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Influence of Repeated Field Applications of Azoxystrobin on Population Diversity of Monilinia Fructicola

Madeline Elizabeth Dowling
Clemson University

INFLUENCE OF REPEATED FIELD APPLICATIONS OF AZOXYSTROBIN ON POPULATION DIVERSITY OF *MONILINIA FRUCTICOLA*

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by
Madeline Elizabeth Dowling
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Accepted by:
Dr. Guido Schnabel, Committee Chair
Dr. Paula Agudelo
Dr. Julia Kerrigan

ABSTRACT

Reduced-risk fungicides are a major component of modern IPM programs, but their site-specific mode of action makes them vulnerable to resistance development. Assessing the resistance risk of a fungicide is critical to establishment of sustainable use protocols for the practitioner. Several controlled laboratory studies indicate that fungicide-induced mutagenesis may be an underappreciated trait in resistance risk assessment. In the brown rot fungus *Monilinia fructicola*, microsatellite instability and transposon movement resulted after prolonged exposure to the fungicide azoxystrobin *in vitro*. In this study, azoxystrobin or propiconazole fungicides were applied weekly to nectarine trees for two years between bloom and harvest, and fungal isolates were investigated for phenotypic and genotypic changes. Results showed no evidence of fungicide-induced microsatellite instability or reduction of sensitivity to fungicides used in the study or to unrelated chemical classes, indicating that fungicide-induced mutagenesis may not occur in field populations as readily as it does *in vitro*. Further research examining larger portions of the genome may be necessary to ascertain the significance of fungicide-induced mutagenesis to fungicide resistance risk assessment.

DEDICATION

I would like to dedicate this work to my Lord and Savior Jesus Christ. Because of Him my life becomes more exciting every day. I can't wait to see what He has for me next. I would also like to dedicate this work to my family: my parents, brother, and grandfather who loved, motivated, encouraged, inspired, and sacrificed to make it possible for me to be where I am today.

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CHAPTER ONE

LITERATURE REVIEW

Brown Rot on Stone Fruits

Stone fruits (*Prunus sp.*) are important agricultural crops consisting of peaches, nectarines, plums, prunes, sweet and sour cherries, and apricots (72). In 2008, the world production value of stone fruits was over 13 billion USD (58). According to the most recent report of the United Nations Food and Agriculture Statistics Division in 2011, the world's top three stone fruit growers, China, Italy, and the United States, produced over 20 million tons of stone fruits together. The most widely grown stone fruits in the world are peaches and nectarines, and the United States generates over 1.2 million tons of these each year (1). Within the United States, California, South Carolina, and Georgia produce the most peaches and nectarines (38). Many diseases such as brown rot, peach scab, bacterial spot, *Armillaria* root and crown rot, fungal gummosis, peach tree short life, phony peach disease, and plum leaf scald threaten stone fruit production (72). One of the most commercially important of these diseases is brown rot (7, 72, 79). This literature review focuses primarily on brown rot of peaches and nectarines in the Southeastern United States.

Pathogen distribution. The first description of a brown rot pathogen was in written by Persoon in 1796, and brown rot has been well documented in Europe and the United States for over 150 years (17, 79). It was later discovered in South America, Japan, and Australia (17). Several *Monilinia* species can cause brown rot, but *Monilinia fructicola* and *Monilinia laxa* are the most common and are distributed worldwide (4, 69, 95). *M. laxa* can be found in Europe and the west coast of the United States, but is not an important

causal agent of brown rot in the Southeastern United States. *M. fructicola* is a major pathogen in the Southeastern USA and California. Though it was introduced to California much later than *M. laxa*, it has become the major brown rot pathogen there (40, 69, 72). This could be due to the ability of *M. fructicola* to sexually reproduce (40). Sexual reproduction in *M. laxa* has rarely been documented (42, 44, 95).

Nomenclature and Taxonomy. The fungus *M. fructicola* is in the kingdom Fungi, phylum Ascomycota, class Leotiomycetes, order Helotiales, and family Sclerotiniaceae. The genus name *M.* comes from the Latin word for necklace, *monile*, because the conidia link together like beads on a string (44). The fungus was first classified as *Ciboria fructicola* by G. Winter in 1883. In 1906, Rehm moved it to the genus *Sclerotinia*, then Edwin Honey grouped it into the *M.* genus in 1928 (20, 79). Edwin Honey also placed *M.* species into two groups: Junctoriae and Disjunctoriae. *M. fructicola* is classed with the pathogenic Junctoriae which produce inoculum around host bloom time and cause blossom infections (43, 44). Technically, *Monilia fructicola* was the asexual form of the fungus. However, in 2011, when the Botanical Code was changed to allow only one name for each fungus, *Monilinia fructicola* was chosen as the name for both asexual and sexual stages of the fungus. (70, 74, 94). The common names "American brown rot," "fruit brown rot," and "peach brown rot" are used to distinguish *M. fructicola* from *M. laxa*, the "European brown rot" (40).

Morphology. *M. fructicola* conidia grow from sporodochia when a hyphal tip expands and a septation forms between the hypha and the newly formed conidium. Another conidium grows out of the first until a chain of conidia forms. Two conidia may

grow from a single parent conidium to create a branch in the chain (37). Conidia are ellipsoid, tapering at the ends in a lemon-like shape with papillae on both ends (4, 88). Typically, spores are blastic, forming in chains at the tip of hyphae, though they can also be arthric, forming when mycelia break apart (2). *M. fructicola* does not contain disjunctors, or spacers, between conidia. As conidia increase in size, pressure builds up between them until they begin to separate from each other (94). Dry weather conditions and disturbances such as wind allow them to completely separate and disseminate (44).

Conidia are multinucleate and the number of nuclei determines the conidial size. Because the number of nuclei decreases with increasing temperature, size is variable, but generally ranges from 12-16 x 8-11 µm (4, 68, 88). While forming in chains, nuclei pass from the first conidia into those later formed through septal pores. Conidia often have four to 10 nuclei, the last to form typically having the fewest nuclei and those first formed containing the most. Conidia typically form a single germ tube, though this may vary among isolates. After conidial dispersal and germ tube formation, nuclei enter the germ tube, but the number of nuclei in the conidium stays fairly constant due to synchronous mitotic division of nuclei (37).

M. fructicola hyphae can be hyaline or pigmented and are septate and multinucleate (69). Primary hyphae are thin-walled and typically grow to be 250 µm or longer with a width of 7 to 10 µm (4). Each septation contains a pore through which nuclei can pass and nuclear movement between hyphal cells has been observed. Hyphal cells often contain up to 40 nuclei. Anastomosis frequently occurs between hyphae at all life stages. *M. fructicola* meets every requirement for heterokaryosis to occur between isolates, though

heterokaryosis has not been widely documented (37, 80). Mycelia can form a network within plant material to form a sclerotized stromata from which apothecia and sporodochia can develop (17, 42, 94, 95).

Apothecia vary in size and proximity to other apothecia (43). Typically there are fewer than 25 apothecia on each mummy (17). Initially, they possess light brownish-grey stalked fruiting bodies tipped by a cup which contains the hymenium surface inside it. The stipe can range from 0.4 to 8.0 cm long. Over time, the cup flattens into a disk, turning dark brown and then black (17, 88). Asci are inoperculate, clavate, and contain unbranched paraphyses and 8 aseptate hyaline ascospores (17, 43, 79, 93).

Disease Cycle and Epidemiology of *Monilinia fructicola*

Below is a summary of the general disease cycle of the *M. fructicola* pathogen. The life cycle varies by geographic location, but this review focuses on the life cycle in the Southeastern United States.

Asexual phase of disease cycle. The asexual form of *M. fructicola* overwinters in sclerotized mummies, peduncles, blighted blossoms, and cankers (40, 44, 87, 101). Sporodochia form on these plant structures and bear greyish-brown conidial masses that become the primary inoculum that infects blossoms (17, 72). However, in the southeastern states, conidia are rarely the primary inoculum (61). Secondary sources of inoculum are conidia forming on new cankers, blighted blossoms, and rotten fruit. Fruit infections are formed when the fungus enters through wounds, trichome sockets, and stomata (40). *M. fructicola* conidia may also utilize appressoria to form latent infections in unripe fruit (62).

Conidia are spread by wind, rain, and insects and may survive for months if they are not scorched by the sun (40).

Sexual phase of disease cycle. Ascospores constitute the major primary inoculum of brown rot in the southeastern states (61). Fruit mummies on the orchard floor typically develop apothecia when the host begins to blossom (40, 44). In one New Zealand study, apothecia appeared 4 days after the first buds began to open. Apothecia formation is expedited when mummies are partially covered with moist soil and shaded by weeds, though filtered light is necessary for hymenium development (95). Exposure to direct sunlight, high temperatures, or low humidity, causes shriveling and disintegration of apothecia, though they may revive during cooler night temperatures and release ascospores (88). Apothecia deteriorate several weeks after their formation, generally releasing all spores within one week. (40, 45).

Changes in humidity or direct contact with water droplets initiates release of ascospores from their ascocarp, then wind or water carries them to infection courts (40). A single apothecium has been observed to discharge 37 million ascospores, and apothecia develop at different rates on a single mummy, providing a continuous stream of ascospores during bloom under the right conditions (45). Near the end of bloom, ascospore production decreases while conidia on blighted blossoms increase until the conidia become the major source of inoculum for future blights (88).

Influence of weather on disease cycle. Blossom blight is most likely when temperatures are mild with high humidity, high precipitation, and cool night temperatures (4). Fruit rot is favored by high humidity and precipitation, though precipitation is not

required for fruit rot to occur (40). Apothecial formation is favored by significant early bloom precipitation. Optimum temperatures for conidia formation vary by isolate, but generally lie between 15°C and 30°C (69), while ascospores favor temperatures of 15-20°C for discharge and can still discharge spores within the range of 7-25°C (45). Spread of conidia is favored by cycles of high humidity followed by low humidity, high temperature, and high winds to disseminate the conidia (40, 94).

Symptoms and Signs. The major symptoms of brown rot are blossom blight, twig cankers, and fruit rot. Blossom blight is initiated by conidia or ascospores that can infect any flower part: petals, stamens, pistil, and sepals (17). Browning anthers or necrotic lesions first indicate the presence of blossom blight three to six days after infection (40, 72). As mycelia grow and spread throughout the flower tissue, they form a meshwork of stroma that encompasses the entire flower and eventually can grow through the pedicel into the branch. In rainy weather, sporodochia burst through the blossom surface and produce conidial tufts (4, 17, 43). In wet weather, blossoms become rotten and soft, while in dry weather they become fragile and brittle. Chemicals released by the fungus prevent the blossom from dropping from the branch and often the blossom becomes attached to the surface of the branch with a gummy exudate (40, 72, 87).

Twig cankers form when the fungus enters a twig through the petiole of a blighted blossom or a fruit spur. The primary phloem of the branch becomes obstructed with *M.* conidia and a gummy exudate that is often released onto the surface of the branch (17, 87). If the cankers form in large branches, they can cause severe stress to the tree that may result in secondary infection and tree death (69). However, cankers are more commonly found

on small twigs that can become girdled at the location of the canker and die from the canker location to the tip of the branch (40, 72). Cankers can be as long as 90 cm and are generally more severe when they form from fruit infection than from blossom infections (87). Cankers are generally a reddish-brown color and are often sunken into the branch (4, 40, 69).

Fruit rots typically occur in mature fruit, and lesions begin as small brown spots that rapidly grow and become softer as the infection progresses (72, 79). Sometimes the rot does not appear until 48 hours after infection, depending on weather conditions. Conidia form in the lesion as soon as 24 hours after the infection begins to show and generally the whole fruit becomes covered with conidia and rot (79). Eventually, this fruit will turn into a mummy (72). As in blossom blight, chemicals released by *M.* anchor the fruit onto the tree and prevent fruit drop. Mummies may remain attached to the tree for several years, providing a secondary source of inoculum (92). Latent infections can occur in immature fruit and eventually cause post-harvest rots (25).

Control of *Monilinia fructicola*

Commercial importance of *M. fructicola*. Brown rot has high commercial importance in the stone fruit industry. As recently as the 1950s, a single rain during fruit maturation could destroy the produce in an entire orchard because of the high incidence of pre and post-harvest brown rot (72, 79). In China, postharvest rots are so serious that some Chinese packaging companies have reported 100% incidence of postharvest rot on their peaches (28). In 2000, a survey of several South Carolina and Georgia orchards listed fruit

rot incidences as high as 32% for orchards controlled using standard commercial protocol, while postharvest incidences were as high as 90% (25). It is very rare to find an orchard with no brown rot. Trees must be planted a minimum of 400 m from any inoculum source for them to be partially safe from infection without fungicide use. Even in this case, some blossom blight will most likely occur (98).

In organic orchards, brown rot is more severe than in conventional orchards (7). Even with the use of copper and sulfur fungicides registered for organic use, the disease incidence is often higher than 75%. In the southeastern United States, organic peach growing is very risky because of the high incidence of brown rot and the often perfect conditions for infection that exist during the summers (69).

Methods of brown rot control. There are several methods available for brown rot control, including cultural, biological, and chemical control. Plant resistance to brown rot has not been effectively bred into any peach cultivar yet (4, 40). The most commonly used cultural methods to control the disease consist of mummy removal, pruning of infected twigs, removal of decaying fruit, and removing alternative hosts. These methods are generally very helpful in reducing inoculum, though the effectiveness of each method depends on geography. Most of these methods are used effectively by growers to decrease inoculum, but high disease incidences still may occur in orchards that use the appropriate cultural controls (98).

Several biological control options for *M. fructicola* have been explored experimentally, but none have high efficacy in field conditions (77). Under laboratory conditions, *Epicoccum nigrum*, *Bacillus subtilis*, *Cryptococcus* spp., *Aureobasidium*

pullulans, Rhodotorural sp. nov. and S*poridiobolus papraroseus* showed some efficacy against postharvest brown rot (46, 52, 77). One of the most effective biocontrol products registered for brown rot to date is Serenade Max, with *Bacillus subtilis* as the active ingredient. It is able to prevent canker formation as much as normal chemical control, but is unable to inhibit fruit rot to the same extent as a fungicide (60).

Chemical control of *M. fructicola* is the most effective method for controlling brown rot. There are several fungicides registered for use on *M. fructicola* in the southeastern United States. The most commonly used classes of fungicides are the methyl-benzimidazole carbamate fungicides (MBC), demethylation inhibitor fungicides (DMIs), and quinone outside inhibitor fungicides (QoIs) (47). Several other fungicides such as sulfur fungicides and captan are registered for brown rot control in the southeast, but they are not widely used because of their inability to control pre-harvest rots. Also, sulfur fungicides can lead to acidification of soil and late season captan applications have been associated with inking on peaches (47, 98). Fungicide resistance is also a critical concern even though fungicides are assessed for risk of resistance development before they enter the market. Manufacturers must test multiple factors including mode of action, cross resistance, selection for resistant isolates, and stress-induced resistance mutations (54). However, resistance still often develops to fungicides meeting all resistance risk criteria.

MBC fungicides were the first truly effective fungicides used on brown rot (98). Their mode of action is to block beta-tubulin formation to halt cell division by preventing microtubule formation, similar to the poison colchicine (21, 76). They were first registered in the 1970s and were widely used until 1982, when resistance became so widespread that

they were removed from the list of fungicides available for control in South Carolina (100). Resistance is based on point mutations located in the beta-tubulin gene of *M. fructicola* (67). After resistance became a significant issue in the 1980s, MBC fungicides from the thiophanate group have been used less frequently (47).

The DMI fungicides were introduced in the 1980s and became the most important fungicides for brown rot control after MBC fungicides were rendered ineffective by resistance development (99). They are highly effective and, along with QoI fungicides, became one of the top selling fungicide classes, making approximately 50% of total worldwide fungicide sales (39). In 2006, they were considered the most important agricultural fungicide class (48). The DMI fungicide mode of action is to inhibit a precursor of sterol synthesis and prevent formation of the fungal cell membrane (33). The DMI fungicides most commonly used for *M. fructicola* control are propiconazole, fenbuconazole, and tebuconazole (47). DMIs are also prone to resistance development and resistance is often due to overexpression of the 14-α-demethylase (CYP51) gene (64, 67). However, DMI fungicides can overcome resistance with higher field rates and are still useful where resistance has not yet developed (13). Rotating with sulfur or respiration inhibitor fungicides is recommended to lower the risk of resistance development (41).

QoI fungicides, also known as strobilurin fungicides, were first discovered in 1977 and then modified to prevent toxicity to mammals and sensitivity to light (9, 39). They were released for use in 1996, and by 2002, the QoI fungicide azoxystrobin was the top-selling fungicide worldwide (10, 78). The QoI mode of action is to block cellular respiration at the cytochrome bc1 complex Qo (Quinone oxidation) site (10, 78).

Strobilurin fungicides are highly effective, and azoxystrobin and several others are considered "reduced-risk fungicides" by the Environmental Protection Agency (10). Reduced-risk fungicides are less toxic to mammals and non-target organisms compared to many older products. However, they generally have a single-site mode of action that allows resistance to develop quickly. QoI fungicides can undergo translaminar movement and flow through plant cells to cover cuticular surfaces of a plant on which they are applied.

Because QoI fungicides have a single-site mode of action, resistance has become prevalent in many fungal pathogens. The G143A mutation in the cytochrome b gene (*cyt b*) causes qualitative resistance to QoI fungicides, and two other point mutations are able to reduce the sensitivity of fungal pathogens to QoI fungicides (49). However, in *M. fructicola*, the G143A mutation may inhibit splicing of an intron just downstream of the G143 site, and lead to production of non-functional *cyt b* mRNA (65). Because of this, the G143 mutation is unlikely to develop in *M. fructicola*.

Stress-Induced Mutagenesis

Mutagenesis is defined as the spontaneous introduction of mutations into DNA (3). It can be random or preferentially target specific areas of the genome (23). Scientists often employ mutagenesis artificially to knock out genes, but natural mutagenesis occurs in response to environmental or chemical stresses (5). Stress-induced mutagenesis has been well documented in bacteria, fungi, plants, and animals (11, 71, 85). A variety of stresses such as ionizing radiation, oxidative stress, temperature stress, starvation, and proteotoxic stress may initiate mutagenesis in an organism (11, 12, 14, 53). One of the most important

of these is oxidative stress because of the availability of reactive oxygen species (ROS) in most cells (34, 66).

Oxidative stress-induced mutagenesis. Oxidative stress is caused by reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radicals, hydroxyl radicals, and singlet oxygen. These mutagenic compounds are natural byproducts in every living organism that generates ATP using the electron transport chain. However, ROS are normally balanced by antioxidants and enzymes such as catalase and superoxide dismutase that convert them into harmless compounds. When this balance is interrupted, oxidative stress occurs (66). Oxidative stress inhibits important cellular functions such as RNA splicing, DNA repair, and DNA methylation (26, 32). It also causes microsatellite instability, transposon jumping, single and double strand breaks, DNA crosslinking, telomere shortening, and insertion and deletion mutations (26). Two characteristic results of oxidative stress are transposon movement and microsatellite instability.

Transposons are present in all organisms to some extent. There are two classes of transposons that move through the genome in different ways. DNA transposons (Class II elements) move by cutting themselves out of the DNA using the enzyme transposase and then reinserting themselves in another location in the genome. Retrotransposons (Class I elements) use reverse transcriptase to copy and paste themselves throughout the genome by forming an RNA intermediate that reverse transcribes itself into DNA. The majority of transposons in the genome are inactive. However, there are active transposons present in many organisms. When inserted into promoter or coding regions, they can silence genes or adjust expression patterns (82, 97).

Oxidative stress can induce transposition. One example is the movement of multiple transposable elements in the rice blast fungal pathogen *Magnaporthe oryzae* after exposure to copper fungicides. The transposons activated by copper stress were different from those activated by heat stress, indicating that the cell may distinguish between stresses to determine which mutagenic pathway to undergo (18). In the bacterial pathogen *Burkholderia cenocepacia*, exposure to ROS resulted in transposon movement in 6 out of the 7 isolates studied (22).

Microsatellites can also be significantly affected by oxidative stress-induced mutagenesis. Microsatellites are present in all eukaryotes, and consist of repeating segments typically 20 to 60 bp long. The repeating segments can be 2 to 5 bp long, and are found throughout the genome (24, 97). One of the most important factors about microsatellite loci is their high polymorphism caused by polymerase slippage during replication. Because the segments repeat, DNA polymerase may miss one or more repeats, "slipping" to shrink or expand the length of the microsatellite region. These slips are typically fixed by mismatch repair (MMR) and sometimes by transcription coupled repair systems (81, 90).

Oxidative stress can inhibit mismatch repair (MMR). For example, sublethal doses of hydrogen peroxide induced stress can inactivate two out of the three major complexes involved in MMR (26). Inhibition of mismatch repair causes high instability in microsatellite regions. In one study of human cell lines, 28.4% of MMR deficient clones altered at microsatellite regions while wild type cells remained unchanged over several generations (34). Even in cells with highly efficient repair systems, exposure to ROS can

result in a 10-fold increase in microsatellite frame shift mutations. In MMR deficient cells, hydrogen peroxide exposure can cause a 340-fold increase in microsatellite frameshift mutations (50).

In *M. fructicola*, oxidative stress-induced mutagenesis has been observed *in vitro* (83, 84). The QoI azoxystrobin produces ROS in fungi. When isolates of the fungus were exposed in 10 consecutive transfers to sublethal doses of azoxystrobin, movement of transposon *Mftc1* was observed. Microsatellite instability was also evidenced in the isolates treated with azoxystrobin as opposed to those treated with propiconazole or left untreated (83). This provides evidence that oxidative-stress induced mutagenesis may be triggered by respiration inhibitor fungicides.

Purpose of this study

Knowledge about potential stress-induced mutagenesis is important for the assessment of resistance risk to a fungicide. The goal of this project was to determine if field exposure to a sublethal dose of the QoI fungicide azoxystrobin can cause stress-induced mutagenesis in the fungus *M. fructicola.* The isolates used for this study were gathered from two orchards: one in South Carolina and one in Georgia from blossoms, cankers, and fruit in 2013 and 2014. The first part of the project focused on the population as a whole. Potential changes in genetic diversity on the population level were assessed over two years in two fungicide-exposed treatments using microsatellite data and compared with the control treatment. The second part of the project focused on mutagenesis of

individual isolates. Matching pairs from blossoms and cankers were identified and potential mutagenesis was assessed at 7 microsatellite loci.

CHAPTER TWO

INFLUENCE OF REPEATED FIELD APPLICATIONS OF AZOXYSTROBIN ON POPULATION DIVERSITY OF *MONILINIA FRUCTICOLA*

Introduction

Monilinia fructicola G. Winter (Honey) is the causal agent of brown rot on peach in the southeastern United States and one of the most devastating diseases impacting stone fruit growers worldwide (7, 72, 79). *M. fructicola* overwinters primarily in fruit mummies and twig cankers (40, 44, 87, 101). In spring, apothecia form on fallen fruit mummies and produce ascospores which may then infect blossoms (17, 61). The fungal mycelium in the blossom may then grow through petioles into the woody tissue of the twigs (87). Twig infections become cankers that often girdle the twigs and decrease the bearing area of the tree (40). Inoculum produced on blossoms and cankers spreads to green and maturing fruit, causing pre-harvest and post-harvest fruit rot that can destroy the produce of an entire orchard if uncontrolled (72).

Extensive chemical control is necessary each season to prevent heavy losses to brown rot (7). Two major fungicides used to control brown rot are the quinone outside inhibitor (QoI) fungicide azoxystrobin and the demethylation inhibitor (DMI) fungicide propiconazole. Representatives of these two fungicide classes have different modes of action and are often rotated to reduce the speed of resistance development to either class (47). QoI fungicides hinder ATP production by inhibiting electron transfer at the quinone oxidation (Qo) site in the cytochrome bc1 complex while DMI fungicides inhibit the biosynthesis of sterols that are essential to fungal cell membrane fluidity (10, 33). Resistance to propiconazole and reduced sensitivity to azoxystrobin have already

developed in some production areas of the southeastern USA and are an increasing concern for growers (47, 64, 65).

To prevent release of fungicides with high risk of resistance development, the fungicide resistance action committee (FRAC) has established guidelines for resistance risk assessment. Many traits correlated with resistance development, such as the FRAC mode of action, FRAC group, cross resistance, and resistant isolate selection must be analyzed before fungicides can be registered (15, 36). Fungicide-induced mutagenesis is not included on this list of traits, but recent research indicates that it may be an important factor in assessment of resistance risk (83, 84).

Exposure of mycelium to sublethal doses of QoI fungicides may induce mutagenesis and increase the risk of resistance development (83, 84). When respiration inhibitor fungicides are used, reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide can be produced (56). This has already been observed for the fungicide SSF126 which has a very similar mode of action to azoxystrobin (6). Multiple studies have already shown that oxidative stress caused by ROS can induce transposon movement, microsatellite instability, DNA double strand breaks, and RNA splicing inaccuracies in other fungi and bacteria (18, 26, 32).

Sublethal exposure of mycelium to fungicides occurs in the field due to incomplete fungicide coverage or dilution of fungicide concentrations in plant tissue. A sublethal dose may counteract resistance development by allowing more genotypes to survive and compete with resistant strains (91). In bacteria, the potential for a sublethal dose of antibiotic to accelerate resistance development through mutagenesis has been documented

(57). A recent *in vitro* study performed on *M. fructicola* showed that transposon movement and microsatellite instability occurred after 12 transfer cycles on medium amended with a sublethal dose of azoxystrobin, while no changes were observed in the same fungal isolates treated with propiconazole or left untreated (83, 84).

The goal of this study was to determine if a sublethal dose of azoxystrobin applied in the field could induce mutagenesis in *M. fructicola*. This was accomplished by examining changes in diversity at the population level and comparing the microsatellite regions of the isolates from the same clonal population before and after azoxystrobin and propiconazole application. Propiconazole was used as a negative fungicide control because it is not a respiration inhibitor and has not shown mutagenic effects *in vitro*. The results of this study should give insights into the potential of fungicide-induced mutagenesis under field conditions and help improve the assessment of fungicide resistance risk.

Materials and Methods

Setup of field trial and fungicide applications. All isolates were collected from treatment blocks of June Princess nectarines in Clemson University's Musser Fruit Research Farm, Seneca, SC and O'Henry peaches in the University of Georgia Horticulture Farm, Watkinsville, GA. The orchard plots used for this experiment were established in 2003 and fungicides, herbicides, and pesticides were applied according to standard commercial protocol before the experiment was begun. Treatment setup for both orchards is shown in Figure 1.1. Some experimental trees died during first year and were replaced with different trees from the same row in 2014. Fungicides were applied at half the rate of

standard commercial practice to ensure survival of some inoculum for analysis. An airblast sprayer was used to apply azoxystrobin (Abound Flowable, 22.9% azoxystrobin, Syngenta Crop Protection, Greensboro, NC) to the field at a rate of 420 g/hectare with 938 L of water/hectare and propiconazole (Tilt, 41.8% propiconazole, Syngenta) at a rate of 141 g/hectare with 938 L of water/hectare. When spraying between fungicide treatment and buffer rows, the nozzles facing the buffer rows were turned off to decrease fungicide contamination of control rows. A total of 12 sprays were applied to the fungicide treatment rows in 2013 and 10 sprays in 2014. No sprays were applied prior to collection of blossom samples, so blossom collection blocks will be referred to as pre-treatment blocks.

Isolate collection and single spore isolations. Isolates were collected from blossoms after petal fall in 2013 and 2014 (March/April) and then from fruit about 12 weeks later in July just before typical harvest time for June Princess nectarines. Only blossoms and fruit exhibiting obvious conidial tufts were used. In 2014, two weather-proof tags printed with the isolate name were attached to the branch on either side of the blossom before each blossom blight sample was collected to guide canker collection later in the season. Criteria for tagged blossom samples included that they be distant from other infected blossoms on the same branch, located on healthy branches, scattered through the tree canopy, and attached to branches with a diameter $\geq$ 1 cm. Nearby healthy blossoms were stripped off the branches to prevent labelling confusion. Spores were collected from symptomatic flowers using sterile, individually wrapped cotton swabs, and gently swirling the cotton tip in newly-formed spore masses. (Thermo Fisher Scientific, Waltham, MA). Five trees were picked from each experimental row and 20 isolates were collected from each of these trees.

The 20 isolates were collected at random from the canopy. Swabs were placed in freezer bags containing solid desiccant and refrigerated at 4°C to prevent premature germination until further use. For single-spore isolations, swabs were tapped over water agar plates and conidia landing on the plate were allowed to germinate in the dark at 22°C for 16 hours. Germinating conidia were removed under the microscope with a scalpel and placed on potato dextrose agar (PDA) in preparation for DNA extraction and permanent storage.

Twig cankers were collected from the Musser Fruit Research Farm on July 2, 2014 and from the UGA Horticulture Farm on July 10, 2014 after 10 and 12 weeks of fungicide sprays, respectively. Cankers ambiguously labelled due to tag sliding or tag loss were discarded. Branch segments (4 to 5 cm in length and about 1 cm in diameter) containing a single canker were sterilized in 10% bleach solution for 1 minute, rinsed for 1 minute in ultra-pure filtered water, then dried in a laminar flow hood on paper towels and placed in the labelled petri dishes. Each dish contained 9.0 cm in diameter filter paper #410 (VWR, Radnor, PA), which was saturated with ultra-pure water to create a moist chamber. The petri dish lids were left closed for 3 days and then propped slightly open to allow conidia formation. After one to two weeks, spores emerged from approximately 20% of the cankers. The remaining cankers were re-moistened with ultra-pure water and, three to five weeks after collection, spores were recovered from nearly 50% of the cankers. Spores were collected using sterile swabs and swabs were stored in a plastic bag with desiccant. Single spore isolations were performed as described above.

Disease Incidence. Approximately one week before spring and summer sampling in 2014, blossom blight and fruit rot disease incidence was determined. For blossom blight,

ten one-year-old branches of approximately the same length were chosen from each of 10 trees and the number of total blossoms and the number of diseased blossoms were counted. For fruit, 100 fruit were examined for all 10 trees in each treatment and the number of fruit symptomatic for brown rot was determined. No data were collected in 2013.

Sensitivity of isolates to various fungicides. The sensitivity of 50 isolates from each of the three field treatments (azoxystrobin, control, and propiconazole) to azoxystrobin, cyprodinil, propiconazole, and iprodione was assessed using discriminatory doses (table 2). Salicylhydroxamic acid (SHAM) was added to azoxystrobin-amended media to inhibit the alternative oxidase activity. Colony diameters were measured after 3 to 4 days of growth in the dark at 22°C.

DNA extraction. DNA was extracted using "the quick and safe method" (19) according to all recommendations except that sample DNA was washed with chilled ethanol and then centrifuged at 12,000 rpm for 3 minutes. The ethanol supernatant was discarded and residual ethanol was evaporated in a laminar flow hood until the pellet was dry. 50 µL TE buffer were added to the DNA, and the tubes were placed in a water bath at 45°C for several hours to ensure that the DNA dissolved. TE buffer was made by mixing a solution of 10mM Tris, pH 8.0 and a solution of 0.1 mM EDTA, pH 8.0. The DNA was then stored in the -20°C freezer.

Fragment sizing analyses. PCR was performed using the microsatellite primers and touchdown PCR method developed by Sydney Everhart (27). The primers used in this experiment were SED, SEF, SEI, SEL, SEN, SEP, and SEQ in Table 1.1. All primers were pig-tailed to prevent poly-A tail formation from interfering with sizing. The CAG fragment

was also added to one primer so that the fragment sizes would be comparable to those described by Everhart et. al. The major difference in the primers and the protocol used in this study is that the fluorophore FAM was attached directly to the primers instead of using the HEX-CAG complementary primer. Direct FAM attachment allows faster throughput of samples. The PCR program used was: an initial cycle of 95°C for 2 minutes and 30 seconds followed by a touchdown cycle repeated 20 times of 95°C for 20 sec, 60°C for 20sec with temperature decreasing by 0.5°C every cycle, and 72°C for 30 sec. The third cycle of 95°C for 20 sec, 50°C for 20 sec, and 72°C for 30 sec was repeated 15 times.

Fragment sizing and sample cleaning was performed in CORE laboratories at Arizona State University and at the Clemson University Genomics Institute. Sizing was performed on 5 samples and 5 loci at each genomics institute to ensure that error would not be induced by using two different machines, but both laboratories gave the same results for all 25 fragments.

Identification of Matching Pairs from Cankers and Blossoms. Isolates from blossoms and corresponding cankers were used for SSR analysis to determine potential change on the multiple-isolate level. To verify identity of complementary blossom and canker pairs collected in 2014, the Multi-drug resistance regulator (MRR1), dihydrofolate reductase (DHFR), and cytochrome P450 (P450) loci were sequenced. MRR1 was first sequenced and only isolate pairs matching at MRR1 were screened at DHFR and those matching at MRR1+DHFR were screened P450. Isolates from a blossom and corresponding canker with identical multi-locus genotypes were described as matching pairs. Primers are shown in Table 1.3. For MRR1, the PCR program began with an initial

treatment of 94°C for 5 minutes, then 40 cycles of 94°C for 30 seconds, 50°C for 1 minute, and 72°C for 2 minutes, and then a final step of 72°C for 10 minutes. The PCR program to amplify the DHFR fragment included an initial cycle of 95°C for 3 minutes; followed by a cycle repeated 34 times of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 1 minute. Last, there was an extension cycle at 72°C for 5 minutes. The program for amplifying the P450 region began with a cycle of 94°C for 3 minutes, then a cycle repeating 35 times of 94°C for 30 sec, 52°C for 40 sec, 72°C for 2 minutes, and then a final extension cycle of 72°C for 12 minutes. Sequencing was performed by CORE laboratories at Arizona State University. Sequences were assembled and compared using DNAstar software (DNAstar Inc., Madison, WI)

Analysis of microsatellite-derived fragment sizes. Fragment sizes were analyzed using Genemapper 4.0 (Life Technologies, Carlsbad, CA) software and also visually assessed to ensure accurate size calling. Statistical analysis of fragment sizes was performed using GenAlex 6.5 (75) to calculate haploid genetic diversity, number of multilocus haplotypes, and number of private alleles by population and by locus. The number of private alleles and multilocus haplotypes was determined for treatment populations of each year and each isolate source separately. The R program Poppr (55) was used to calculate the genotype accumulation curve to ensure that isolates were resolved from each other.

Results

Disease incidence. Disease incidence was determined before and after fungicide applications in 2014. Pre-treatment blossom blight incidences in azoxystrobin, control, and propiconazole blocks, were 29.4%, 23.9%, and 23.3%, respectively, and were not significantly different from each other according to the Tukey HSD test (α=0.05; p = 0.8). Disease incidences on fruit in azoxystrobin, control, and propiconazole treatment blocks were 5.3%, 38.3%, and 2.7%, respectively (Fig. 1.2). No significant difference in disease incidence was found between the azoxystrobin and propiconazole treatment (α= 0.05; p= 0.6), but disease incidence was significantly lower than in the control (α=0.05; p<0.0001). No disease incidence data were collected in experimental year 2013.

Assessment of Population-Level Phenotypic and Genotypic Response

Overall genotypic variation at five loci. For the population-level study, a total of 35 isolates were obtained from both blossoms and fruit of five trees (7 isolates per tree) of each treatment. Isolates of *M. fructicola* collected from blossoms and fruit in 2013 and 2014 were analyzed for changes in population diversity before and after application of azoxystrobin and propiconazole fungicides. All microsatellite loci (SEF, SEL, SEN, SEP, and SEQ) were polymorphic, with an average of 5.7 alleles at each locus and 3.7 effective alleles (the number of alleles weighted by frequency) at each locus. An average of 87.6% and 84.7% of the isolates from blossoms and fruit, respectively, were unique (not clones).

Sensitivity of field populations to fungicides. Relative growth on media amended with a single discriminatory dose was investigated to test the sensitivity of *M. fructicola*

isolates from all treatments to azoxystrobin and propiconazole as well as chemically unrelated fungicides iprodione and cyprodinil (Fig. 1.3). According to the LSD and AV tests, sensitivity to azoxystrobin (α=0.05, p=0.385), propiconazole (α=0.05, p=0.121), iprodione (α=0.05, p=0.181) and cyprodinil (α=0.05, p=0.300) did not vary among treatments. Experiments for the isolates collected in 2014 are in progress.

Haplotype detection using microsatellite multi-locus variability. A genotype accumulation curve was generated using pooled data from all 313 isolates. This dataset contained 220 multi-locus haplotypes. The number of multi-locus haplotypes increased in a near linear fashion with each additional locus, but did not plateau even with 5 loci. As expected, the number of clones decreased relative to the number of haplotypes detected. Preliminary data for isolates from fruit collected in 2014 indicates that the addition of two more loci may be sufficient to distinguish all haplotypes (data not shown).

Calculation of haploid genetic diversity**.** Haploid genetic diversity is a measurement of allelic population variation. It was used in this study to test if diversity increased in response to fungicide treatment. All haploid genetic diversity values were calculated from clone-corrected data (Fig. 1.4). For pre-treatment blossom isolates collected from each treatment block, the haploid genetic diversities were not significantly different for collection years 2013 (p>0.420) and 2014 (p>0.1457). Likewise, the same values for post-treatment fruit isolates from control, azoxystrobin, and propiconazole treatments were not significantly different for 2013 (p=0.420) or 2014 (p=0.146).

Identification of private alleles. The number of private alleles measures the number of alleles in a population that are not found in any other population. The number of private

alleles found in a single treatment population ranged from one to eight (Table 1.4). Analysis of blossom isolates indicated that the number of private alleles was variable between treatment populations prior to fungicide exposure in both years. Analysis of fruit isolates indicated that the number of private alleles was consistently greater in the azoxystrobin-treated population than in the propiconazole-treated population.

Identification of unique multilocus haplotypes. Treatment populations from each isolate source and collection year were pooled and the number of unique multilocus haplotypes within each treatment population and pre-treatment population was determined. The values ranged from 24 to 29 and indicated little variation in number of unique multilocus haplotypes among treatment population, isolate source and collection year (Table 1.4).

Quantification of alleles by population. The number of alleles across loci indicates the potential for diversity in the population. There was no significant difference in the number of alleles among treatment population, collection year, or isolate source (Fig. 1.5). With regard to isolates from blossoms, there were no differences among treatment populations in 2013 ($p>0.479$) and 2014 ($p>0.255$). Likewise, no significant differences in number of alleles among treatment populations from fruit were detected in 2013 ($p=.383$) or 2014 ($p=0.395$). The control populations from fruit for 2013 and 2014 have not been analyzed yet.

Calculation of number of effective alleles by population. The effective number of alleles gives an overall representation of population evenness by weighting each allele based on its frequency in the population. For 2013 blossom isolates, there was no

significant difference in the number of effective alleles among all pre-treatment populations ($p>0.403$). The same was true for the two pre-treatment populations compared to the control population of 2014 blossom isolates ($p>0.098$), but the population from the propiconazole-treated block was significantly different in number of effective alleles compared to the azoxystrobin treatment population ($\alpha=0.05$, $p=0.0498$). For the 2013 ($p>0.323$) and 2014 ($p>0.550$) fruit isolates, there was no significant difference in the number of effective alleles between treatment blocks. (Fig. 1.6)

Assessment of Isolate-Level Genotypic Response

Isolate differentiation using gene-sequence variability. To determine if the isolates collected from a blossom and its corresponding canker were clones, three regions: MRR1, DHFR, and P450 were sequenced. These regions were highly variable with average percent variability between non-matching isolates at 1.1%, 1.8%, and 0.97% for MRR1 (2250bp), DHFR (1000bp), and P450 (1750bp), respectively. Only genetically matching pairs were used for subsequent microsatellite analysis to detect potential genetic changes due to field exposure of fungicides. The MRR1+DHFR+P450 sequence analysis identified 8 unique pairs with matching multi-locus genotypes for each of the treatments at the Musser Farm. The only two genetically identical non-complementary pairs were present in the control and propiconazole treatments. Three pairs of matching multi-locus genotypes at MRR1+DHFR+P450 were observed in the control and azoxystrobin populations at the UGA Horticulture Farm, and none of these matched non-complementary isolates. In total, 93% of the isolates sequenced matched only their complementary isolate

at the accumulation of all 3 regions. Several spores isolated from a single canker differed genetically, and the same phenomenon was observed for spores isolated from a single blossom.

Comparison of matching complementary pairs at microsatellite loci. The 8 matching pairs for each of the three treatments from the Musser Farm and 3 matching pairs from the two treatments at the UGA Hort Farm were analyzed for their microsatellite profiles. All pairs from the two locations had unique profiles, including the two pairs that were genetically identical at MRR1+DHFR+P450. Within each complementary pair, we did not detect differences in fragment sizes at any of the seven microsatellite loci (Table 1.5).

Discussion

The purpose of this study was to investigate, on the population and individual isolate level, whether frequent exposure of *M. fructicola* to azoxystrobin could accelerate genetic changes and generate associated phenotypes. Previous studies indicated that long-term and constant exposure of *M. fructicola* to the QoI fungicide azoxystrobin, induced microsatellite instability and transposon movement *in vitro*, but it is unknown if such effects can occur under field conditions (83, 84). This study utilized the same microsatellite loci that responded to azoxystrobin-induced oxidative stress *in vitro* and detected azoxystrobin-induced changes on the molecular level in *M. fructicola*. Most mutational events occurring in microsatellite regions are caused by polymerase slippage, but are corrected by the mismatch repair (MMR) system of the fungus (81, 90). MMR accuracy may be affected by reactive oxygen species (ROS) generated by QoI fungicides,

such as azoxystrobin (29, 89). Even small doses of ROS can completely disable several key complexes involved in MMR, and the number of retained mutations resulting from polymerase slippage increases dramatically in response to oxidative stress (26, 50, 51).

High polymorphism at each of the five microsatellite loci used in this study allowed differentiation of genotypes of *M. fructicola* within treatment populations. The same loci were used to characterize the movement of *M. fructicola* within and between tree canopies (27). Whether the five loci used in this study provide sufficient resolution of population diversity is not known, but a preliminary screening of the 2014 fruit population showed that six and seven loci may not increase differentiation. Comparisons of haploid genetic diversity, number of alleles, and multi-locus haplotype number between treatments all indicated that increased mutagenesis did not occur at the population level at the loci examined in this study following exposure to either propiconazole or azoxystrobin. For example, during both years, haploid genetic diversities of azoxystrobin and propiconazole-treated isolates differed by less than ±0.05, even less than the difference in diversity between untreated controls of 2013 and 2014. These results do not support the hypothesis that repeated application of azoxystrobin may increase genetic diversity under field conditions.

Analysis of individual canker and blossom isolates collected before and after consecutive fungicide applications allowed an investigation on the single-isolate level, whereby many of the unknown variables encountered in the population level study were eliminated. These variables included the unknown extent of genetic diversity within the population and potential gene flow between trees and rows. To ensure that the canker

isolate was the clonal match of the blossom isolate, three regions highly variable in their nucleotide composition were used for fingerprinting. This method differentiated 93% of all isolates from blossoms based on sequence variations in the combined MRR1+DHFR+P450 gene sequences. This novel method of fingerprinting *M. fructicola* isolates is a highly sensitive method to distinguish individuals within a population and thus will be useful for future population characterizations. Consistent with our population analysis, comparison of blossom and canker pairs revealed no changes in microsatellite loci. However, the microsatellite loci distinguished the two complementary pairs of isolates that were not differentiated by MRR1+DHFR+P450, indicating that all of the blossom/canker pairs were unique individuals.

The ability to differentiate individual isolates from each other not only allowed us to match pairs for mutagenesis analysis but also revealed some insights into the epidemiology of *M. fructicola*. The high genotypic diversity of isolates documented in this study supports the hypothesis that sexually produced ascospores are the primary inoculum source for blossom infections. This was unexpected, considering that in both experimental years no apothecia were detected during bloom on fruit mummies on the orchard floor or in nearby peach dump sites located between brush piles at the edge of woods (Schnabel personal communication). In a former study, however, apothecia were detected on fallen mummies in orchards in the Southeastern United States where mummies were not removed to dump sites (61). We also found multiple genotypes colonizing a single blossom, indicating that multiple infections can lead to blossom blight, agreeing with former understanding of *M. fructicola* life cycle. Our study also revealed that multiple genotypes

may colonize a single canker. Studies about blossom infections are rare, but it is assumed that blossoms may be infected by more than one spore, and that these spores may infect any flower tissue, though no recent studies appear to have researched this phenomenon (17, 40). Whether a single or multiple genotypes may eventually cause twig canker was not investigated in this study.

Although the results of this study do not support azoxystrobin-induced mutagenesis at our microsatellite loci, they cannot exclude the possibility of fungicide-induced mutagenesis elsewhere in the genome. Though microsatellite regions are highly mutable, they represent only a small proportion of the genome and only a few were screened in the study. Other mutations could still have occurred that were not observed at the loci used in this study. The number of private alleles was consistently greater in the azoxystrobin treatment for both years, but private allele numbers were highly variable even between populations that were not treated, indicating that this variation is not related to fungicide-induced mutagenesis. It is possible that for detectable levels of mutagenesis at microsatellite loci to occur, the mycelium has to be constantly exposed to a high dose of fungicide as was the case in the *in vitro* observations. In this field trial, exposure was frequent but may only have lasted for a short time due to degradation and/or dilution of spray material in plant tissue. Also, only mycelium and spores on blossoms or fruit surfaces were directly exposed to fungicide, while mycelium in plant tissue may not have been exposed at all due to limited or no systemic activity of both fungicides in woody tissue (8, 10, 16, 86). It is also possible that antioxidants produced in field conditions might quench reactive oxygen species. One study indicates that peach fruit can respond to *M.* infection

by producing caffeic acid, a phenolic compound that appears to induce upregulation of an antioxidant within the fungus (63). Similar phenolic-induced quenching of ROS has been documented in the rice-blast fungus *M. grisea* and is believed to limit alternative oxidase activity of the fungus in vivo (96). *M. fructicola* can also undergo AOX *in vitro*, but not in vivo, indicating that ROS quenching may also occur in it.

Even though disease incidence values indicated a large decrease of disease in the fungicide treatments compared to the control, the number of genotypes in the control and fungicide treatments did not decrease significantly. This is the opposite of the initial experimental hypothesis that fungicide treatments would select for less sensitive genotypes and thus cause a reduction of genotypes. But it appears that a large amount of genotypes survived fungicide treatments but they were inhibited in their ability to cause an epidemic. The implications of this result are interesting because they appear to differ from our current understanding on how low dose applications may influence population dynamics. Current models indicate that the application of low doses of fungicides results in decreased diversity and selection for resistant isolates, though potentially at a slower rate than high fungicide doses (30, 31, 35, 73, 91). It is also possible that the resolution of our technique to distinguish genotypes was not enough to detect a reduction of genotypes.

The assessment of phenotypic characteristics of treatment populations revealed no changes in population responses to fungicide sensitivity. The sensitivity of fungicide-exposed field populations to azoxystrobin and propiconazole *in vitro* was not different compared to the control, indicating that no selection for resistance had occurred during the season. This is consistent with our genotypic data described above and supports the

argument that fungicide treatments did not reduce the population by killing off the most sensitive individuals but rather prevented existing genotypes from progressing. No changes in sensitivity to cyprodinil and iprodione were detected *in vitro*. Both fungicides belong to chemical classes unrelated to those used for field applications. This lack of change indicates that no resistant genotypes had randomly arisen in response to increased mutagenesis. Again, this is consistent with the overall conclusion of this study that azoxystrobin does not accelerate mutagenesis under field conditions, but it stands in contrast to findings in other systems where pathogen populations subjected to site-specific fungicides were predisposed to developing resistance to unrelated fungicides (59).

In conclusion, genotypic and phenotypic analysis of *M. fructicola* populations that were exposed to consecutive applications of azoxystrobin and propiconazole in the field did not provide evidence of increased mutagenesis. The analysis of individual isolates collected before and after consecutive fungicide applications concurred with the population data, also indicating that no mutagenesis took place. Additional comparison of isolate pairs at the whole genome level would ensure detection of mutational events, and would indicate conclusively if mutagenesis occurred in these isolates.

Table 1.1 Microsatellite primers used in this study

Primer name	Primer Sequence (5'-3')[a]
SEF	**CAGTCGGGCGTCATCA**GACTATAGAGTTTTCTACGGATGG
	GTTTTGTCTCTCAACTTTTAAATCAGCC
SEL	**CAGTCGGGCGTCATCA**GAGTATAACCAACCCAACGGC
	GTTTAGAGATGGAGTCAGGAGTGTTG
SEN	**CAGTCGGGCGTCATCA**TGCGTGTCATGTCGTCC
	GTTTCGAGGCTTAACTTCCGTGC
SEP	**CAGTCGGGCGTCATCA**TAGGCCACAGCTGATACCG
	GTTTATCAATTGGTTTGGGTCCTTG
SEQ	**CAGTCGGGCGTCATCA**GGAGGTGGATGGTGGGTAG
	GTTTGGCTGTGGGTTGAGTGAG
SED	**CAGTCGGGCGTCATCA**TTGGCATGGCATTTGGAGC
	GTTTCCATTTTATTCATATCCAACGCCC
SEI	**CAGTCGGGCGTCATCA**CTCAAGCGGTGGCTCAAAG
	GTTTAACCACCACGACCACGAC

[a]CAGTCGGGCGTCATCA is the CAG sequence described in Everhart et al. (27). The GTTTT sequence at the beginning of the reverse primers is pig tailing to prevent a poly A tail from obscuring the true fragment length.

Table 1.2 Discriminatory doses and media used to test for resistance development after a season of spraying fungicide.

Fungicide	Discriminatory dose	Media
Azoxystrobin	0.3 ppm + 75 ppm SHAM[a]	PDA
Propiconazole	0.01 ppm	PDA
Cyprodinil	0.05 ppm	CzA
Iprodione	0.3 ppm	PDA

[a]PDA was amended with SHAM to prevent alternative oxidase activity

Table 1.3 Primers used to obtain sequences used for fingerprinting and comparing isolates collected from cankers and blossoms

Primer locus	Primer type	Primer Sequence (5'-3')
DHFR	Forward	TGGCATATTTCGCGAGGGTT
	Reverse	ATAGGAACGGGACCTGTGGA
MRR1 fragment 1	Forward	TGTCTTACCATCCTGTCATAC
	Reverse	TCATTGTCTCAGTACCTCGATC
MRR1 fragment 2	Forward	TGCAGTCATGCGCTCTGATG
	Reverse	TGTCTTACCATCCTGTCATAC
P450	Forward	TCACGAAACACACCCGTCT
	Reverse	CCAGGCGTTATTGGCTATG

Table 1.4 Allelic variations at 5 microsatellite loci of fruit and blossom populations collected in 2013 and 2014.

Year and Isolate Source	Treatment	Sample size[a]	Alleles per locus[b]	Effective alleles per locus[b]	Private alleles[a]	Unbiased haploid genetic diversity[b]	Unique multilocus haplotypes[a]
2013 Blossom blight	Azoxystrobin	31	6.00	3.88	6	0.736	28
	Control	31	5.60	4.04	3	0.764	27
	Propiconazole	31	5.20	3.41	3	0.714	27
2014 Blossom blight	Azoxystrobin	32	5.20	3.27	3	0.693	27
	Control	32	5.60	3.57	2	0.701	29
	Propiconazole	32	6.60	4.96	6	0.811	29
2013 Fruit rot	Azoxystrobin	30	6.20	3.98	8	0.744	24
	Propiconazole	30	5.80	3.45	6	0.697	26
2014 Fruit rot	Azoxystrobin	32	5.80	3.61	3	0.709	27
	Propiconazole	32	5.40	3.72	1	0.741	28

[a]before clone-correction

[b]clone-corrected

Table 1.5. Comparison of matching canker and blossom isolates at seven microsatellite loci.

Treatment	Isolate Pair[a]	Microsatellite Loci						
		SED	SEF	SEI	SEL	SEN	SEP	SEQ
Azoxystrobin	3.1.6 B,C	131	126	110	137	227	250	136
	3.1.8 B,C	145	131	114	147	227	254	134
	3.2.17 B,C	131	117	114	137	231	247	138
	3.2.7 B,C	141	131	114	143	231	247	134
	3.2.8 B,C	131	117	104	143	227	254	132
	3.4.1 B,C	131	131	114	147	223	247	132
	4.1.4 B,C	135	144	114	137	231	254	130
	4.5.11 B,C	131	126	104	137	231	231	138
Propiconazole	9.2.18 B,C	131	117	114	143	231	254	134
	9.5.3 B,C	131	131	114	137	223	254	136
	9.5.6 B,C	131	144	104	143	235	231	136
	9.5.7 B,C	131	144	114	137	231	254	138
	10.1.8 B,C	131	139	114	137	227	265	134
	10.2.2 B,C	145	117	114	147	223	254	134
	10.3.2 B,C	145	117	110	147	227	254	132
	10.5.17 B,C	135	123	114	147	227	257	138
	10.5.7 B,C	127	126	114	143	227	254	140
Unsprayed Control	6.1.8 B,C	141	139	114	147	223	254	138
	6.2.13 B,C	133	135	114	143	231	254	146
	6.3.18 B,C	131	117	114	137	227	254	140
	6.4.4 B,C	141	123	102	143	231	231	134
	7.1.1 B,C	131	117	110	143	227	254	138
	7.2.17 B,C	131	117	98	137	227	254	138
	7.3.12 B,C	131	117	114	143	235	250	140
	7.5.4 B,C	131	127	104	137	227	250	138
UGA Azoxystrobin	Azo82 B,C	127	149	110	143	227	254	125
	Azo13 B,C	117	106	114	137	223	254	130
	Azo93 B,C	127	127	114	137	227	254	138
UGA Control	Con176 B,C	141	135	117	143	223	254	136
	Con66 B,C	106	131	98	136	231	254	134
	Con91 B,C	106	126	114	143	227	250	134

[a]B and C represent isolates from a matching pair

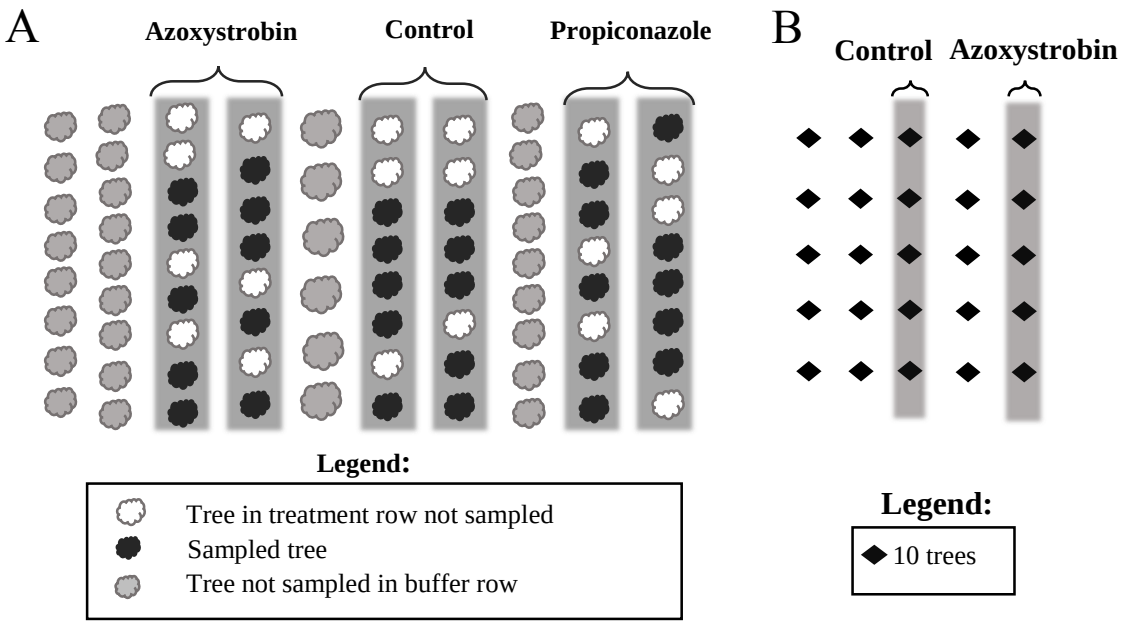

Figure 1.1 Schematic of the experimental setup for collections at **A,** Musser Fruit Research Farm, Seneca, SC and **B,** University of Georgia Horticulture Farm, Watkinsville, GA. At the Musser Farm, 20 isolates were collected from each of the 10 trees per treatment marked black. At the Georgia Hort Farm, 150 isolates were collected at random from trees of each treatment.

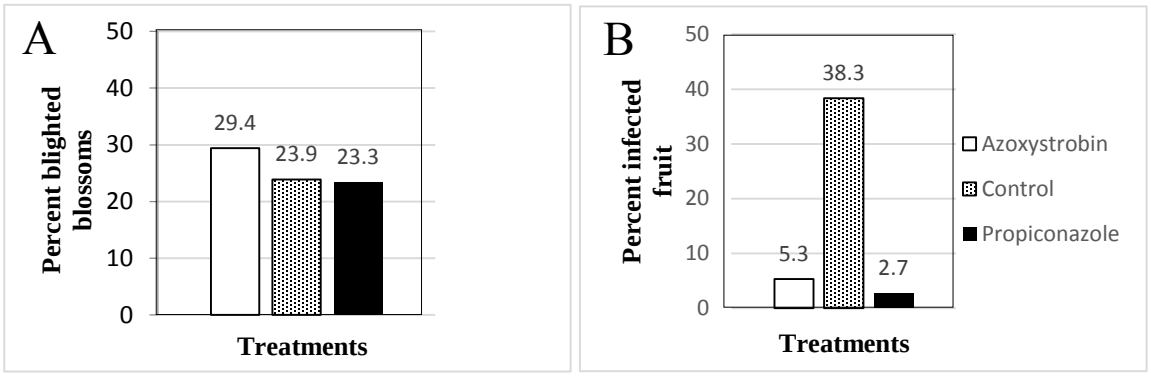

Figure 1.2 Incidence of blossom blight (**A**) and fruit rot (**B**) incidence in treatment blocks. Fruit rot incidence data were collected after 10 applications of fungicides.

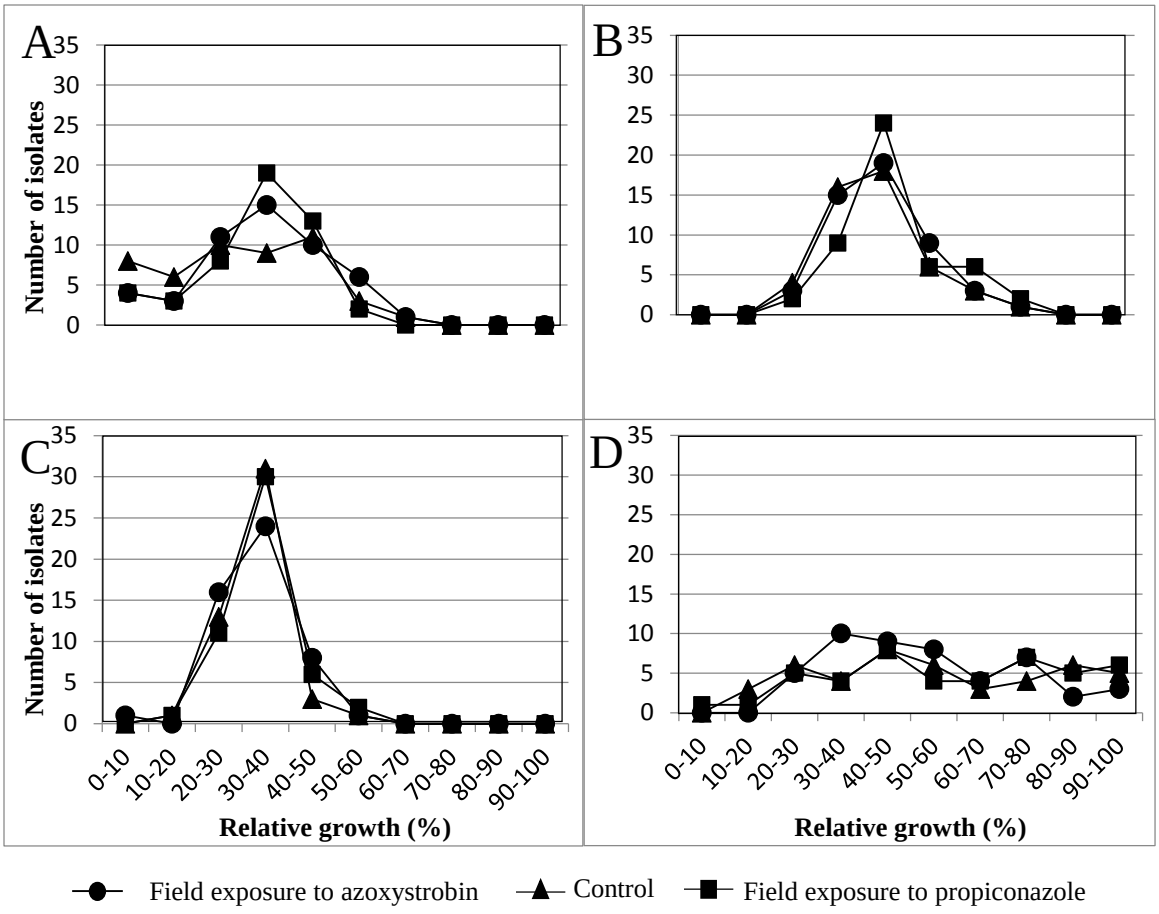

Figure 1.3 Sensitivity of *M. fructicola* isolates unexposed or exposed to 10 consecutive applications of azoxystrobin or propiconazole to azoxystrobin (**A**), propiconazole (**B**), iprodione (**C**), and cyprodinil (**D**). Each assay was performed with 50 isolates

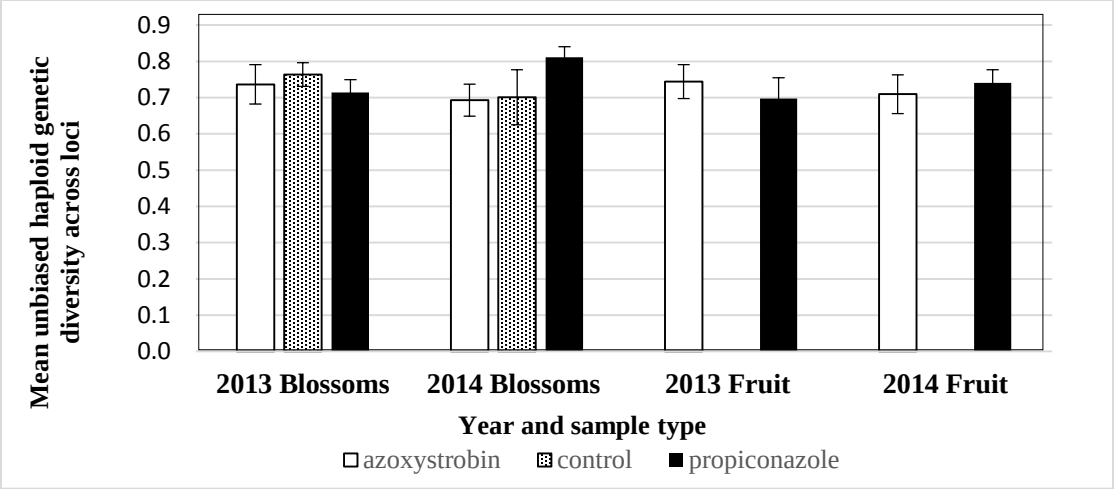

Figure 1.4 Unbiased haploid genetic diversity by *M. fructicola* population. Error bars represent the standard error of the mean unbiased haploid genetic diversity across loci measured by GenAlex 6.5 (75). All data was clone-corrected before haploid diversities were calculated.

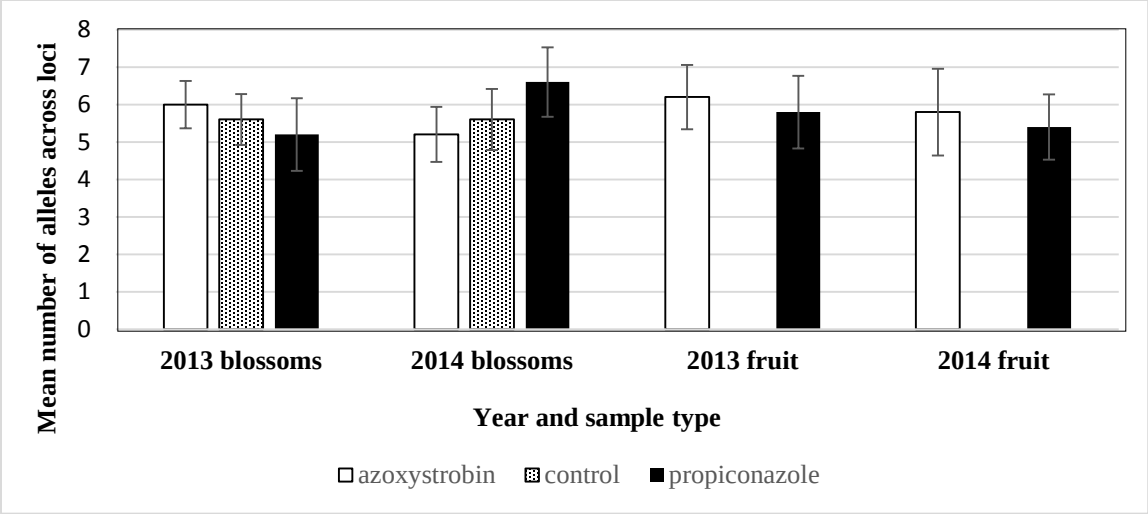

Figure 1.5 Mean number of alleles averaged across loci for each isolate source and collection year. Error bars represent the standard error and were calculated using GenAlex 6.5 (75).

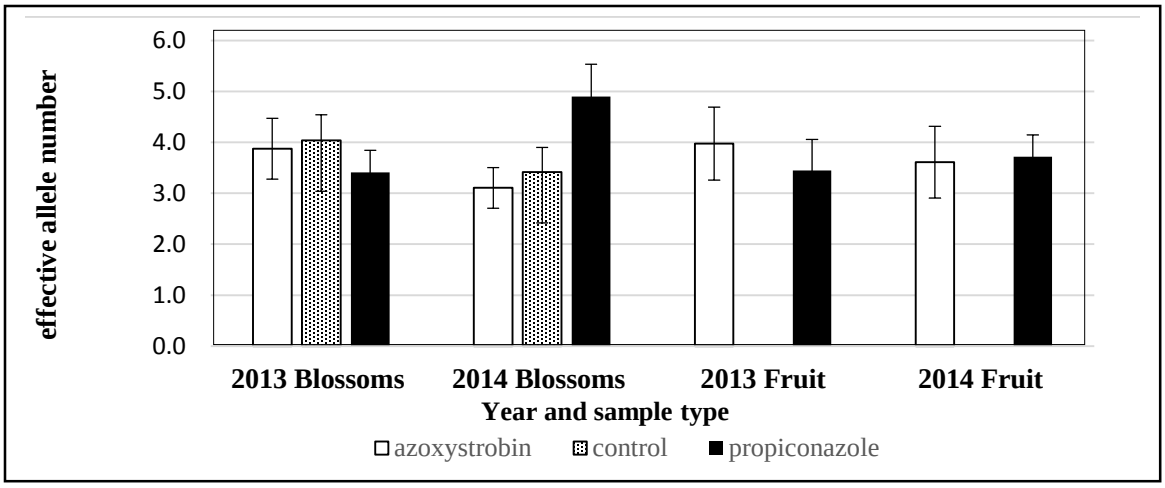

Figure 1.6 Number of effective alleles in treatment populations from each isolate source and collection year. Error bars indicate standard error values calculated using Genalex 6.4 (75)

CHAPTER THREE

CONCLUSION

Azoxystrobin-induced mutagenesis previously observed *in vitro* with microsatellite markers was not reproducible under field conditions in this two year study. Neither comparison of pathogen populations nor comparison of individual isolates obtained from field treatments before and after fungicide applications revealed signs of mutagenesis. More detailed, genomic analysis such as SNP or GBS analysis and comparison of transposon placement before and after fungicide application may be necessary to determine whether or not azoxystrobin and other QoI fungicides increase the rate of mutagenesis in fungi. Several other interesting results were obtained from the study, including evidence that low rates of fungicides may cause a decline in disease incidence but not a decline in the number of genotypes present in the population. This lack of selection pressure may be useful information for the design of anti-resistance management strategies, since it indicates that selection for resistant genotypes decreases when a lower dose is applied. We also observed that blossoms are infected by multiple genetically diverse spores given the wide variety of genotypes found in blossoms. This supports the hypothesis that blossom infections likely derived from ascospores. Single blossoms and cankers were infected by more than one genotype, which sheds light on a formerly unknown aspect of the life cycle of *M. fructicola.* The sequence analysis of the multidrug resistance regulator 1, dihydrofolate reductase region, and cytochrome P450 region differentiated *M. fructicola* isolates within the same field and thus provides an additional, powerful, new tool to characterize populations.

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