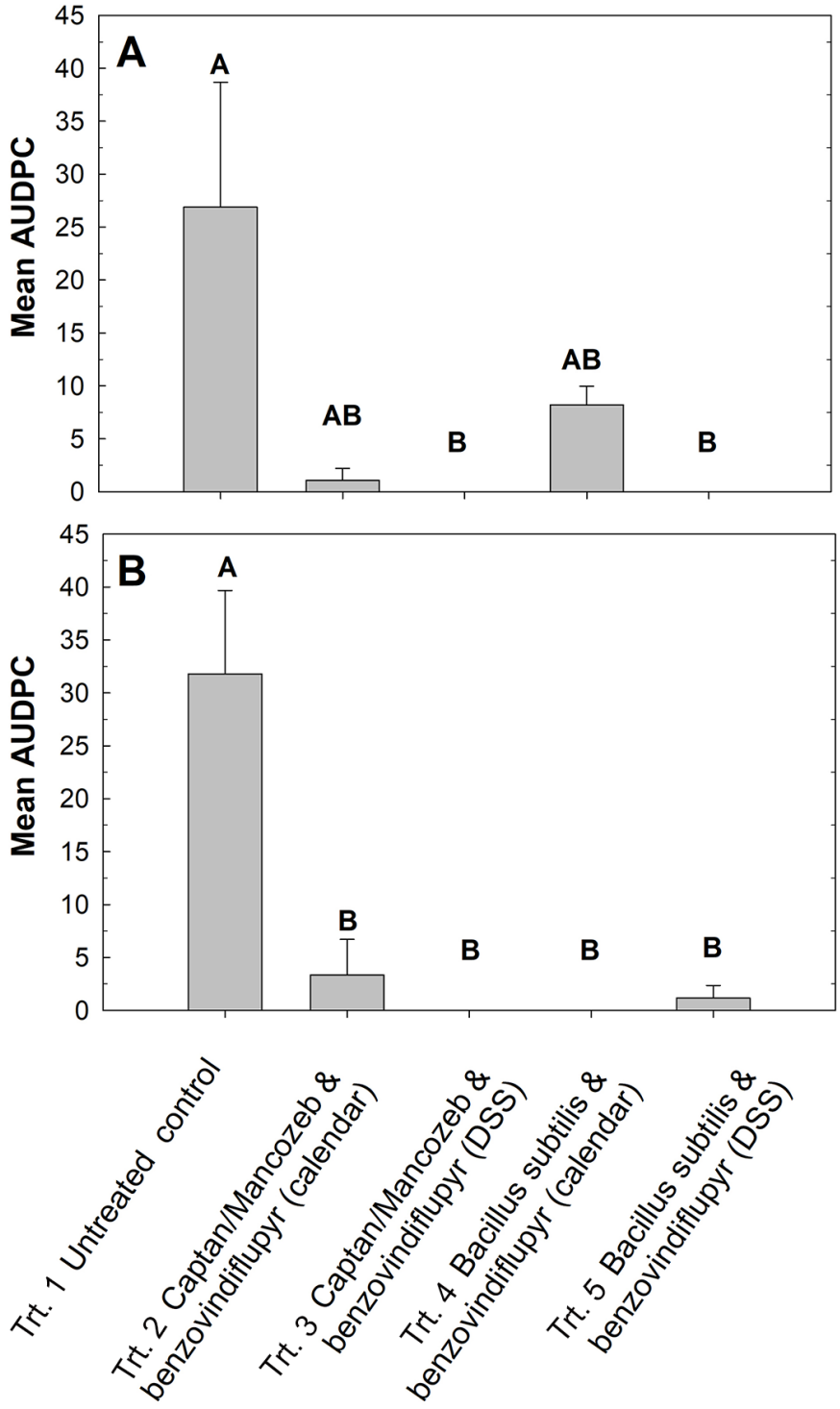

Figure 4.4. Mean area under the disease progress curve for incidence of apple scab on terminal leaves for the (A) vertical axis planting in 2020 and (B) the super spindle planting in 2020. Within each graph, different letters above bars indicate significant differences between means based on Tukey HSD test ($P < 0.05$).

An additional late season rating was completed in 2020 on terminal leaves due to high late season rainfall and an observed increase in terminal leaf apple scab lesions in order to further capture differences between programs (Supplementary Table S4.2). On 24 August 2020, the untreated controls reached approximately 5% disease incidence in both orchards. Again, in both orchards, disease incidence for all programs (trts. 2-5) were statistically different from the untreated control. In both cases, the treatment of Serenade Opti alone (trt. A) was not statistically different from the untreated control, with 2.34% incidence and 3.91% incidence in the vertical axis and super spindle orchards, respectively ($P > 0.05$; Supplementary Table S4.2).

Supplemental Table 4.2. Late season 2020 disease incidence ratings expressed as a percentage in vertical axis and super spindle orchard on terminal leaves taken on 24 August 2020.

Trt	Program	Vertical Axis Terminal	Super Spindle Terminal
1	Untreated control	5.00 a[z]	4.61 a
2	Mancozeb, captan & benzovindiflupyr - calendar	0.16 c	0.31 c
3	Mancozeb, captan & benzovindiflupyr -DSS	0.00 c	0.94 c
4	*B. subtilis,* benzovindiflupyr- calendar	0.47 bc	0.78 c
5	*B. subtilis,* benzovindiflupyr- DSS	0.00 c	1.25 bc
A	*B. subtilis* -DSS	2.34 ab	3.91 ab
B	Benzovindiflupyr -DSS	0.469 bc	1.72 bc

[z] The incidence of apple scab symptoms on terminal leaves is expressed as the mean of eight distal leaves from 10 shoots from each of four replicate plots. And that means denoted with the same letters indicate a lack of significant difference based on Tukey HSD test ($\alpha = 0.05$).

SDHI fungicide sensitivity assessment. In 2019, isolates were successfully cultured from leaf lesions for all management programs with 8-32 isolates per treatment. Differences in isolate number between programs reflect success with conidial isolation and overall disease incidence. In 2020, the incidence of apple scab symptoms on leaves and fruit was so low (< 3%) that collection of leaf lesions was not possible with the need to continue season long disease assessments. Indeed, there were no leaf lesions observed in many of the management program plots in both orchards (Table 4.4). In 2019, there were no treatments with a mean %RG >50, which could suggest a potential shift toward quantitative fungicide resistance. Across all of the programs, the mean %RGs for benzovindiflupyr ranged from approximately 20% to 30% (Figure 4.5).

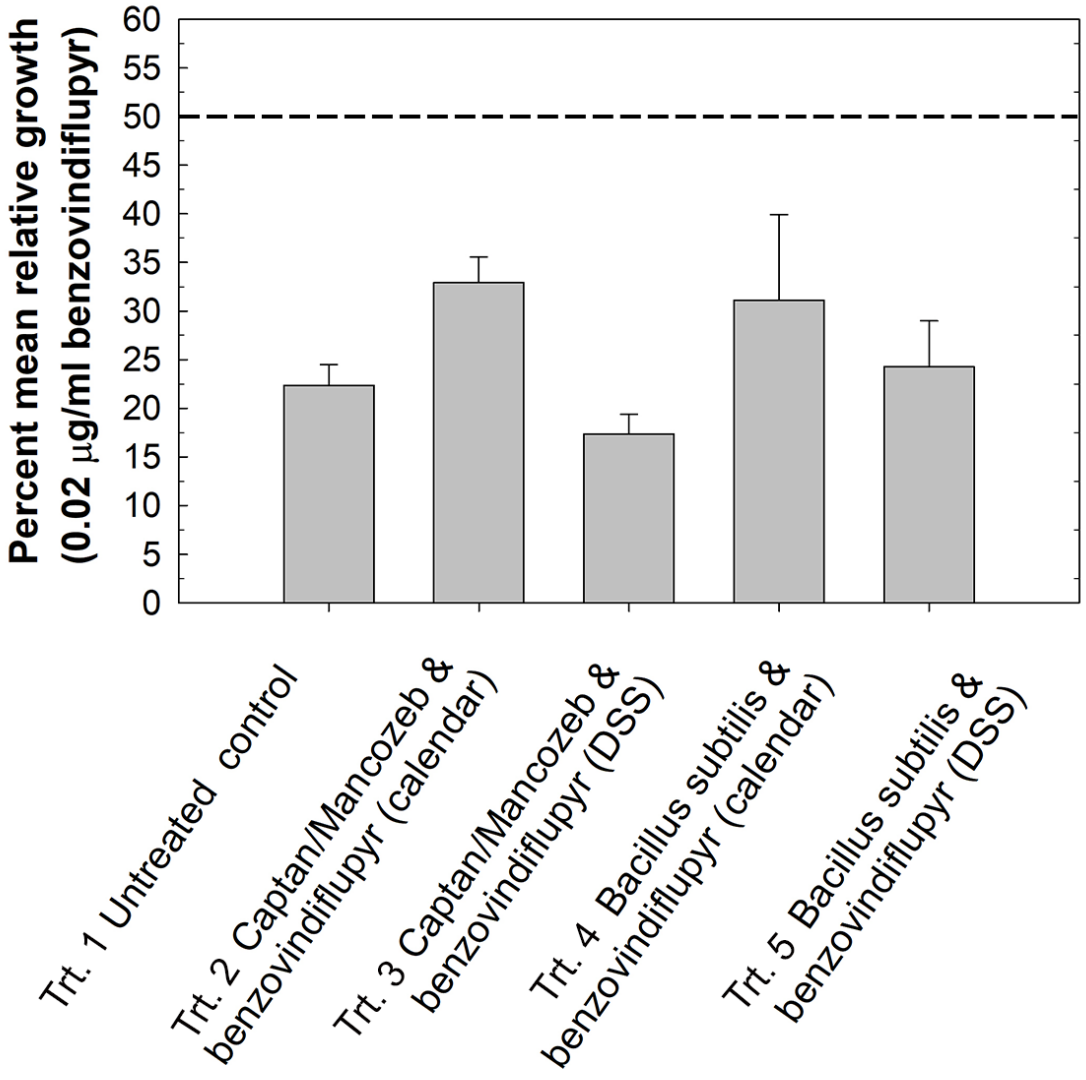

Figure 4.5. Sensitivity of apple scab isolates from leaves of the 2019 vertical axis planting between programs. Conidial germination assays were completed on 10x the EC_{50} value of benzovindiflupyr to calculate mean percent relative growth. The dotted line represents the 50% relative growth threshold for a potential resistance shift.

Discussion

With a history of successional development of resistance to single-site fungicides in the apple scab pathogen *V. inaequalis*, multi-site protectant fungicides such as mancozeb and captan have become integral components of commercial management programs (Agnello et al. 2019; Villani et al. 2019; Jurick II and Cox 2017). While multi-site fungicides are effective for both disease and fungicide resistance management, continued use of these fungicides may not be sustainable in future commercial markets (Beckermann et al. 2015b). In this work, we established a framework by which biopesticides could be substituted for synthetic multi-site protectant fungicides and still effectively manage apple scab as well as reduce selection for fungicide resistance. Specifically, we wished to identify the conditions that would enhance the efficacy of the biopesticide, *B. subtilis* QST 713, as a rotation partner for the single-site SDHI fungicide, benzovindiflupyr, without compromising disease control or selection for fungicide resistance. This proposed management paradigm would capitalize on horticultural practices that reduce disease pressure, reduce drying time of fruit and foliage, increase fungicide application precision, and improve fungicide coverage through use of super-spindle training systems and disease forecasting to achieve sustainability as a commercial practice.

Over the two years of the study, we found that there is indeed a possibility for curtailing multi-site fungicide use and the potential for biological controls to be used in substitution as a rotational partner with SDHIs in modern super spindle production systems when applications are timed using disease forecasting. The two years of the study presented radically different apple scab management challenges in the regard to disease pressure resulting from seasonal precipitation patterns with the 2019 season representing a high disease pressure season. Irrespective of these seasonal challenges, we didn't observe any significant differences between

any of the management programs in development of apple scab symptoms on fruit or leaves for vertical axis or super spindle orchards. There were slight observed numerical differences in AUDPC between the programs where applications of the biological control, *B. subtilis* QST 713 were used in place of mancozeb and captan as a rotational partner, especially in the vertical axis orchard under high disease pressure in the 2019 season. However, these slight numerical observations in AUDPC were not statistically significant ($P > 0.05$), and in turn, likely not commercially or biologically relevant. Along these lines, management programs with applications of *B. subtilis* QST 713 had the lowest observable levels of disease when applied in the super spindle orchard and when application were timed using the NEWA DSS. In support of these observations, Boland (1997) found that the efficacy of biological controls was better in environments less conducive for pathogen development. We did note differences in tree canopy relative humidity between in the super spindle and vertical axis orchard in the 2019 season where there was considerably more rainfall. While such an observation is not surprising, it may be that training for reduced canopy density may only be beneficial in more temperate apple production regions or seasons when there is considerable precipitation in the spring in regards to disease incidence. Canopy pruning is also a recommended cultural practice to reduce humidity within the canopy, and in turn, the development of apple scab (MacHardy 1996).

Although there were no statistical differences in apple scab development between the management programs in either orchard or year, we observed that the programs where SDHI applications were timing using the NEWA DSS (trts. 3 + 5) had lower levels of apple scab symptoms on leaves and fruit than programs where SDHIs were applied on a calendar schedule. While these observations were more apparent during the 2019 season when there was considerable rainfall, there were some indications of this trend of improved control using the

DSS despite the low disease incidence observed in 2020. Prior evidence for the impact of disease forecasting was noted by Shtienberg and Elad (1997) who found that use of forecasting improved the efficacy of biopesticides against *B. cinerea*. The use of disease forecasting to time the application of SHDI fungicides for apple scab could help maximize opportunities to target the germination of *V. inaequalis* conidia, which we have found to be the stage most sensitive to SDHI fungicides (Ayer et al. 2019a, Villani et al. 2016a). As many apple scab infection periods may be predicted by the NEWA DSS, SDHI fungicides or other single-site fungicides should likely be applied only prior to severe infection periods. Such periods would include those defined by our threshold conditions for the current study, where of more than 15% ascospore release and more than 35 hours of leaf wetness was predicted. Materials such as biopesticides, which may often be less potent during bouts of high disease pressure (Rosenberger et al. 2000; Strickland and Cox 2020; Yoder et al. 2007; Yoder et al. 2014b; Yoder et al. 2016), could be used prior to infection periods with lower levels of predicted leaf wetness or between infections to ensure coverage and resistance management.

Fungicide sensitivity testing was only able to be completed after one year, where all treatment's mean relative growth was <50% on benzovindiflupyr. The lack of multiple years of testing is due to the absence of leaf lesion development in 2020 to due complete control afforded by the management programs. While one might to be tempted to suggest that achieving near complete control would be one of the best means of ensuring fungicide resistance management, the effect of selection on potentially unobserved lesions would never been known. Moreover, we would not expect to see fungicide resistance after a single year of fungicide use (Ayer et al. 2020), and therefore we cannot make robust conclusions about fungicide resistance management in our study. However, other studies have demonstrated the ability of biological controls to

manage fungicide-resistant pathogens such as *Penicillium expansum* on apple (Chand-Goyal and Spotts 1997; Erampalli and Brubacher 2006) and *B. cinerea* on grape (Hovinga and Derpmann 2020). Due to the multi-site mode of action of biopesticide active ingredients, it is postulated that they could be an important tool for reducing resistance selection (Jacobsen et al. 2004). Similar studies have investigated the integration of fungicides with biological controls and their effect on disease control and/or fungicide resistance management across a wide range of pathosystems (Elmer and McGovern 2004; Jacobsen et al. 2004; Kiewnick et al. 2001; Korsten et al. 2007; Lima et al. 2008; Shtienberg and Elad 1997). For example, Rotolo et al. (2018) found that *Bacillus spp.* or *Aureobasidium spp.* alone afforded little control of *B. cinerea* in grapes but including them in rotations with the SDHI fungicide, fluopyram, improved control, reduced the presence of SDHI-resistant conidia, and resulted in reduced fungicide residue. However, for the present pathosystem, a longer term investigation into the ability of biological controls to play a role in resistance management would be imperative.

While specific fungicides and biopesticides were used in this proof-of-concept study, there is potential for the management concepts presented here to be implemented with any number of SDHI fungicides and *Bacillus*-based biopesticides, if not others with different modes of action. Indeed, many SDHI fungicides and other biopesticides would likely be as effective under the same use practices (Yoder et al. 2014a; Sundin and Outwater 2017). Growers may also be hesitant to replace multi-site protectant fungicides for biological controls given their history of diminished disease control (Rosenberger et al. 2000; Strickland and Cox 2020; Yoder et al 2007; Yoder et al. 2014b; Yoder et al. 2016). However, as first step toward adoption, we were able to demonstrate that rotations with biopesticides provided the same level of control as industry standard programs rotating SDHIs with captan and mancozeb even during seasons like

2019 with considerable rainfall. A central question surrounding the potential for adoption, although more of an academic curiosity than a practical consideration for apple growers, is the relative contributions of benzovindiflupyr relative to that of *B. subtilis* or captan mixed with mancozeb to disease control. In this regard, we added two programs in 2020 that had applications consisting of only *B. subtilis* QST 713 (trt. A) or benzovindiflupyr applications (trt. B), which were not significantly difference in mean AUDPC (Supplementary Table S4.1). Similarly, we observed no differences between the programs where benzovindiflupyr was rotated with captan and mancozeb mixes (trts. 2 + 3) and the benzovindiflupyr only program (trt. B) in 2020 (Supplementary Table S4.1). Other than the potential for resistance management, these observations call into question the value of captan, mancozeb, or *B. subtilis* QST 713 applications in dry years. Perhaps differences between these treatments would have been more pronounced in a season with higher levels of rainfall and higher disease pressure. Levels of control would likely be similar to what was reported for biopesticide-only programs in previous studies (Rosenberger et al. 2000; Strickland and Cox 2020; Yoder et al. 2016).

Additional refinements to the concept of replacing multi-site fungicide applications with biopesticides could include shorter application intervals for biopesticides, or applications of single-site fungicides from fungicide classes other than SHDIs allowing for multiple modes of action and improved fungicide resistance management. At minimum, applications of multi-site protectant fungicides as a rotational partner in management programs could be replaced with a biopesticides later in the season when apple scab disease pressure is lower due to warmer weather and pre-harvest intervals are much longer for multi-site protectant fungicides (MacHardy 1996). Additionally, the changing climate and the likelihood of increased drought periods interspersed with times of heavier rain (Sweet et al. 2017) may further allow for

practicality of implementation of a similar management plan in the near future.

There are several reasons an apple grower might decide to implement the management paradigms like those described in our study to curtail use of multi-site protectant fungicides. These include better economic incentives to gain access to specialty domestic and export markets with fungicide use restrictions, intrinsic motivation for the grower to move towards sustainability, or extrinsic regulations (Lefebvre et al. 2015). However, due to the low prices of the multi-site protectant fungicides like mancozeb and captan, their replacement with biopesticides, which are often more expensive, may increase production costs (Rosenberger 2003) and pose a barrier to implementation. In contrast, specialty markets such as Red Tomato Eco Apple® (https://redtomato.org/) may provide an economic incentive for growers to become certified in their management practices and receive a larger return on investments. In the future, additional policies and regulations might be put into place that may restrict use of multi-site protectant fungicides, and producers might be best served to begin implementing alternatives prior to these changes. Indeed, this has occurred in other countries (Pesticide Action Network 2020) and may become a factor to consider in the United States in the future.

In conclusion, the present study establishes a foundational framework for replacing the use of synthetic, multi-site protectants with biological controls, with increased potential for sustainable disease management when implemented with modern planting systems, disease forecasting through DSSs, and rotations of highly effective single-site fungicides. These concepts are feasible to implement and amenable for future refinement as the industry moves towards increased sustainability in tree fruit production and the management of fungal diseases.

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CHAPTER 5

CONCLUSIONS

The overarching goal of this work was the better understand ways to sustainably use succinate dehydrogenase inhibitors to manage apple scab, caused by *Venturia inaequalis,* through understanding efficacy, fungicide resistance development, and integrated management plans.

In chapter 2, we look at baseline sensitivity of *V. inaequalis* to four SDHI fungicides; fluxapyroxad, benzovindiflupyr, inpyrfluxam, and pydiflumetofen. Our results indicate high *in vitro* efficacy of these compounds against both mycelial growth and conidial germination, with higher activity against conidial germination. We also observe a strong correlation in cross-sensitivity between the six tested SDHI fungicides, indicating that when a loss of field efficacy is observed to one fungicide, similar is to be expected in loss of efficacy to the others. Lastly, this chapter also develops primers for screening of mutations in the *VisdhC* and *VisdhD* genes. At this time, there have been no isolates with mutations in the *VisdhC* or *VisdhD* genes identified. Altogether, this chapter develops framework for future phenotypic and genotypic monitoring for resistance to SDHI fungicides, which is a valuable tool in understanding how to use SDHI fungicides better and more sustainably, as well as when not to use them if fungicide resistance develops.

In chapter 3, we question common fungicide application practices with the goal to identify practices that will reduce the selection pressure for resistance to develop. Specifically, this chapter looks at application of SDHI fungicides at a high dose, a low dose, in a mixture with a second single-site fungicide, and in a mixture with a multi-site fungicide. This is done through a four-year selection, repeated measures study where SDHI fungicide sensitivity is analyzed at

the end of each season. We found, that while there were variations between years and orchards, the treatment of low doses of the SDHI resulted in more isolates with a shift towards a reduction in sensitivity than the treatment of high doses. Further, use of mixtures in active ingredients resulted in few differences in sensitivity. This work supports the use of higher doses in fungicide applications as well as use of multi-modes of action to reduce selection pressure. This chapter aims to ensure SDHI fungicides remain an effective tool in apple scab management for a sustainable period of time through delaying the development of fungicide resistance.

In chapter 4, we look to determine how biopesticides could be used in an apple scab management plan with SDHI fungicides without use of synthetic multi-site fungicides captan and mancozeb. We found integration of biopesticides with SDHIs resulted in adequate apple scab management, with increased efficacy seen in orchards less conducive to disease and when using disease support systems. This chapter provides framework for more sustainable management plans, and an option for growers if regulations on use of mancozeb and captan were to become stricter. Even if that does not occur, this chapter give growers an alternative to use of synthetic broad-spectrum fungicides mancozeb and captan, to increase the sustainability of their practices.

This research contributes to more sustainable agriculture, specifically through increasing understanding of management of apple scab, caused by *Venturia inaequalis*, through use of SDHI fungicides. There are many questions yet to be explored about management of apple scab with SDHI fungicides that could be further pursued. Many questions can be addressed once qualitative resistance to SDHI fungicides is observed, which may occur after continuing selection pressure with extension of the work done in chapter 3. The first, is characterizing different mutations, as it has been reported in other fungi that there can a variety of mutations in the *sdhB*, *sdhC*, and *sdhD* genes. Documenting which ones exist in *V. inaequalis,* and the extent

which they confer resistance would be the priority once resistance appears. It would be important to consider cross-resistance of *V. inaequalis* isolates with differing *sdh* point mutations between the main SDHI fungicides. In addition, looking into fitness costs associated with each mutation would be helpful in understanding persistence of resistance in orchards. Furthermore, the work for chapter 2 on quantitative and multi-fungicide resistance can be elaborated on to better understand the mechanism for these shifts in sensitivity, as well as the quantitative shifts seen in chapter two. This could give better insight in how to manage these populations. Experiments that could address this may involve use of RNAseq analysis of *V. inaequalis* in response to flooding with different fungicides to better understand fungal response. This could answer questions on if and how detoxification is occurring. Finally, the work done in chapter 4 could be continued to better understand the ability of biopesticides to serve a role in fungicide resistance management over the years. Other management plans that could be further explored include low-spray management plans and management plans that incorporate more or different single-site fungicides and biopesticides.

In conclusion, this work has contributed to the body of knowledge surrounding SDHI fungicide use and development of fungicide resistance. Fungicide resistance to single-site fungicides will inevitably be a continual problem in high fungicide input crops, therefore continual research in this area is crucial as well as understanding status of resistance and new products in development.

APPENDIX

APPLE DISEASE EXTENSION AND OUTREACH

Throughout my graduate experience at Cornell, I have participated in and developed considerable extension and outreach programming. These efforts have been some of the most rewarding experiences of my doctoral training. All of my research projects have immediate translational aspects, a fact that I have greatly enjoyed, as it has led to more immediate translation to practice with positive impacts on growers' livelihoods. Presentations at grower's conferences and expos, written blog posts/columns, extension fact sheets, and elementary/middle school educational programs have led to hundreds of stakeholder contact hours with educational enrichment and impacts on sustainable apple production. The active learning goals of this programing ranged from increased knowledge on plant pathogens and plant pathology to specific recommendations for applied chemical, biological, and cultural disease management, in order to best identify practices for fungicide resistance management and reductions in the spread of antimicrobial resistance. The following vignettes are examples of some of my written extension work directed to help the apple industry of New York with basic apple disease biology and management. These include a NYS IPM apple scab fact sheet (Figure A1-A3) and different blog and publication outlets used to create disease forecasting alerts and management recommendations to apple growers across the state of New York (Figure A4-A6). In addition to my extension work, I have also had a high interest in education outreach with the local community. These activities were coordinated through programs like the Graduate Student School Outreach Program (GRASSHOPR), Expanding Your Horizons (EYH), and Science, Technology, Engineering, and Math (STEM) nights with the local public-school systems.

Audiences ranged from elementary to high school students (with an indirect extension to parents) with learning goals broadly focused on increased exposure to different career paths in the sciences, more specifically focused on plants and plant microbes. Through these outreach events, I have helped create foundational educational materials and experiments that can be used by graduate students in the future.

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New York State Integrated Pest Management Program | Cornell Cooperative Extension

TREE FRUIT

Apple Scab

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Apple scab, one of the most devastating fungal diseases of apple, occurs worldwide, wherever apples are grown. The fungal pathogen, *Venturia inaequalis*, can also infect crabapple, hawthorn, mountain-ash, firethorn, and loquat.

Symptoms

On leaves, initial symptoms will appear as light, olive green lesions that become velvety as the fungus develops conidia, the asexual infectious spores. With time, lesions may darken to brown and become bumpy as symptoms progress. Depending on apple cultivar and other factors, scab lesions can vary in their appearance on leaves (Fig. 1). On fruit, lesions start out small and slowly enlarge, similar to symptoms on leaves, sporulating with conidia that contribute to secondary infections. As fruit mature, lesions may crack, deform the fruit, and contribute to reduced storage life and rots (Fig 2).

Disease cycle and epidemiology

Cool, wet weather favors apple scab infections, and, therefore, this disease is more problematic in temperate climates. Initial infection requires 6 to 23 hours of leaf wetness when temperature ranges between 42°F and 75°F, with colder temperatures requiring longer durations of leaf wetness. Symptoms will appear approximately two weeks after initial infection when conducive weather conditions prevail.

Primary apple scab infections are caused by ascospores, the spores resulting from sexual reproduction. Ascospores develop in pseudothecia, microscopic fungal structures that grow from the previous year's scab lesions on the overwintering leaf litter in the fall, winter, and spring. In spring, ascospores are forcibly discharged from the pseudothecia, in coordination with apple tree growth.

Figure 1. Symptoms of apple scab on leaves showing early, light olive-green symptomatic lesions (left), brown sporulating lesions (center), and darkened, raised lesions (right). Photos: K. Ayer, Cornell University.

Figure A1. Apple scab fact sheet (page 1). https://ecommons.cornell.edu/handle/1813/43072.2

Figure 2. Symptoms of apple scab on fruit showing symptomatic lesions on developing fruit (left), sporulating, expanding lesions as fruit matures (center), and advanced lesions resulting in cracked fruit (right). Photos: K. Ayer, Cornell University.

From green tip through tight cluster until shortly after petal fall, ascospores continue to mature and release with rainfall. Wind disperses ascospores to the susceptible young leaves and young fruit of apple trees to cause primary infections.

Apple scab lesions produce a superficial olive green to almost black, microscopic layer of conidia (Figs. 1 and 2). Conidia cause secondary infections on newly developing leaves and fruit during wetting events. Both primary and secondary apple scab lesions give rise to conidia. The repeated cycles of secondary infections throughout the growing season can, if unmanaged, result in high levels of apple scab on leaves and fruit. Infected leaves may drop prematurely and heavily infected trees may defoliate, reducing vigor and potentially decreasing return bloom. Infected leaves in the leaf litter will serve as sources of inoculum in the following season, developing pseudothecia that will overwinter and mature to form ascospores in the spring (Fig 3).

Management

Based on the disease cycle of apple scab, management should aim to limit primary (ascospore) infection as well as reduce overwintering inoculum. Orchard scouting in the fall can provide useful insight to better inform management decisions. An integrated management approach incorporating cultural tactics, resistant varieties, and fungicides to prevent primary infection is the most sustainable and effective at managing this disease and achieving season-long control.

Cultural practices to reduce overwintering inoculum include leaf shredding with a flail mower and urea applications to fallen leaf litter. These will increase the rate of decomposition of overwintering leaf litter and reduce carryover inoculum. To create a less conducive environment for apple scab, prune trees to create an open canopy. This will increase air circulation, hasten drying time and reduce leaf wetness time, as well as improve fungicide spray penetration into the tree canopy, all important factors in reducing apple scab infection in the spring.

Cultivars of apple trees vary in their susceptibility to apple scab. Highly susceptible cultivars include cvs. Jersey Mac, McIntosh, Cortland, and Jonagold while more moderately susceptible cultivars include cvs. IdaRed, Gala, Crispin, and Northern Spy. 'Honeycrisp' is relatively resistant to apple scab. Resistant apple cultivars include cvs. Enterprise, Liberty, William's Pride, Prima, Redfree, Goldrush, Dayton, Pristine, and CrimsonCrisp. Breakdown of host resistance by the emergence of *V. inaequalis* strains that can overcome the main resistance gene used in apple breeding has been reported, especially in areas that rely almost exclusively on host resistance to manage scab.

Use of fungicides to prevent apple scab infection of fruit is essential when growing susceptible cultivars. The most effective fungicides include single-site products belonging to the succinate dehydrogenase inhibitors, quinone outside inhibitors, and demethylation inhibitors (Fungicide Resistance Action Committee (FRAC) groups 7, 11, and 3, respectively). To prevent the emergence of fungicide resistance in *V. inaequalis* and conserve the efficacy of these products, rotate the modes of action or FRAC groups according to label directions. Other tactics

Figure A2. Apple scab fact sheet (page 2). https://ecommons.cornell.edu/handle/1813/43072.2

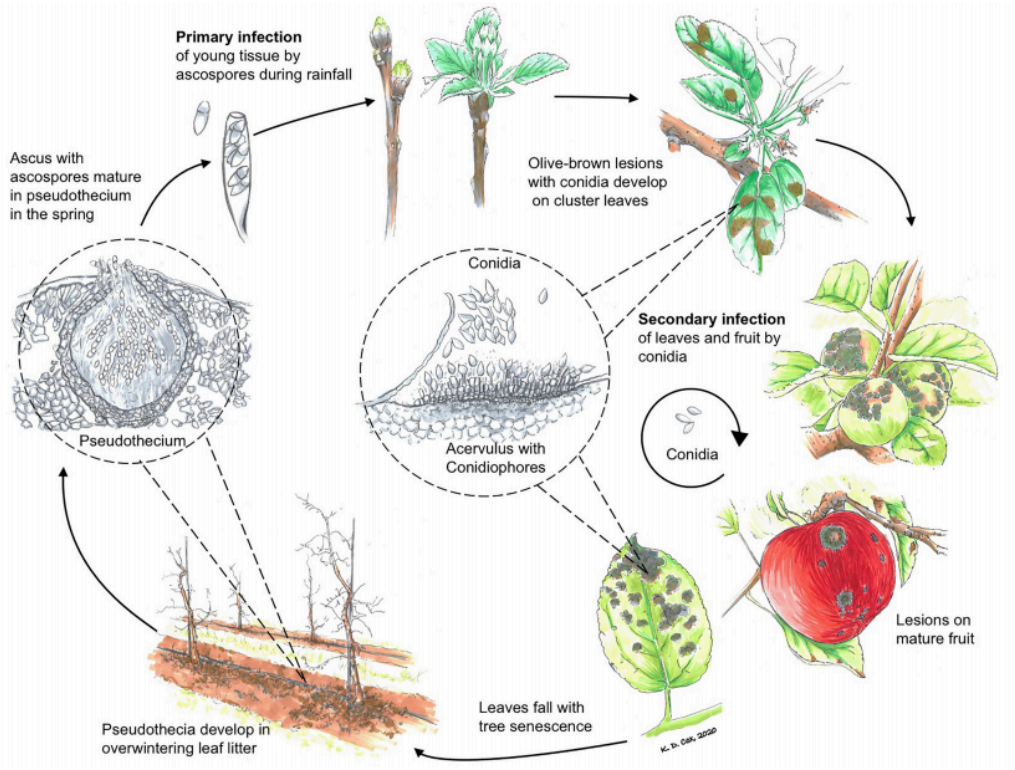

Figure 3. Apple scab disease cycle for *Venturia inaequalis* on apple. Illustration: K. Cox, Cornell University.

to prevent fungicide resistance include applying a mixture of two single-site fungicides or applying a single-site fungicide with a multi-site, protectant fungicide. Organic management options include lime-sulfur, organic copper, or biopesticide products.

Applications may be required throughout the growing season from green tip through cover sprays on highly susceptible cultivars, integrated with cultural practices to achieve adequate control. The apple scab ascospore maturity model and the infection risk disease forecasts on the Network for Environment and Weather Applications (NEWA) online at newa.cornell.edu can be used to ensure appropriate timing of fungicide applications. Refer to the Cornell Pest Management Guidelines for Commercial Tree Fruit Production for specific fungicide products and application timing for managing apple scab, as many products exist with many different chemical modes of action.

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Figure A3. Apple scab fact sheet (page 3). https://ecommons.cornell.edu/handle/1813/43072.2

DISEASES

Weekly Apple Scab Update for NY (5/18 to 5/23/20)

Below are apple scab predictions for NY apple regions based on the NEWA disease forecast system (http://newa.cornell.edu/index.php?page=apple-diseases). Information is kept concise. Alerts will also be posted to Twitter @FruitPathology with updates occurring throughout the week, which would allow notifications to send to mobile device. The various outputs are explained below table.

	Hudson Valley	Finger Lakes	Wayne County	Niagara County	Champlain Valley
Infection Predicted	NONE	LOW May 18th (Current)	LOW May 18th (Current)	LOW May 18th (Current)	NONE
Maturity	100%	90%	81%	70%	63%
Discharge	97%	6%	7%	9%	27%

* Predictions are regional; the model works best under local conditions. Always check weather and crop stage before making a management decision.

Infection predicted:

- **"Low"**: <10% ascospores discharged; **"Moderate"**: 10-20% ascospores; **"High"**: >20% ascospores discharged; **"None"** – no infection predicted for the week;
- **"Date"**: An infection event is predicted for the date listed. If a multi-day infection event is predicted, the first full date of the infection will be listed

Ascospore maturity: The ascospore maturity during the predicated infection event. If no infection event is predicted, the maturity by the end of the week is listed.

Discharge: The percent ascospore discharge during the predicted infection event(s). If no infection event is predicted, the cumulative ascospore discharge by the end of the week is listed.

Weekly Blossom Blight Update for NY (5/18 to 5/23/20)

Below are blossom blight predictions for NY apple regions based on the NEWA disease forecast system (http://newa.cornell.edu/index.php?page=apple-diseases). Information is kept concise. Alerts will also be posted to Twitter @FruitPathology with updates occurring throughout the week, which would allow notifications to send to mobile device. The various outputs are explained below the table.

	Hudson Valley	Finger Lakes	Wayne County	Niagara County	Champlain Valley
Infection Risk	HIGH May 18th (Current)	MODERATE May 23rd	LOW May 23rd	LOW May 23rd	HIGH May 23rd
Highest EIP	120	86	22	21	118
Highest 4-Day DH	490	355	152	142	489

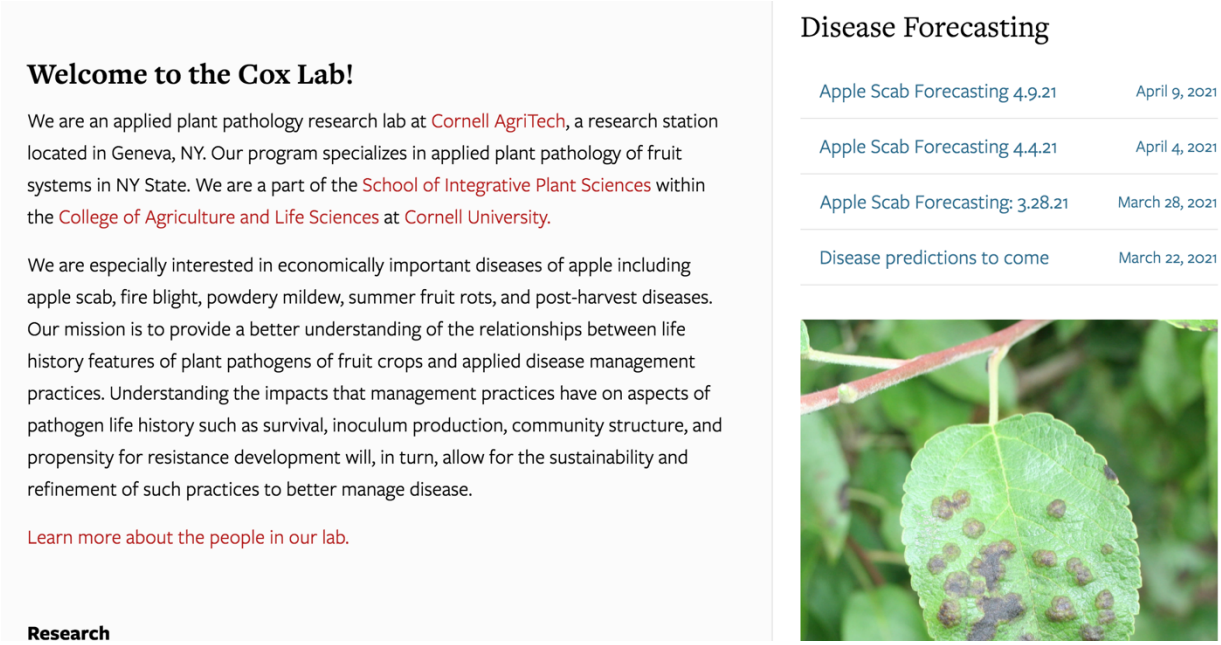
* Predictions are regional; the model works best under local conditions. Always check weather and crop stage before making a management decision.

Infection risk:

- **"Low"**: EIP and 4-day DH accumulation at/below 75 and 300, respectively; **"Moderate"**: EIP and 4-day DH accumulation between low and high-risk values; **"High"**: EIP and 4-day DH accumulation at or above 100 and 400, respectively with moisture predicted. **"None"**: little to no risk predicted for the week;
- **"Date"**: The date of highest risk for the week is listed.

Highest EIP & 4-Day DH: The highest EIP value and 4-day DH accumulation for the week is listed.

Figure A4. Screenshot of disease predictions for the weekly Scaffolds publications. http://www.scaffolds.entomology.cornell.edu/2020/SCAFFOLDS-5-18-20.pdf



Welcome to the Cox Lab!

We are an applied plant pathology research lab at Cornell AgriTech, a research station located in Geneva, NY. Our program specializes in applied plant pathology of fruit systems in NY State. We are a part of the School of Integrative Plant Sciences within the College of Agriculture and Life Sciences at Cornell University.

We are especially interested in economically important diseases of apple including apple scab, fire blight, powdery mildew, summer fruit rots, and post-harvest diseases. Our mission is to provide a better understanding of the relationships between life history features of plant pathogens of fruit crops and applied disease management practices. Understanding the impacts that management practices have on aspects of pathogen life history such as survival, inoculum production, community structure, and propensity for resistance development will, in turn, allow for the sustainability and refinement of such practices to better manage disease.

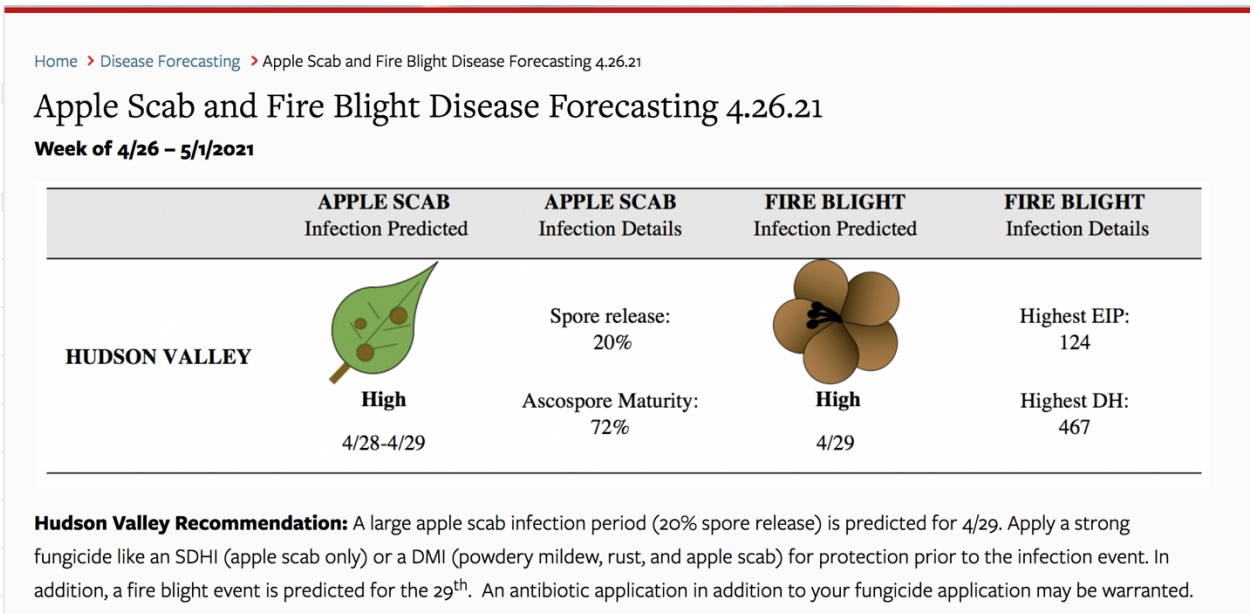
Learn more about the people in our lab.

Research

Disease Forecasting

Apple Scab Forecasting 4.9.21	April 9, 2021
Apple Scab Forecasting 4.4.21	April 4, 2021
Apple Scab Forecasting: 3.28.21	March 28, 2021
Disease predictions to come	March 22, 2021

Figure A5. Screenshot from the Cox Lab Blog with disease forecasting updates posted weekly for both apple scab and fire blight during peak management season.
https://blogs.cornell.edu/coxlab/



Home › Disease Forecasting › Apple Scab and Fire Blight Disease Forecasting 4.26.21

Apple Scab and Fire Blight Disease Forecasting 4.26.21

Week of 4/26 – 5/1/2021

	APPLE SCAB Infection Predicted	APPLE SCAB Infection Details	FIRE BLIGHT Infection Predicted	FIRE BLIGHT Infection Details
HUDSON VALLEY	**High** 4/28-4/29	Spore release: 20% Ascospore Maturity: 72%	**High** 4/29	Highest EIP: 124 Highest DH: 467

Hudson Valley Recommendation: A large apple scab infection period (20% spore release) is predicted for 4/29. Apply a strong fungicide like an SDHI (apple scab only) or a DMI (powdery mildew, rust, and apple scab) for protection prior to the infection event. In addition, a fire blight event is predicted for the 29th. An antibiotic application in addition to your fungicide application may be warranted.

Figure A6. Example of the apple scab disease forecasting and recommendations provided weekly. This was done for the Champlain Valley, Wayne County, Niagara County, Finger Lakes, Capital Region, Hudson Valley, and Long Island.
https://blogs.cornell.edu/coxlab/2021/03/28/apple-scab-disease-forecasting-week-of-3-28-21/