ABSTRACT

LIBERATOR, KELLY LORRAINE. The Effect of the 2-aminoimidazoles on Monilinia fructicola (an ascomycete) and Interactions with Selected Fungicides. (Under the direction of Dr. David F. Ritchie).

Monilinia fructicola is the causal agent of blossom blight and brown rot of peach in the southeastern United States. Fungicides are heavily relied on to control the disease and fungicide resistance or decreased sensitivity has developed in this pathosystem to many of the major fungicide groups. The 2-aminoimidazoles (2-AIs) are a group of novel compounds based on the marine natural product oroidin and a lead 2-AI analog was evaluated to determine its effect on M. fructicola. The effective concentration to inhibit growth by 50% (EC₅₀) was calculated as 1.97 ug/ml in conidial germination assays and 11.37 and 9.83 in mycelial growth assays with North Carolina M. fructicola isolates. The 2-AI is positively charged and versions of the analog with a neutral charge showed a loss of activity in conidial germination assays, indicating that charge is important for 2-AI efficacy. The effect of the 2-AI on M. fructicola isolates with different fungicide resistance phenotypes was determined in mycelial growth assays. The EC₅₀s were 5.52 and 5.69, 4.93, 10.07, and 18.97 ug/ml for fungicide sensitive, benzimidazole resistant, demethylation inhibitor resistant, and quinone outside inhibitor reduced sensitivity phenotypes. Synergistic and antagonistic interactions between the 2-AI and propiconazole were found in mycelial growth assays, indicating the 2-AIs should be further tested in field trials for their ability to increase the activity of commercial fungicides. Lastly, scanning and transmission electron microscopy studies showed changes in gross morphology and ultrastructure in 2-AI treated M. fructicola conidia.
The Effect of the 2-aminoimidazoles on *Monilinia fructicola* (an ascomycete)
and Interactions with Selected Fungicides

by
Kelly Lorraine Liberator

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APPROVED BY:

__________________________________  __________________________________
Dr. David F. Ritchie                Dr. Christian Melander
Committee Chair

__________________________________
Dr. Gary Payne
DEDICATION

I dedicate this work to my family, friends, and ever-patient husband. Thank you for always believing in me and encouraging me. I wouldn’t be where I am today without you.
BIOGRAPHY

Kelly Lorraine Liberator was born on June 12, 1983 and grew up in Madison, CT. In August of 2001 she began her undergraduate degree at University of Maryland. Initially intending on pursuing a degree in International Business, her interests led her to join the Environmental Science program and specialize in soil science. During her sophomore year she began working as a research assistant with Dr. Jose Costa in his wheat breeding program and gained an appreciation and taste for working on field trials. Her work with Dr. Costa led her to have a strong interest in agricultural research and a desire to continue in this increasingly important field. In 2005 she graduated from University of Maryland.

Kelly then relocated to the triangle area of North Carolina where she began working at BASF as a contract greenhouse and field technician in their Herbicide and Fungicide Biology groups. At this time Kelly’s passion for agricultural research and crop protection was cemented. After 6 months she gained full time employment in BASF’s Global Insecticide Group working on the plant propagation team. She gained fundamental knowledge of entomology and the BASF organization in her role. In 2008 she returned to the Fungicide Biology group as an Agricultural Biologist working in the seed treatment group. It was here that she was introduced to plant pathology and decided that she wanted to dedicate herself to the study of plant diseases and how to prevent them.

In the Fall of 2009 Kelly entered the Plant Pathology program at North Carolina State University to pursue a Master of Science under the direction of Dr. David Ritchie. Her
project involved researching the 2-aminoimidazoles for potential use in fungal disease management.
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I would like to thank my parents, who have always supported me in my life. Thank you to my mother, who taught me about patience and the importance of thinking and not judging. And to my father, thank you for teaching me to work hard and always try my best. And to my brother and sister, thank you for blazing the trail and being the kind of people I could aspire to be like.

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1. CHAPTER I:  

Literature Review

1.1. Peach

*Prunus persica*, commonly known as the peach tree, belongs to the family Rosaceae and is native to China. It is a member of the stone fruit family and is related to plums, almonds, apricots and cherries. European countries such as France, Spain, Greece and Italy as well as Iran and China are large producers of peaches (Bassi and Monet, 2008). In the United States California, Georgia and South Carolina are the largest peach producers (United States Department of Agriculture National Agricultural Statistics Service, 2012).

In 2011 there were 112,680 acres of fruit bearing peach trees in the U.S. that produced 1,034,000 tons of peaches valued at $596,000,000. North Carolina contained 1,100 acres of fruit bearing peach trees that produced 5,300 tons valued at $5,150,000 in 2011 (United States Department of Agriculture National Agricultural Statistics Service, 2012).

Production of peach fruit begins with flower-bud initiation the preceding summer and continues through harvest the following growing season. After pollination, fertilization, and seed development occur, fruit set and growth begins. Fruit growth occurs in three phases consisting of cell division, pit hardening, and final swell that primarily involves cell enlargement. Peaches are climacteric and will produce a burst of ethylene accompanied by increased respiration as they ripen, which continues after harvest (Wills et. al., 1998; Trainotti et. al., 2007).
1.2. *Monilinia fructicola*: Taxonomy, Geographic Distribution, Economic Importance, and Biology

*Monilinia fructicola* (Wint.) Honey is the causal organism of brown rot of peach in the Southeastern U.S. This species is one of several necrotrophic ascomycete fungi that cause brown rot of stone and pome fruits. Brown rot fungi were first described on fruit in Europe by Persoon in 1796 (Byrde and Willetts, 1977). The teleomorph of this fungus was first described as *Ciboria fructicola* by G. Winter on mummified peaches from Pennsylvania in 1883 (Batra, 1991). In 1906 the species was transferred to the genus *Sclerotinia* by Rehm and then to *Monilinia* in 1928 by E. Honey (Batra, 1991). The anamorph was formally described as *Monilia fructicola* by Batra in 1991 and this is the currently accepted name (Batra, 1991).

While the related species *Monilinia fructigena* and *Monilinia laxa* are common in Europe, *M. fructicola* is generally considered a New World species and is commonly found in Australia, New Zealand, the U.S., Canada, Argentina, Bolivia, Brazil, Peru, and Central America (Byrde and Willets, 1977). *M. fructicola* is currently listed as a quarantine pathogen in Europe (European and Mediterranean Plant Protection Organization, 2011). However, with the first report from France in 2001, *M. fructicola* has since been reported in Austria (eradicated), Germany, Hungary, Italy, Poland, Romania, Slovenia, Spain, Switzerland, Austria, Serbia, and the Czech Republic (Baker et. al., 2011; Bossard et. al., 2006; Duchoslavová et. al., 2007; Hilber-Bodmer et. al., 2010; Petróczy and Palkovics, 2006; Vasic et. al., 2012).
M. fructicola causes both blossom blight and fruit rot of peach. Blossom blight is an important disease in areas of California whereas fruit rot is the primary concern in the southeast U.S. Blossom blight results when spores of the fungus infect flowers during the spring. Although it is considered rare, sexual ascospores are produced from apothecia that form on fallen ‘mummies’. Asexual conidia are produced from mummies, infected twigs, or stem cankers (Byrde and Willets, 1977). Diseased blossoms turn dark brown and exhibit a blighted appearance and infection can spread to young fruit, twigs and stems (Byrde and Willets, 1977). Fruit rot can take place following blossom blight or at any time during fruit development and is the most potentially economically destructive part of the disease. Blighted blossoms provide inoculum for the fruit rot phase but conidia from thinned fruit on the orchard floor, aborted fruit in the tree, and diseased fruit on nearby wild species also may serve as inoculum sources (Landgraf and Zehr, 1982). Diseased fruits develop small brown spots caused by growing mycelia. Sporogenous hyphae form conidiophores which break through the peach skin. Infections spread from diseased to healthy fruits within the tree or tree-to-tree by wind-blown rain, insects, and air movement (Byrde and Willets, 1977). Very advanced infections result in the entire fruit surface being covered in mycelia, conidiophores, and conidia which cause the fruit to shrivel into what is known as a ‘mummy’ (Byrde and Willets, 1977).
1.3. **Fungicide Mode of Action and Resistance**

1.3.1. **Benzimidazole Fungicides**

Benomyl was first registered in 1969 in the U.S. by E.I. du Pont de Nemours and Co. (DuPont) and thiophanate-methyl was first registered in 1973. Benzimidazole and thiophanate fungicides (BZI) are part of the methyl benzimidazole carbamate (MBC) group of fungicides that affect mitosis and cell division at the site of β-tubulin assembly in mitosis. They inhibit microtubule assembly by binding to the heterodimeric subunit, the tubuline molecule (Davidse, 1986). MBC fungicides also block nuclear division (Clemons and Sisler, 1971; Davidse, 1986; Davidse, 1973; Hammerschlag and Sisler, 1973).

Resistance in *M. fructicola* to the BZI fungicides was found in Michigan cherry orchards (Jones and Ehret, 1976) and South Carolina peach orchards by 1976 (Zehr et. al., 1991) and by 1977 in California (Szkolnik et. al., 1978). Benzimidazole resistance is associated with point mutations that cause changes in amino acid sequences at the BZI binding site, or β-tubulin gene (Koenraadt et. al., 1992). Ma et. al. (2003) reported a point mutation in codon 198 results in the replacement of glutamic acid with alanine in highly resistant isolates of *M. fructicola* from California (Ma et. al., 2003). The 198A allele has also been associated with BZI resistant *M. fructicola* isolates from South Carolina (Zhu et. al., 2010).

Resistance to the BZI fungicides has been shown to be persistent. Zehr et. al. (1991) reported that benomyl-resistant strains of *M. fructicola* persisted in commercial orchards, where 7.7% to 14.9% of the isolates collected from blossoms after BZI application during bloom were resistant (Zehr et. al., 1991). Resistant strains also persisted long after benomyl
use was discontinued in commercial orchards that used customary cultural practices. Such commercial management practices may allow resistant strains to persist while selection pressure promotes development of ecologically fit strains (Zehr et. al., 1991).

### 1.3.2. Quinone Outside Inhibitor Fungicides

The quinone outside inhibitor (QoI) group of fungicides was first sold in 1996 upon Syngenta’s release of azoxystrobin and BASF’s release of kresoxim-methyl (Bartlett et. al., 2002). QoI fungicides inhibit electron transport in the mitochondrial respiratory chain at complex III, also known as cytochrome bc$_1$ complex, by binding to the cytochrome b site (Qo-site) (Bartlett et. al., 2002). Cytochrome b is situated in the inner mitochondrial membrane of fungi. Inhibitors bind to cytochrome b and prevent electron transfer between cytochrome b and cytochrome c$_1$. This stops ATP production and thereby disrupts the energy cycle inside the fungus (Bartlett et. al., 2002).

QoI fungicides are currently very important for brown rot management in the southeast because they are the only other fungicides as effective as DMIs at controlling the disease (Schnabel et. al., 2004). However, populations of *M. fructicola* from New York and South Carolina have exhibited a shift toward decreased sensitivity to QoIs recently. Isolates collected from six counties in New York in 2005 and 2007 showed a shift in decreased sensitivity to the active ingredients boscalid and pyraclostrobin in Pristine fungicide (Villani and Cox, 2008). Amiri et. al. (2010) reported a 3-fold, 2-fold, and 5-fold increase in mean EC$_{50}$s of isolates collected from South Carolina to azoxystrobin, pyraclostrobin and boscalid, respectively, from 2006 to 2008. In addition, the mean EC$_{50}$ to the mixture of pyraclostrobin
+ boscalid increased 2-fold over the same period (Amiri et. al., 2010). This trend is not limited to the U.S. as QoI decreased sensitivity shifts have also been reported in *M. fructicola* populations from Brazil (May-De Mio et. al., 2011).

### 1.3.3. Demethylation Inhibitor Fungicides

Sterol biosynthesis inhibitors (SBI) fungicides are the largest class of fungicides and were first introduced for agricultural use when Bayer launched triadimefon in 1973. Many compounds in this group possess both eradicative and curative activity. Eradicative fungicides prevent new infections and curative fungicides prevent existing infections from spreading and surviving. Sterol biosynthesis targets also allow for synthesis of many active ingredients that have favorable toxicological and environmental profiles and are safe for plants (Kuck and Vors, 2007). The demethylation inhibitor (DMI) group of compounds is identified as SBI class I. The DMIs inhibit the 14α-demethylase (*CYP51*) enzyme, which is involved in the production of sterols in fungi. Sterols are critical for membrane stabilization, structure and function and are thereby vital for the formation of functional cell walls (Kato, 1986). The primary sterol in most ascomycete and basidiomycete fungi is ergosterol (Kato, 1986). Impaired ergosterol biosynthesis impedes formation of new membranes and causes deterioration in existing membranes (Kuck and Vors, 2007). DMIs specifically target sterol 14α-demethylase. The cytochrome P-450 enzyme catalyzes the first oxygenation step during 14α-demethylation and is the primary site of action. SBI fungicides exhibit fungistatic action when fungi can remove C-4 methyl groups in the presence of 14-α methyl groups and fungicidal action when they cannot. Filamentous fungi usually have distorted and
excessively branched and swollen hyphae and germ tubes when they are exposed to SBI fungicides (Buchenauer, 1987).

The DMI fungicides were first introduced in the 1970s and by the 1980s had replaced BZI fungicides as the primary crop protection product used to control brown rot. However, as with other fungicide classes, prolonged use of DMIs can lead to an evolution of resistant populations (Brent and Holloman, 1988). Resistance has been reported in many parts of the US, starting with South Carolina (Zehr et. al., 1999) and Georgia (Schnabel et al., 2004). From 1993 to 1995 Zehr et. al. (1999) studied the effect of prolonged use of propiconazole in peach orchards. They found that when a previously unexposed orchard was sprayed 29 times with propiconazole over a three year period there was a decrease in sensitivity to the fungicide and an increase in *M. fructicola* isolates less sensitive to propiconazole (Zehr et. al., 1999). In 2006 DMI resistant isolates of *M. fructicola* were found in New Jersey (Burnett et. al., 2010). In 2005 and 2007 236 *M. fructicola* isolates from New York were tested for sensitivity to propiconazole and febuconazole and all isolates showed a shift in sensitivity toward DMI resistance. In addition, isolates collected in 2007 showed a stronger shift, indicating an increased buildup of resistance over time (Villani and Cox, 2008). DMI resistance has also been reported in other parts of the world such as Brazil (May-De Mio et. al., 2011).

DMI resistance is multigenic and quantitative and is likely due to three different mechanisms (De Waard et. al., 1993; Koller et. al., 1997). First, resistance has been associated with mutations in the DMI target gene *CYP51* that alter protein structure and reduce fungicide binding affinity (Delye et. al., 1997; Delye et. al., 1998). Secondly,
overexpression of the *CYP51* gene has been linked to DMI resistance (Hamamoto et. al., 2000; Luo and Schnabel, 2008; Ma et. al., 2006b). Lastly, certain energy-dependent drug efflux mechanisms have been associated with reduced sensitivity to DMI fungicides (Hayashi et. al., 2003; Nakaune et. al., 1998).

In 2008 Luo and Schnabel sequenced the *CYP51* gene (Mf*CYP51*) and upstream flanking sequence of DMI-resistant and sensitive field isolates from Georgia and conducted expression analysis on them. They found that sensitive and resistant isolates had the same amino acid sequences in Mf*CYP51* protein coding region, suggesting that field resistance was not due to mutations in the gene. However, expression of the Mf*CYP51* gene was 5-fold to 11-fold higher in DMI-resistant isolates than in sensitive isolates. Further analysis showed that sensitive and resistant isolates had the same number of copies of the Mf*CYP51* gene, which eliminated this as a reason for more mRNA in resistant isolates. This study thus proved that increased expression of the Mf*CYP51* gene could be a potential mechanism for DMI field resistance in *M. fructicola*. They also identified a large insert, named ‘Mona’, upstream of Mf*CYP51* in only DMI resistant isolates. ‘Mona’ contains a predicted promoter sequence and the authors concluded that the insert causes overexpression of Mf*CYP51* and is linked with the resistance phenotype (Luo and Schnabel, 2008). A further study identified ‘Mona’ in all of the sampled DMI resistant isolates of *M. fructicola* collected from Georgia, South Carolina, New York and Ohio from 2003-2007. While the nucleotide composition and location of ‘Mona’ were the same in all isolates, they possessed different copy numbers of ‘Mona’ elements. The authors suggested that DMI resistant isolates evolved from a common ancestor and the ‘Mona’ insertion event probably occurred in the 1970s or 1980s soon after
DMIs were introduced (Luo et. al., 2008). However, Villani and Cox reported the presence of ‘Mona’ in only half of the sampled population of DMI-resistant isolates from New York in 2005 and 2007 (Villani and Cox, 2008). Only 3 of 16 populations of M. fructicola isolates resistant to febuconazole and propiconazole collected from 2005 to 2009 in New York possessed the ‘Mona’ element in addition to some sensitive isolates as well. This led the authors to suggest that ‘Mona’ likely contributes only some quantitative resistance to the DMI fungicides (Villani and Cox, 2011).

1.4. Novel Molecules Based on Secondary Metabolites of Caribbean Sea Sponges

Because crop protection products are needed to control many plant diseases and resistant pathogen populations evolve, the search for new chemistries and molecules is critical. Sea sponges of the genus Agelas (Family Agelasidae) are prevalent in Caribbean coral reef communities and produce unique secondary metabolites that are primarily composed of brominated pyrrole alkaloids (Braekman et. al., 1992). In 1981 Faulkner and Clardy described the structure of the first dimeric pyrrole-imidazole alkaloid, sceptrin, from Agelas sceptrum (Walker et. al., 1981). Then in 1986 Rinehart isolated ageliferin, a bicyclic, nonsymmetrical dimer from Agelas conifera (Keifer et. al., 1986). These biologically active natural products generated interest due to their anti-predatory, antimicrobial, and antibiofilm effects. Crude organic extracts of Agelas species have anti-predatory effects on the bluehead wrasse (Thalassoma bifasciatum), a type of generalist fish (Pawlik et. al., 1995). Sceptrins, ageliferins, and oxysceptrins from Agelas conifera were shown in 1991 to have antiviral and antimicrobial effects by Keifer et. al. (1991). Ageliferin compounds inhibited Bacillus
**B. subtilis**, *Escherichia coli*, and Herpes simplex virus, type 1. In addition, an ageliferin compound produced an EC$_{50}$ of 21 ug/ml in barnacle settlement assays, indicating moderate settlement inhibition and potential antifouling activity (Keifer et. al., 1991).

In addition to antimicrobial effects, the ability of compounds to prevent or disperse biofilms is of interest because they would have many applications in the medical, agricultural, and other industries. Bacterial biofilms are communities of surface attached bacteria encased and protected by an extracellular matrix of biomolecules (Donlan and Costerton, 2002; Musk and Hergenrother, 2006). Biofilms are responsible for 50-80% of microbial infections in humans and are insensitive to many microbicides and host defenses (Costerton et. al., 1999; Mah and O’Toole, 2001). In addition, bacteria in biofilms have been estimated to be more than 1000-fold more resistant to conventional antibiotics than those living in a planktonic state (Rasmussen and Givskov, 2006).

Feeding deterrent studies by Lindel et. al. (2000) have shown that the structure activity relationship of synthetic alkaloids related to oroidin, another secondary metabolite of *Agelas* species, is highly dependent on the 2-aminoimidazole (2-AI) moiety (Lindel et. al., 2000). It has been shown that an imidazole or 2-AI head is necessary to maintain antibiofilm activity in analogs based on oroidin (Richards et. al., 2008a). The biologically active natural product bromoageliferin is another member of the oroidin class of molecules and is also characterized by a 2-AI subunit (Al Mourabit and Potier, 2001). Huigens III et. al. (2007) investigated if a 2-AI structural motif and bicyclic core in bromoageliferin was responsible for its biological activity. Two bromoageliferin analogs, trans-bromoageliferin analog 1 (TAGE) and cis-bromoageliferin analog 2 (CAGE) were synthesized as well as 4-(3-
aminopropyl)-2-aminoimidazole as a control. They determined that TAGE and CAGE produced IC\textsubscript{50} values of 100 \textmu M – 190 \textmu M in assessments of anti-biofilm activity against \textit{Pseudomonas aeruginosa}. However, they also determined that TAGE and CAGE significantly reduced planktonic growth of \textit{P. aeruginosa} at 500 \textmu M and 400 \textmu M, respectively. This indicated that TAGE and CAGE may have both bactericidal and biofilm inhibition activity that led to a decrease in \textit{P. aeruginosa} biofilms formation (Huigens III et. al., 2007). The ability of 2-AI derivatives to control fungal biofilms has also been investigated. A second generation 2-AIT library was synthesized and two lead compounds inhibited and dispersed biofilms of \textit{C. albicans} at 37.2 \textmu M and 24.7 \textmu M and those formed by \textit{Cryptococcus neoformans} (Rogers et. al., 2010a).

Manipulation of the compound structure has allowed researchers to enhance the effect of the 2-AIs. Richards et. al. (2008) found that an increase in the chain length from 6 to 12 carbons increased biofilms inhibition activity of 2-AI analogs by more than a full order of magnitude (Richards et. al., 200b). Synthesized analogs of dihydrosventrin were able to inhibit and disperse bacterial biofilms across order, class, and phylum by replacing the pyrrole subunit with a triazole subunit (Rogers and Melander, 2008). A second generation library of 2-aminobenzimidazole (2-AIBs) analogs based on a lead compound were screened against methicillin-resistant \textit{Staphylococcus aureus} (MRSA) (ATCC BAA-44) for antibiotic activity. The second generation lead compound 33 was more effective than methicillin, gentamycin, and streptomycin and as effective as ciprofloxacin. Lead compound 33 was also proven to have bactericidal rather than bacteriostatic action, which is preferred when treating bacterial infections (French, 2006; Huigens III et. al., 2010; Kaka et. al., 2006). In addition,
S. aureus ATCC BAA-44 did not show any drug resistance to the 2-AIBs and their relative potencies to ATCC BAA-44 and antibiotic sensitive strains was equal (Huigens III et. al., 2010). This demonstrated that structure manipulation could increase antibiotic activity and that the mechanism of action of the 2-AIBs is likely different than that of conventional antibiotics and could be used in cases where resistance has developed (Huigens III et. al., 2010). Combination therapy with 2-AIBs and conventional antibiotics was also investigated, and compound 33 with vancomycin produced a synergistic two-fold increase in antibiotic activity against ATCC BAA-44. Whereas the MIC for compound 33 was 12.5 uM, the synergistic action was achieved at a sub-MIC concentration of 2 uM (Huigens III et. al., 2010). Synergism was also shown for a lead 2-AIT compound with non-microbicidal antibiofilm properties when combined with tetracycline. This compound was able to resensitize antibiotic resistant Escherichia coli without having any bactericidal effects (Rogers et. al., 2010b).

Mechanism of action of marine natural products and 2-AI derivatives has been investigated although research to elucidate the exact mechanism is ongoing. Bernan et. al. (1993) reported that the marine natural product sceptrin, isolated from Agelas mauritiana, caused a release of potassium ions from E. coli and lysis of red blood cells. Sceptrin also exhibited antibiotic activity on E. coli. Workers concluded that sceptrin likely disrupts cell membranes of prokaryotic and eukaryotic cells. They also observed the formation of spheroplasts in cell walls preceding membrane damage (Bernan et. al., 1993). Sceptrin, bromoageliferin, and dibromoageliferin were shown to inhibit voltage-dependent calcium entry in a cell line of phaeochromocytoma PC12 from rat adrenal medulla (Bickmeyer,
Mechanisms of action studies on non-microbicidal antibiofilm compounds have also been investigated. A 2-ABI derivative capable of inhibiting and dispersing gram-positive biofilms was shown to operate via a Zn(II)-dependent mechanism. NMR data demonstrated that the compound binds directly to Zn(II) (Rogers et al., 2009). In addition to being associated with pathogenesis of gram-positive bacterial infections, Zn(II) is also an important regulator of biofilms formation (Conrady et al., 2008). Another lead 2-AI compound (CR6) is an oroidin derivative and was found to have antifungal effects. It contains a 2-aminoimidazole heterocycle linked to an 11 carbon alkyl chain. It is also positively charged (Ballard et al., 2008).

Biofilms caused by bacterial plant pathogens may also be controlled using 2-AI compounds. In vitro experiments done with a copper-resistant strain of *Xanthomonas euvesicatoria* indicated that bacterial growth in broth solutions and bacterial recovery from treated leaf discs were reduced in treatments containing a 2-AI analog combined with copper (Worthington, et al., 2012). In addition, mixtures of the 2-AI with copper decreased bacterial spot foliar disease in 3 years of field trials conducted with copper-resistant *X. euvesicatoria* (Worthington, et al., 2012). Compounds with the ability to enhance control of bacterial plant diseases could extend the life and increase the effectiveness of currently available products.

### 1.5. Synergism in Agricultural Fungicides

The 2-AI based molecules have proven synergistic action with several antibiotics in controlling medically relevant antibiotic resistant bacteria (Huigens III et al., 2010; Rogers...
et. al., 2010b). This has important implications for the agricultural industry, where fungicide resistant populations are constantly evolving. Mixing fungicides can widen the disease control spectrum of a product, may result in synergistic interactions between products, and could delay the selection of resistant individuals in the population (Gisi, 1996).

The 2-AI compound CR6 is a positively charged molecule that may have similar effects on fungi as certain cationic surfactants, which have been used in agriculture to increase disease control and efficacy of fungicides. In general, surface active agents such as surfactants are used in combination with fungicides because they reduce the air-liquid and solid-liquid interfacial energies and make the contact angle between them close to zero. This in turn increases contact between the foliar fungicide spray and the leaf surface and usually increases efficacy. Surfactants are also known to increase cuticular penetration of the leaf surface because it acts as a cosolvent or solubilizing agent in the spray mixture and can increase permeability (Backman, 1978; Forsyth, 1964). The fungicidal effects of different types of surfactants alone on fungi have been studied. In experiments with species of Monilinia, Puccinia, and Alternaria cationic surfactants were found to have the greatest fungicidal effects, followed by anionic and then non-ionic surfactants. The mechanism of action is irreversible damage to cell membranes, causing leakage of cellular constituents, culminating in cell death (Forsyth, 1964).

Much research has focused on the effect of the commercial fungicide-surfactant dodine (n-dodecylguanidine acetate) on fungi alone and in combination with other fungicides. Dodine has systemic and contact capabilities and its toxicity relies on altering fungal membrane permeability. In addition dodine blocks certain vital anionic sites on the
cell surface and can interfere with specific enzyme functions (Brown and Sisler, 1960). The toxicity of dodine on many fungi, including *M. fructicola*, is very well characterized. For example, in 1964 Forsyth showed that dodine produced an EC$_{50}$ of 0.9 ppm against *M. fructicola* conidia in slide germination experiments (Forsyth, 1964).

Synergistic effects between cationic surfactants, such as dodine, and conventional fungicides have also been investigated. De Waard and Nistelrooy (1982) investigated potential synergists and antagonists of fenarimol using SBI resistant and sensitive *Aspergillus nidulans*. They showed synergistic action between cationic agents, including dodine, and fenarimol against SBI sensitive and resistant strains of the fungus. The authors proposed that the synergism mechanism likely was accumulation of the cationic agent in cell membranes that lead to increased uptake of fenarimol. In addition, dodine was negatively cross-resistant with SBI fungicides and thus mixtures of fenarimol with dodine could be beneficial for resistance management (De Waard and Nistelrooy, 1982).

### 1.6. Two-Component Regulatory Systems

Current research suggests that the 2-AI compounds may target two-component regulatory systems in bacteria. Two-component regulatory systems have been described in Eubacteria, Archaea, and some eukaryotic organisms over the last decade. These act as stimulus-response coupling systems and allow organisms to sense and respond to changes in their environment (Stock et. al., 2000). The classic two-component regulatory system consists of a membrane bound histidine protein kinase (HK) and response regulator (RR).
First the HK senses an environmental stimulus and then the RR mediates a cellular response to that stimulus (Stock et. al., 2000).

Signal transduction occurs via an autophosphorylation reaction. Phosphoric groups are transferred from adenosine triphosphate (ATP) to histidine residues in the histidine kinases. The histidine protein kinases then transfer the phosphate group to an aspartic acid residue on the response regulator. Lastly, the response regulator activates an attached output domain which leads to stimulation or expression of target genes. The activity of a response regulator is controlled by the level of phosphorylation. Thus, quantitative changes in phosphorylation reactions lead to changes in the response (Stock et. al., 1989).

A less common bacterial phosphorelay system involves a multistep His-Asp-His-Asp relay mechanism (Appleby et. al., 1996). This type of system usually involves a hybrid kinase, or a histidine kinase with an attached receiver domain. First, phosphate is shuttled from the histidine residue in the transmitter to the aspartate residue in the receiver domain. The phosphate is then relayed to a histidine residue on a histidine phosphotransfer domain, which, importantly, may be on the same or a separate protein. Employment of separate histidine phosphotransfer proteins predominates in Eukaryotes (Thomason and Kay, 2000). Lastly, the response is produced when the phosphate is shuttled from the histidine phosphotransfer domain to a downstream response regulator protein (Thomason and Kay, 2000). Multi-step phosphorelays may be advantageous to the organism due to flexible signaling connections between hybrid kinases and response regulators. This is particularly true in cases when separate histidine phosphotransfer proteins are involved (Thomason and Kay, 2000).
While it is relatively rare in prokaryotes, nearly all HK’s in eukaryotes are hybrid kinases. For example, only 5 of the 30 known HK’s in E. coli are hybrid HK’s (Mizuno, 1997). Phylogenetic analysis indicates that the HK1b subfamily contains most of the prokaryotic protein kinases and the RB family contains most of their associated receiver domains. The same applies to eukaryotic phosphorelay genes. This indicates that an ancestral prokaryotic hybrid kinase may have transferred kingdoms and evolved into eukaryotic phosphorelay genes (Grebe and Stock, 1999). However, histidine phosphotransfer domains are not present in eukaryotic hybrid kinases (Thomason and Kay, 2000). Another difference involves RR domains. While transcription factor RRs are very common in prokaryotes, this is very rare in eukaryotes. SKN7 in Saccharomyces cerevisiae is the only known eukaryotic RR with DNA binding (Brown, 1993). In both prokaryotes and eukaryotes gene expression is regulated by two-component systems. However, eukaryotes also use two-component systems to regulate other signaling pathways. The eukaryotic system involves more steps in the regulation process and may assist in transmitting signals from the cytoplasm to the nucleus (Stock et. al., 2000).

Two-component regulatory systems allow organisms to sense and respond to a wide variety of environmental changes, stressors, and growth conditions. This includes chemotaxis, nitrogen regulation, phosphate regulation, osmoregulation, sporulation, secretion of degradative enzymes, virulence, and quorum sensing (Stock et. al., 1989; Wolanin et. al., 2002). In Eukarya, two-component regulatory systems help regulate hormone-dependent developmental processes (Thomason and Kay, 2000). The SLN1 gene in S. cerevisiae was
the first discovered eukaryotic yeast phosphorelay gene. *SLNI* is involved in adaptation to osmotic stress and regulates the HOG MAP kinase cascade (Ota and Varshavsky, 1993).

Histidine protein kinases and two-component regulatory systems are particularly interesting since they represent a possible target for antimicrobial therapy. They are ubiquitous in bacteria and present in some eukaryotes, but absent in mammals. Thus, inhibitory molecules could be used for human diseases. Also, compounds that could interfere with HK systems could allow the pathogen to be destroyed by the host immune system instead of direct toxic action. This could lead to less resistance development in pathogen populations (Wolanin et. al., 2002). Certain bacteria regulate resistance to antibiotics via two-component regulatory systems. For example, the Vancomycin resistance system is present in *Streptococcus pneumoniae* (*VncS/VncR*) and *Enterococcus faecalis* (*VanR/VanS*) (Evers and Courvalin, 1996; Novak et. al., 1999). In addition, many bacteria and some fungi utilize two-component regulatory or phosphorelay systems for expression of virulence factors and pathogenesis (Stock et. al. 2000).

Phosphorelay targets are being identified in Eukaryotes with gene knockout studies. Dimorphism, or the transition from yeast to hyphal growth, is important for virulence in *Candida albicans* (Corner and Magee, 1997). Studies have shown that mutants of the histidine kinase *CaHK1* exhibit reduced hyphal development on solid medium as well as reduced virulence in a mouse systemic infection assay (Yamada-Okabe et. al., 1999). In addition, a mutant of *CaSSK1* response regulator in *C. albicans* also shows reduced hyphal growth and avirulence in a mouse infection model (Calera et. al., 2000). Homologs of the histidine kinase *CaNIK-1* have been found in the filamentous fungi *Neurospora crassa* and
*Aspergillus nidulans.* Similar to *C. albicans*, studies have shown reduced hyphal development in NIK-1 mutants of *N. crassa* (Alex et al., 1996). In addition, the HK gene *Mfos-1* has been isolated from *M. fructicola*. Deletion of this gene resulted in reduced virulence on prune and increased osmotic sensitivity. Such studies indicate that HKs also be important for the virulence of plant pathogenic fungi (Ma et al., 2006a).

Histidine kinase inhibitors have been investigated against the human pathogen *Pseudomonas aeruginosa*, which causes pulmonary infections in cystic fibrosis (CF) patients. Bacterial colonies form an exopolysaccharide coat called alginate in the lungs of CF patients. The alginate coat inhibits contact between bacterial cells and antibiotics, thereby increasing resistance to therapeutic drugs. Certain aromatic structures were found that inhibit the HKs *AlgR2/AlgR1* that control alginate gene expression. Histidine kinase inhibitors such as these could be used in combination with conventional antibiotics to increase their effectiveness (Matsushita and Janda, 2002).

A limited number of two-component inhibitors have been investigated to date. Compounds including salicylanilides (Macielag et al., 1998), triphenylalkyl derivatives, cyclohexenes, benzoaxines (Barrett and Hoch, 1998), and hydrophobic tyramines (Barrett et al., 1998) are currently being investigated (Stock et al., 2000). Analogs based on aromatic structures, peptide inhibitors, and phosphohistidines are also under investigation (Matsushita and Janda, 2002).
1.7. **Project Objectives**

The studies in this project were designed to quantify the antifungal effects of the 2-AI CR6 on *M. fructicola*, investigate the role of compound structure on activity, evaluate the potential to use 2-AIs to control fungicide resistant isolates, and identify potential synergistic interactions between CR6 and DMI fungicides such as propiconazole. The first objective involved quantifying the effects of CR6 on germination of conidia and mycelial growth of *M. fructicola*. Our working hypothesis was that CR6 would inhibit germination of conidia and mycelial growth in a dose dependent manner. Identifying a new compound with activity against fungi could result in development of new products for plant disease control. The second objective was to investigate the role of the compound charge in the efficacy of CR6. The CR6 compound is positively charged and there are instances where charge has played an important role in the efficacy of fungicides, such as copper and dodine acetate. Our working hypothesis was that non-charged versions of CR6 would not inhibit growth of *M. fructicola* as strongly as the positively charged version. The ability of CR6 to inhibit mycelial growth of *M. fructicola* isolates with DMI, QoI, and BZI resistant phenotypes was the focus of our third objective. There is no research to suggest that the mechanism of action of CR6 is similar to that of DMIs, QoIs, or BZIs so we hypothesized that CR6 would equally inhibit growth of all fungicide resistant phenotypes tested. New fungicides without cross-resistance to known chemistries could be used to control fungicide resistant populations. Our fourth objective was to investigate if CR6 could be used to increase the efficacy of propiconazole against both DMI sensitive and DMI resistant isolates. This involved determining if mixtures of CR6 and propiconazole would result in synergistic interactions when used in mycelial
growth assays. The DMIs are a very important group of fungicides for the control of brown rot and products that could increase their efficacy and longevity of use and could allow growers to continue using DMIs against sensitive populations and where resistance has developed. Lastly, we conducted scanning and electron microscopy studies to examine changes in gross morphology and ultrastructure in *M. fructicola* conidia treated with CR6.
1.8. Literature Cited


Bactericidal activity of orally available agents against methicillin-resistant


2. CHAPTER II:

The Effect of the 2-aminoimidazoles on *Monilinia fructicola* (an ascomycete) and Interactions with Selected Fungicides
2.1. Introduction

Fungicides are used to control many plant diseases caused by fungi, including brown rot of peach caused by *Monilinia fructicola* (Winter) Honey. Host resistance in peach cultivars is not available so growers rely heavily on fungicides to control the disease. However, fungicide-resistant populations of *M. fructicola* have developed several times in the last 40 years, rendering certain products completely ineffective or reducing their efficacy to an unacceptable level. Benomyl was first introduced in 1969 in the U.S., and by 1976 resistance was reported in Michigan cherry orchards (Jones and Ehret, 1976) and South Carolina peach orchards (Zehr et al., 1991) and in California peach orchards by 1977 (Szkolnik et al., 1978). Resistance to the benzimidazole fungicides (BZIs) caused by point mutations in the β-tubulin gene have been persistent in orchards long after benomyl use was discontinued (Koenraadt et al., 1992; Zehr et al., 1991). Demethylation inhibitor fungicides (DMIs) were first introduced in the 1970s and had replaced the BZI fungicides by the 1980s due to BZI resistance development. DMIs specifically target sterol 14α-demethylase at cytochrome P-450 (Buchenauer, 1987). However, prolonged use of the DMIs led to a buildup of resistant *M. fructicola* populations. Resistance has been reported in isolates from New Jersey (Burnett et al., 2010), Georgia (Schnabel et al., 2004), South Carolina (Zehr et al., 1999), New York (Villani and Cox, 2008), and Ohio (Luo et al., 2008). Resistance is multi-gene and quantitative and likely due to mutations in the DMI target gene *CYP51*, overexpression of *CYP51*, and certain energy-dependent drug efflux mechanisms (Delye et al., 1998; De Waard et al., 1993; Hamamoto et al., 2000; Hayashi et al., 2003; Koller et al., 1997; Luo and Schnabel, 2008; Ma et al., 2006b; Nakaune et al., 1998). More recently, the
quinone outside inhibitor (QoI) fungicides were introduced in 1996 and inhibit electron transport in the mitochondrial respiratory chain at complex III by binding to the cytochrome b site (Bartlett et al., 2002). Reduced sensitivity to the QoIs has since been identified in populations of *M. fructicola* from New York and South Carolina (Villani and Cox, 2008; Amiri et al., 2010).

Because crop protection products are needed to control many plant diseases and resistant pathogen populations often develop, the search for new chemistries and molecules is critical. Sea sponges of the genus *Agelas* (Family Agelasidae) are prevalent in Caribbean coral reef communities and produce unique secondary metabolites that are primarily composed of brominated pyrrole alkaloids (Braekman et al., 1992). These biologically active natural products generated interest due to their anti-predatory, antimicrobial, and antibiofilm effects (Pawlik et al, 1995). A group in the North Carolina State University Chemistry Department has synthesized a library of 2-aminoimidazole (2-AI) compounds, including the oroidin derivative analog CR6 (MW = 274). This compound is positively charged and contains a 2-aminoimidazole heterocycle linked to an 11 carbon alkyl chain (Ballard et al., 2008; K. Liberator & C. Melander, personal communication). New compounds such as the 2-AIs could be developed into fungicides or be used in combination with commercially available products to enhance disease control. We quantified the fungicidal effects of the 2-AI CR6 on *M. fructicola*, investigated the role of compound structure on activity, evaluated the potential to use 2-AIs to control fungicide resistant isolates, identified synergistic interactions between CR6 and propiconazole, and examined CR6-treated conidia with scanning and electron microscopy.
2.2. Materials and Methods

2.2.1. 2-Aminoimidazole synthesis

All compounds were synthesized by the Melander laboratory in the North Carolina State University Chemistry Department according to previously published methods (Ballard et. al., 2008).

2.2.2. Monilinia fructicola isolates

Two M. fructicola isolates, 10-0 and 1-4, were isolated from diseased peach collected from Candor, North Carolina in 2008 and 2009, respectively (Table 1). M. fructicola isolates 99.2A3.04, GADL3-03, Bmpc7, Dmap2-08, Mf.Pdt3-07, Mf.Pdt9-07, Mf.Bpc15-07, and FRf1-08 were supplied by the laboratory of Guido Schnabel at Clemson University (Table 1). Isolates 99.2A3.04 and GADL3-03 were sensitive to all fungicides tested. Bmpc7 and GADL3-03 were resistant to DMI fungicides, Mf.Pdt3-07 and Mf.Pdt9-07 were resistant to BZI fungicides, and Mf. Bpc15-07 and FRf1-08 had reduced sensitivity to QoI fungicides.

2.2.3. Standard procedure for production of conidia

Isolate 10-0 was stored on PDA at 4ºC before use. A 5 mm plug of isolate 10-0 was transferred to potato dextrose agar (PDA) and incubated for seven days at 25ºC in the dark. Subsequently, a 5 mm plug was taken from the outer growing edge of each 7-day-old colony and transferred to the center of a V8 agar petri dish. The cultures were then incubated at 25ºC for 7 days with a 12 hour photoperiod. After incubation each petri dish was flooded with 5 ml of sterile deionized water (SDW) and scraped with a rubber policeman to dislodge
conidia. The resulting conidial suspension was then filtered through four layers of sterile cheesecloth and vortexed. A hemocytometer was used to determine the conidial density and the suspension was adjusted to $1 \times 10^6$ conidia ml$^{-1}$. All conidial suspensions were prepared immediately before use.

2.2.4. **Standard procedure for conidial germination assays**

All suspensions were thoroughly mixed and a 20 ul aliquot was immediately transferred to the surface of a clean and sterile glass slide. Two repetitions per treatment were prepared and all slides were placed in a moist chamber and incubated in the dark for 12 hours. After incubation a glass coverslip was placed on each drop of conidial suspension and germinated and non-germinated conidia were counted under the microscope. A total of 50 conidia were observed for each replication and a percent germination was calculated for each. A conidium was considered germinated when the length of the germ tube exceeded half the minor diameter of the conidium.

2.2.5. **Standard procedure for mycelial growth assays**

Isolates were stored on PDA at $4^\circ$C before use. A 5 mm plug of each isolate was transferred to PDA and incubated for 7 days at $25^\circ$C in the dark. A 3 mm plug taken from the growing edge of each 7-day-old culture was transferred to the center of each petri dish. All petri dishes were incubated at $25^\circ$C for 4 days in the dark. At the end of the incubation period colony diameters were measured in two perpendicular directions for each repetition and averaged.
2.2.6. Conidial germination assay: selection of CR6 from 2-aminoimidazole library

2-AI analogs coded as 1D3, 1C10, 1C9, 2A5, 2B2, 2B3, CR6, and 3H5 were included in the experiment. The 2-AI active ingredients were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions at concentrations of 100 mM. The 2-AI stock solution was added SDW to make a 1000 uM working solution. An equivalent volume of DMSO was added to SDW to make a 1000 uM working solution for the control. Individual 1000 ul conidial suspensions were prepared in 1.5 ml microcentrifuge tubes by combining the appropriate amount of 2-AI or DMSO working solution with conidial suspension, 8% sucrose, and SDW. The final suspensions contained 1 x 10^5 conidia, 1% sucrose, and 0 and 100 uM of each 2-AI analog. Analogs 2B2, 2B3, CR6, and 3H5 were subsequently tested at 0, 25, and 50 uM as well. The standard procedure for conidial germination assays was then followed. The experiment was conducted once.

Arcsine square root percent transformation was applied to all percent germination values. Analysis of variance was performed on transformed values and followed by the Student-Neuman-Keuls post-hoc test for means separation using Agricultural Research Manager (ARM version 8, Gylling Data Management; Brookings, SD). An alpha of 0.05 was used.

2.2.7. Conidial germination assays: sensitivity of North Carolina isolate to CR6

CR6 active ingredient was dissolved in DMSO to achieve a concentration of 27,409 ug/ml. CR6 stock solution was added to SDW to make a 274.1 ug/ml working solution. An equivalent amount of DMSO was added to SDW to make a 274.1 ug/ml DMSO working
solution for the control. Standard procedure for conidial production was followed. Individual 1000 ul conidial suspensions were prepared in 1.5 ml microcentrifuge tubes by combining the appropriate amount of CR6 or DMSO working solution with conidial suspension, 8% sucrose, and SDW. The final suspensions contained $1 \times 10^5$ conidia, 1% sucrose, and 0, 0.548, 1.096, 1.644, 2.192, 2.741, 3.289, or 3.837 ug/ml CR6. The standard procedure for conidial germination assays was followed. The experiment was repeated three times.

The % germination for each treatment was transformed to % relative germination of the control. A log$_2$ transformation was applied to all CR6 concentrations. The mixed procedure (SAS; Cary, NC) was used to generate a cubic equation and dose response curve to model % relative germination associated with increasing rates of CR6.

### 2.2.8. Mycelial growth assays: sensitivity of North Carolina isolates to CR6

Sensitivity to CR6 was determined with mycelial inhibition assays. CR6 active ingredient was dissolved in DMSO to achieve a concentration of 27,409 ug/ml. CR6 stock solution was added to SDW to make CR6 working solutions and DMSO was added to SDW to make a control working solution. A 1:100 dilution was prepared by incorporating 1 ml of each working solution into 100 ml of autoclaved PDA cooled to 60ºF. Final concentrations of CR6 amended PDA included 0, 3.4, 6.9, 13.7, and 27.4 ug/ml. Fifteen to 20 ml of PDA was dispensed into each petri dished and allowed to solidify. Isolates 10-0 and 1-4 were used in the experiments. Standard procedure for mycelial growth assay was followed. Six
repetitions per isolate per concentration were included and the experiment was repeated three times.

Measurements of mycelial growth were transformed to relative growth of the control for each isolate. A log₂ transformation was applied to all concentrations of CR6. Using the mixed procedure (SAS; Cary, NC) linear regression analysis was used to determine the effective concentration of each fungicide required to inhibit fungal colony growth by 50% (EC₅₀). EC₅₀ was estimated for each strain and fungicide by solving the equation for x:

\[ 0.50 = B_0 + B_1 \cdot x \implies x = (0.50 - B_0)/B_1 \]

Estimated standard errors for confidence intervals were obtained via the delta method (Casella and Berger, 2002).

Let \( n = (0.50 - B_0) \) be the numerator and \( d = B_1 \) be the denominator

\[ \text{Estimated SE}(x) = (x^2) \cdot \left( \frac{\text{Var}(n) / (n^2)}{n^2} + \frac{\text{Var}(d) / (d^2)}{d^2} - 2 \cdot \text{Cov}(n,d) / (n \cdot d) \right) \]

2.2.9. Conidial germination assays: charged and non-charged analogs

Non-charged 2-AI version #1 was created using single atom replacement and non-charged 2-AI version #2 was created via chemical modification, or acylation (Figure 4). CR6, CR6 non-charged version #1, and CR6 non-charged version #2 active ingredient were each dissolved in DMSO to achieve a concentration of 27,409 ug/ml. Stock solutions were added to SDW to make 274.1 ug/ml CR6 working solutions. DMSO was added to SDW to make a 274.1 ug/ml DMSO working solution for the control. Standard procedure for conidial production was used. Individual conidial suspensions were prepared in 1.5 ml microcentrifuge tubes by combining the appropriate amount of CR6 or DMSO working
solution with conidial suspension, 8% sucrose, and SDW. For each compound-concentration combination the resulting suspensions contained 1 x 10^5 conidia, 1% sucrose, and 0, 6.9, 13.7, and 27.4 ug/ml of the analog. The standard procedure for conidial germination assays was followed. The experiment was repeated three times.

Analysis of variance was conducted with proc GLM (SAS, Cary, NC) followed by the Student-Neuman-Keuls post-hoc test for means separation. An alpha of 0.05 was used.

2.2.10. Mycelial growth assays: confirmation of fungicide resistant phenotypes

Sensitivity to propiconazole (Orbit 3.6E; Syngenta Crop Protection, Greensboro, NC), azoxystrobin (Abound; Syngenta Crop Protection, Greensboro, NC), and thiophanate-methyl (Topsin M 70 WP; Elf Atochem North America, Inc., Philadelphia) was assessed by incorporating formulated material into SDW which was added to autoclaved PDA cooled to 60°F. Final concentrations of propiconazole included 0, 0.0001, 0.001, 0.01, 0.1, 1.0, and 10 ug ai/ml. Final concentrations of azoxystrobin included 0, 0.001, 0.01, 0.1, 1.0, and 10 ug ai/ml. Salicylhydroxamic acid (SHAM) was added at 100 ug/ml to all azoxystrobin amended PDA in order to suppress the alternative oxidative pathway. Final concentrations of thiophanate-methyl included 0, 0.0625, 0.125, 1.0, 4.0, 8.0, 50.0, 100.0, and 500.0 ug ai/ml. Fifteen to 20 ml of PDA was dispensed into each petri dish and allowed to solidify. The isolates GADL3-03 and 99.2A3.04, which were known to be sensitive to all fungicides tested, were included in each assay. Bmpc7 and Dmap2-08 were tested for sensitivity to propiconazole, Mf.Bpc15-07 and FRF1-08 were tested for sensitivity to azoxystrobin, and Mf.Pdt9-07 and Mf.Pdt3-07 were tested for sensitivity to thiophanate-methyl. Standard
procedure for mycelial growth assays was followed. Three repetitions per isolate per concentration were included and the experiment was conducted once.

Measurements of mycelial growth were transformed to relative growth of control for each isolate. A $\log_{10}$ transformation was applied to all concentrations of propiconazole and azoxystrobin and a $\log_2$ transformation was applied to all concentrations of thiophanate-methyl. To determine the effect of thiophanate-methyl on BZI-sensitive and BZI-resistant isolates we used the mixed procedure (SAS; Cary, NC) to generate equations for parallel quadratic curves with separate slopes for each isolate. The same procedure was used to generate linear curves for DMI-sensitive isolates and parallel quadratic curves with separate slopes for DMI-resistant isolates. The mixed procedure (SAS; Cary, NC) was used to generate linear regression equations with separate slopes and separate intercepts for QoI-sensitive and QoI-resistant isolates.

2.2.11. Mycelial growth inhibition assays: sensitivity of fungicide resistance phenotypes to CR6

Sensitivity to CR6 was determined by incorporating technical grade material into DMSO to achieve a concentration of 27,409 ug/ml. CR6 stock solution was added to SDW to make working solutions that were incorporated into autoclaved PDA cooled to 60°F. Final concentrations of CR6 amended PDA included 0, 1.7, 3.4, 6.9, 13.7, and 27.4 ug/ml. Fifteen to 20 ml of PDA was dispensed into each petri dished and allowed to solidify. Isolates GADL3-03, 99.2A3.04, Bmpc7, Mf.Pdt3-07, and Mf.Bpc.15-07 were included. Standard
procedure for mycelial growth assays was followed. Three repetitions per isolate and concentration combination were included and the experiment was repeated three times.

Measurements of mycelial growth were transformed to relative growth of control for each isolate. A log₂ transformation was applied to all concentrations of CR6. For Mf.Bpc15-0, 99.2A3.04, GADL3-03, Mf.Pdt3-07 and Bmpc7 mycelial growth inhibition was regressed on log₂ CR6 concentration and jointly modeled using proc GLM (SAS; Cary, NC). EC₅₀ was estimated for each strain and fungicide by solving the equation for x:

\[ 0.50 = B0 + B1 \times x \Rightarrow x = (0.50 - B0)/B1 \]

Estimated standard errors for confidence intervals were obtained via the delta method (Casella and Berger, 2002).

Let n = (0.50-B0) be the numerator and d = B1 be the denominator

Estimated SE(x) = (x²)*(Var(n)/(n²) + Var(d)/(d²) - 2*Cov(n,d)/(n*d))

2.2.12. Mycelial growth inhibition assays: determination of synergism between CR6 and propiconazole

Synergism between propiconazole and CR6 was determined with mycelial inhibition assays. Propiconazole (Orbit 3.6E; Syngenta Crop Protection, Greensboro, NC) formulated material was diluted in SDW as a working solution. CR6 active ingredient was dissolved in DMSO to achieve a concentration of 27,409 ug/ml and CR6 stock solution was then diluted in sterile deionized water to create working solutions. Working solutions of propiconazole and CR6 were incorporated into autoclaved PDA cooled to 60°F. Final concentrations of propiconazole alone included 0, 0.01 and 0.1 ug/ml and final concentrations of CR6 alone
were 0, 0.428, 0.856, and 3.426 ug/ml. Each rate of propiconazole was also combined with each rate of CR6. Fifteen to 20 ml of PDA was dispensed into each petri dish and allowed to solidify. The isolates GADL3-03, 99.2A3.04, Bmpc7, and Dmap2-08 were included. Standard procedure for mycelial growth assays was followed. Three repetitions per isolate and product combination were included. The experiment was repeated three times.

The Abbott method and Gowing formula were used to assess synergism and antagonism (Kossman and Cohen, 1996). The observed joint effect of inhibition was compared to the expected level of inhibition from individual replications of different combinations of CR6 and propiconazole. For each date and appropriate combination of fungicide levels, the estimated inhibition was calculated. Letting C1 be the estimated inhibition due to CR6 and C2 the estimated inhibition due to propiconazole, the expected joint inhibition was C1+C2(1-C1). The observed inhibition was then compared to the expected inhibition with the hypothesis test t-test (SAS; Cary, NC). A two-tailed t-test was performed to identify both synergistic and antagonistic interactions.

2.2.13. **Scanning and transmission electron microscopy**

The standard procedure for conidial production was used to prepare all conidial suspensions. CR6 active ingredient was dissolved in DMSO to achieve a concentration of 27,409 ug/ml. CR6 stock solution was added to SDW to make a 274.1 ug/ml working solution. An equivalent volume of DMSO was added to SDW to make a working solution for the control treatment. Three hours prior to fixation individual conidial suspensions were prepared in 15 ml centrifuge tubes by combining the appropriate amount of CR6 or DMSO
working solution with conidial suspension, 8% sucrose, and SDW. The resulting suspensions contained $1 \times 10^5$ conidia, 1% sucrose, and 0 (for DMSO control), 2.7, and 6.9 ug/ml of CR6. Another identical conidial suspension was prepared one hour before fixation containing 0 ug/ml CR6. Conidial suspensions were kept at 25°C in the dark and shaken occasionally before fixation. Lastly, a *M. fructicola* culture growing on V8 agar was fixed for SEM to examine gross morphology of an undisturbed colony.

For scanning electron microscopy techniques, tubes containing conidia were spun at 2,500 RPM for 10 minutes, the supernatant discarded and the sample resuspended with 3% GTA in 0.1M Na cacodylate buffer pH 6.8 at 4°C. Five squares of agar containing mycelia and conidia from the plated fungus were excised and placed in a vial containing the above fixative on ice. All samples were stored at 4°C for several days.

A small aliquot of each conidial sample was placed in a syringes containing 1 ml of 0.1M Na cacodylate buffer pH 6.8 and filtered through a 0.45µ Nucleopore filter. The filter was removed and placed in a vial of the same buffer at 4°C. The filters were washed in three 30-minute changes of buffer, and then dehydrated in a graded (30, 50, 70, 95, 100 X 3 changes) ethanol series at 4 degrees C, warming to room temperature in the 100% ethanol. All samples (filters and agar blocks) were critical point dried in liquid CO$_2$ for 15 minutes at critical point using a Tousimis Samdri-795 (Tousimis Research Corporation, Rockville, MD) and immediately mounted on stubs with double-stick tape and silver paint. Samples were coated with a total of approximately 60Å of Au/Pd in a Hummer 6.2 sputtering system (Anatech USA, Union City, CA). Samples were viewed using a JEOL JSM-5900LV.
Scanning Electron Microscope (JEOL U.S.A, Peabody, MA) at 15kV. Digital images were captured using a JEOL Digital Scan Generator imaging system.

For transmission electron microscopy techniques the tubes were spun at 2,500 RPM for 10 minutes and the fixative was removed. The sample pellets were transferred to microcentrifuge tubes for the remainder of the processing. The samples were washed in three 15-minute buffer washes (spun between solution changes) then post-fixed in 2% osmium tetroxide in the same buffer for 1 hour at 4°C, followed by 3 more changes of cold buffer as above. The tubes were spun again and washed with a brief dH₂O rinse, spun one more time and embedded in 2% agarose for completion of the processing. The pellet containing the cells was cut off and divided into 1mm³ blocks in cold dH₂O and then the blocks were placed in cold 30% ethanol for dehydration in a graded (30, 50, 70, 95, 100 X 3 changes) series, warming to room temperature in the 100% ethanol. Blocks were embedded in Spurr’s Resin (Ladd Research, Williston, VT) in BEEM capsules at 70°C overnight. Blocks were hand-trimmed and thin-sectioned at 75-80nm using an LKB NOVA Ultramicrotome (Leica Microsystems, Buffalo Grove, IL). Sections were stained with 4% uranyl acetate and Reynold’s lead citrate. Samples were viewed and photographed using a JEOL JEM-1200EX (JEOL U.S.A, Peabody, MA) at 80kV. Photographs were initially recorded using Kodak 4489 Electron Microscope film (Eastman Kodak Co., Rochester, NY), developed and then digitized using an Epson Perfection 4870 Photo (Epson America Inc., Long Beach, CA) flatbed scanner at 1200dpi, and processed to a positive image in Adobe Photoshop (Adobe Systems Inc., San Jose, CA).
2.3. Results

2.3.1. Conidial germination assays: selection of CR6 from 2-aminoimidazole library

Analogs 1D3, 1C10, 1C9, and 2AS at 100 uM allowed conidial germination statistically equivalent to the control (Table 2). Analysis of variance of percent germination with 100 uM of 2B2, 2B3, CR6, and 3H5 revealed that all analogs completely inhibited germination (Table 3). The same analogs used at 25 uM and 50 uM resulted in percent germination ranging from 0 - 96.5% (Table 4). Analog CR6 was found to be most active at 25 uM and allowed 2.36% germination of conidia, which was statistically lower than other analogs tested at 25 uM. Analog CR6 was thus selected and used for further experiments.

2.3.2. Conidial germination assays: sensitivity of North Carolina isolates to CR6

The cubic equation and dose response curve generated to describe the relationship between % relative conidial germination and CR6 concentration allowed us to calculate an EC50 of 1.97 ug/ml for *M. fructicola* isolate 10-0 (Figure 1). There was considerable variation among the three runs of the experiment. However, the cubic model was associated with an R² of 0.80 and we concluded that the model explained the dose response relationship sufficiently well.

2.3.3. Mycelial growth assays: sensitivity of North Carolina isolates to CR6

Statistical analysis concluded that linear regression slopes for isolates 10-0 and 1-4 were not significantly different and that separate intercepts should be used for regression
lines (Figure 2). The EC$_{50}$s for isolate 1-4 and 10-0 were 11.37 ug/ml and 9.83 ug/ml, respectively (Figure 3). The 95% CIs for isolate 1-4 and 10-0 were 9.66-13.40 ug/ml and 8.38-11.52 ug/ml, respectively (Figure 3).

2.3.4. Conidial germination assays: charged and non-charged analogs

Percent germination of conidia treated with 6.9, 13.7 ug/ml of non-charged versions #1 and #2 and 27.4 ug/ml of non-charged version #2 did not differ significantly from the check (Table 5 and Figure 5). Non-charged version #1 at 27.4 ug/ml and all three concentrations of CR6 allowed significantly less conidial germination than the control (Table 5 and Figure 5). All three concentrations of CR6, however, were associated with significantly less percent conidial germination than 27.4 ug/ml of non-charged version #1 (Table 5 and Figure 5).

2.3.5. Mycelial growth assays: confirmation of fungicide resistant phenotypes

The EC$_{50}$s for propiconazole were 0.032, 0.037, 0.624, and 0.468 ug/ml for isolates 99.2A3.04, GADL3-03, Bmpc7, and Dmap2-08, respectively (Table 6 and Figure 7). Isolates GADL3-03 and Dmap2-08 were considered resistant to propiconazole. For thiophanate-methyl the EC$_{50}$s for isolates 99.2A3.04, GADL3-03, Mf.Pdt3-07, and Mf.Pdt9-07 were 0.324, 0.317, 2173.95, and 0.286 ug/ml, respectively (Table 6 and Figure 6). Only isolate Mf.Pdt3-07 was resistant to thiophanate-methyl and this was used in further experiments. Lastly, the azoxystrobin EC$_{50}$s for isolates 99.2A3.04, GADL3-03, Mf.Bpc15-07, and FRf1-08 were 0.084, 0.079, 0.275, and 0.443 ug/ml, respectively (Table 6 and Figure
8). Only isolate Mf.Bpc15-07 showed consistent growth and reduced sensitivity to azoxystrobin thus it was included in further experiments. The growth of isolate FRf1-08 was erratic and could not be used for further testing.

2.3.6. Mycelial growth inhibition assays: sensitivity of fungicide resistance phenotypes to CR6

All regressions were significant at \( P < 0.001 \) and all isolates showed increased inhibition as CR6 concentration increased (Figure 9). The EC\(_{50}\)s for isolates Mf.Pdt3-07, GADL3-03, 99.2A3.04, Bmpc7, and Mf.Bpc15-07 were 4.93, 5.52, 5.69, 10.07, and 18.97 ug/ml of CR6, respectively (Table 7 and Figure 10). The 95% CIs were 3.17-7.66 ug/ml, 3.49-8.74 ug/ml, 3.53-9.18 ug/ml, 5.80-17.47 ug/ml, and 9.93-36.34 ug/ml for isolates Mf.Pdt3-07, GADL3-03, 99.2A3.04, Bmpc7, and Mf.Bpc15-07, respectively (Table 7 and Figure 10). However, the 95% CIs for isolates Mf.Pdt3-07, GADL3-03, 99.2A3.04, and Bmpc7 overlapped, indicating that there were no significant differences between EC\(_{50}\)s of these isolates. In addition, the 95% CIs for isolates Bmpc7 and Mf.Bpc15-07 overlapped, also indicating a non-significant difference in sensitivity.

2.3.7. Mycelial growth inhibition assays: determination of synergism between CR6 and propiconazole

Synergistic and antagonistic interactions were found using a 2-tailed t-test and categorized as strong when \( t < 0.01 \) and weak when \( t < 0.05 \) (Table 8). There was evidence for synergism at \( t < 0.01 \) between 0.01 ug a.i./ml propiconazole plus 0.428 ug/ml and 0.856
ug/ml CR6 and 0.1 ug a.i./ml propiconazole plus 0.856 ug/ml CR6 for isolate 99.2A3.04. In addition, propiconazole at 0.01 ug a.i./ml plus CR6 at 3.426 ug/ml produced a synergistic interaction at $t < 0.05$ for isolate 99.2A3.04. The combination of 0.01 ug a.i./ml propiconazole plus 0.428 ug/ml CR6 for isolates GADL3-03 and Bmpc7 also produced a synergistic interaction at $t < 0.05$. There was evidence for antagonism between 0.1 ug a.i./ml propiconazole plus 3.426 ug/ml at $t < 0.01$ for isolates 99.2A3.04 and GADL3-03. This same propiconazole and CR6 combination produced an antagonistic interaction at $t < 0.05$ for isolate Bmpc7. Boxplots show the differences between $C_{obs}$ and $C_{exp}$ (Figure 11).

**2.3.8. Scanning and transmission electron microscopy**

Images taken with the scanning electron microscope revealed that the conidia in the control treatment had germinated, but the conidias treated with 2.7 and 6.9 ug/ml of CR6 had not germinated or formed only very short germ tubes. The pores in the Nucleopore filters in untreated samples remained visible and unclogged whereas those in treated samples appeared clogged or covered in a substance (Figure 12). In addition, the treated conidia appeared to have a rough surface and irregular depressions whereas the control conidias had a smoother surface texture (Figure 12).

Transmission electron microscopy images revealed that control conidia contained many lipid bodies, whereas those treated with 6.9 ug/ml of CR6 had very few (Figure 13). Ribosomal aggregation could also be seen in CR6-treated conidia, while ribosomes in control conidia appeared normal (Figure 14). Conidia treated with CR6 also displayed cytoplasmic vacuolization, disorganization, and coagulation (Figure 14).
2.4. Discussion

There is often a relationship between structure and activity of antimicrobial compounds. Thus, when new antimicrobial compounds are being developed the structure of the compound often has to be altered to increase activity against target organisms. For example, Lindel et. al. (2000) tested the marine natural product oroidin and various analogs of the compound in fish anti-feeding experiments. They found that the pyrrole moiety was needed for feeding deterrent activity because compounds lacking it lacked activity. In addition, they found that compounds with an imidazole group in addition to a pyrrole and side chain section were most active but that functionalized imidazoles alone were not active (Lindel et. al., 2000). In addition, structure activity analysis on a 50-compound library based on oroidin and found analogs containing a 2-aminoimidazole motif and a dibrominated pyrrolecarboxamide subunit had the most anti-biofilm activity against *P. aeruginosa* (Richards et. al., 2008). Eight analogs in the 2-AI library based on oroidin were screened and we found that the compound coded CR6 was most active (Table 4). Structural alterations to 2-AI analogs could affect their mechanism of action or ability to bind at the site of action, so it is not surprising that there is a range in activity of the 2-AIs against *M. fructicola*. Further studies are needed to elucidate which structural characteristics are most important for 2-AI efficacy against *M. fructicola* and other fungi.

Certain concentrations of CR6 were inhibitory to *M. fructicola* conidial germination and mycelial growth. The EC$_{50}$ was 1.96 ug/ml for isolate 10-0 based on the cubic model that described the dose response relationship (Figure 1). There was considerable variability among the three repeats of the conidial germination experiments. It is possible that the
system we used to measure conidial germination affected the ability of CR6 to interact with *M. fructicola* conidia. Also, the concentrations used ranged from only 0 – 3.847 ug/ml in increments of 0.548 ug/ml. Perhaps a more accurate assay should be used when working with such a small range of concentrations. The EC₅₀'s calculated from mycelial growth assays with North Carolina isolates 10-0 and 1-4 were 9.83 and 11.37 ug/ml, respectively (Figures 2 and 3). Thus, in *M. fructicola* CR6 is more inhibitory to conidial germination than mycelial growth. Examples in differences in sensitivity between conidia and mycelia can be found in the literature. For example, studies demonstrated that conidial germination was particularly sensitive to azoxystrobin because it disrupts energy production and greatly impacts energy-demanding stages of fungal development such as spore germination (Bartlett et. al., 2002; Godwin et. al., 1994). In contrast, triazole fungicides, which inhibit ergosterol biosynthesis, do not prevent spore germination because spores contain a supply of ergosterol needed for germination and therefore do not need to synthesize it (Bartlett et. al., 2002). In comparison to CR6, EC₅₀'s associated with most modern fungicides are much lower. For example, a mycelial growth study conducted with fungicide sensitive *M. fructicola* isolates from South Carolina indicated that EC₅₀'s for azoxystrobin, propiconazole, and thiophanate-methyl ranged from 0.08-0.13 ug/ml, 0.003-0.013 ug/ml, and 0.11-0.14 ug/ml, respectively (Luo and Schnabel, 2008). Our experiments with fungicide sensitive isolates indicated EC₅₀'s ranging from 0.079-0.084 ug/ml, 0.035-0.045 ug/ml, and 0.317-0.324 ug/ml for azoxystrobin, propiconazole, and thiophanate-methyl, respectively (Table 6). Although CR6 is not as inhibitory at low concentrations as most modern fungicides, structure activity relationship studies could allow researchers to develop an analog specifically designed for fungicidal
action. Conversely, CR6 could be used as an additive to increase the effectiveness of other products rather than being used alone.

The lead 2-AI compound CR6 is a positively charged molecule that may have similar effects on fungi as certain cationic surfactants, which have been used in agriculture to increase disease control and efficacy of fungicides. In general, surface active agents such as surfactants are used in combination with fungicides because they reduce the air-liquid and solid-liquid interfacial energies and make the contact angle between them close to zero (Backman, 1978; Forsyth, 1964). Fungal spores are negatively charged and cationic surface active agents react at the cell surface (Douglas et. al., 1959; Hannan, 1961; Somers and Pring, 1966). In experiments with species of _Monilinia, Puccinia_, and _Alternaria_ cationic surfactants were found to have the greatest fungicidal effects. The mechanism of action is likely irreversible damage to cell membranes causing leakage of cellular constituents, culminating in cell death (Forsyth, 1964).

Much research has focused on the effect of the commercial fungicide-surfactant dodine (n-dodecylguanidine acetate) on fungi alone and in combination with other fungicides. Dodine has systemic and contact capabilities and its toxicity relies on altering fungal membrane permeability. In addition, dodine blocks certain vital anionic sites on the cell surface and can interfere with specific enzyme functions (Brown and Sisler, 1960). The toxicity of dodine on many fungi, including _M. fructicola_, is very well characterized. For example, in 1964 Forsyth showed that dodine produced an EC$_{50}$ of 0.9 ppm against _M. fructicola_ conidia in slide germination experiments (Forsyth, 1964). Somers and Fisher investigated the effect of dodine on _Neurospora crassa_ and found positively charged dodine
cation reacted with surface carboxyl and phosphate groups of the spores in solution (Somers and Fisher, 1967). The anionic carboxyl and phosphate groups located near the cytoplasmic membrane of many fungal spores are considered binding sites for cationic fungicides (Somers, 1966). The same is true for the effect of dodine on *M. fructicola* (Brown and Sisler, 1960). When investigating the mechanism of action of dodine, Brown and Sisler (1960) found that a 20-fold increase in dodine concentration was required to maintain efficacy if the pH of the medium was lowered from 7.8 to 5.1. However, the authors reported that pH was not likely influencing toxicity by altering the charge of the guanidine group (Brown and Sisler, 1960).

We conducted experiments to determine if the positive charge of CR6 was important for its efficacy against *M. fructicola* conidia. The normal CR6 compound was assayed against non-charged version #1, which was altered by a single atom replacement, and non-charged version #2, which was manipulated via acylation (Figure 4). The results indicate that there was a loss of activity that occurred with the loss of charge (Table 5 Figure 5). Both non-charged version #1 and #2 inhibited conidial germination significantly less than the normal positively charged CR6. Out of all rates tested of the modified compounds, non-charged version #1 at 27.4 ug/ml was the only treatment that was associated with significantly less conidial germination than the control. These data indicate that the charge of CR6 is important for its efficacy and likely plays a role in its mechanism of action against fungal conidia. Although single atom replacement and acylation techniques were chosen to eliminate the charge of CR6 because they created minimal structural alterations to the compound, it is possible that these changes altered the binding ability of CR6 independent of
charge. Further studies should be conducted to verify that the change in charge was the only reason for reduced activity of non-charged versions.

Rogers et. al. (2010) also designed experiments to investigate the possible mechanism of action of a positively charged 2-AI compound against bacteria. They found that levels of Ca(II) cations in their media modulated activity and that the addition of CaCl$_2$ to the media suppressed resensitization and antibiofilm effects of the 2-AI against MRSA strain BAA-44. The addition of 25 g/L of CaCl$_2$ decreased biofilms inhibition from 100% to 40% with 100 uM of the 2-AI (Rogers et. al., 2010). A similar phenomena was observed between Zn(II) and a 2-aminobenzimidazole. When growth medium was supplemented with 200 uM of ZnCl$_2$, the 2-ABI was not able to inhibit biofilm formation by several gram-positive bacteria. However, a $^1$H NMR binding study detected that the 2-ABI was binding directly to Zn(II) (Rogers et. al., 2009). Authors investigating mixtures of positively charged miconazole with Ca(II) and Mg(II) found that these metal cations reduced growth inhibition of Candida albicans (Cope, 1980). Considering that cationic products interact with negatively charged sites on fungal spore surfaces, the authors hypothesized that the decrease in activity of miconazole could be due to competition with Ca(II) and Mg(II) for negatively charged binding sites (Cope, 1980). Studies need to be conducted to determine if the addition of metal cations suppress the fungicidal effects of CR6 on M. fructicola as well. It is possible that certain metal cations interact directly with the CR6 compound, as with Zn(II), or that they compete for binding sites on the cell surface, as with Ca(II).

The 2-AI based molecules have proven synergistic action with several antibiotics for controlling medically relevant antibiotic resistant bacteria (Huigens, III et. al., 2010; Rogers
et. al., 2010). For example, a 2-AIT compound was shown to have a synergistic effect on *Staphylococcus aureus* biofilm dispersion when mixed with novobiocin. The effect was, however, dependent on antibiotic concentration (Rogers et. al., 2010). In addition, authors demonstrated that the 2-AI produced a synergistic effect and controlled growth of multi-drug resistant *Escherichia coli* when combined with tetracycline. Growth of both resistant and sensitive strains of *E. coli* were reduced by 99% when 50 uM of tetracycline was combined with 150 uM of the 2-AI. The 2-AI at 150 uM alone used by Rogers et. al. (2000) did not inhibit bacterial growth, indicating a non-microbialidal mechanism (Rogers et. al., 2010). The 2-AI, CR6, used in our study did slightly inhibit mycelial growth of *M. fructicola* when applied alone and at low concentrations. For example, 0.856 ug/ml of CR6 reduced radial growth of isolate 99.2A3.04 by 13.6%. There are many 2-AI analogs in the library produced by the Melander laboratory so it is possible that certain analogs might be more directly antifungal than others. In addition, target sites on fungi and bacteria are often very different so analogs that are not toxic to bacteria still could be fungicidal. Other cationic compounds have proven synergistic effects, such as Cu(II) combined with Polycide, a quaternary ammonium compound, to control *P. aeruginosa* biofilms (Harrison et. al., 2008). Because Cu(II) is an electrophile, it may displace similar transitional metal ions such as Fe (III) and result in toxicity (Harrison et. al., 2007).

Potential synergistic effects between 2-AIs and conventional fungicides have important implications for the agricultural industry, where fungicide resistant populations of pathogens are constantly evolving. Mixing fungicides can widen the disease control spectrum of a product, may result in synergistic interactions between products, and could
delay the selection of resistant individuals in the population (Gisi, 1996). Synergism is a particularly attractive result of mixing products because the same level of disease control could be achieved with lower rates of each product. In our studies we found both synergism and antagonism between different concentrations of propiconazole and CR6 when used to control mycelial growth of DMI-sensitive and resistant isolates (Table 8). As in experiments done by Rogers et. al. (2010), this indicates that synergism is dependent on dose or ratio of products in the mixture (Rogers et. al., 2010). Synergistic and antagonistic interactions in our experiments demonstrate that the relationship between products is impacted by dose and fungal isolate (Table 8). Strong and weak synergism was most often detected with 0.01 ug/ml of propiconazole mixed with 0.428 ug/ml of CR6. In fact, for the DMI-sensitive isolate GADL3-03 and the DMI-resistant isolate Bmpc7, this was the only rate combination that resulted in synergism. This may imply that synergism is more likely to occur when the propiconazole component is not providing a very high level of control on its own. For example, 0.01 ug/ml of propiconazole alone inhibited growth of 99.2A3.04 and GADL3-03 by 13% and 18%, respectively. The same concentration actually increased growth of DMI-resistant isolate Bmpc7 by 6%. Conversely, CR6 at 0.428 ug/ml alone did not inhibit growth of any isolate. However, when combined these concentrations were able to provide more control than statistically expected according to the Abbott Method/Gowing Equation (Kossman and Cohen, 1996). Rogers et. al. (2010) also found synergistic interactions between a 2-AI and conventional antibiotics when 150 uM of the 2-AI alone did not inhibit bacterial growth (Rogers et. al., 2010). Harrison et al. (2008) also found antagonism against P. aeruginosa biofilms when high rates of Cu(II) and Polycide were combined, whereas
lower concentrations resulted in synergism (Harrison et. al., 2008). As with the combination of CR6 and propiconazole, more is not necessarily better. In addition, the statistical procedure and its strength to measure synergism are important. The Abbott approach/Gowing formula was used in our experiments. This procedure does not distinguish between synergistic and additive action well when a high effective dose of either component is used (Kossman and Cohen, 1996). In our synergism experiments, however, the greatest % inhibition due to any one concentration of CR6 or propiconazole was 46%, which we deemed sufficiently low.

There are multiple examples in the literature where positively charged cationic surfactants and metals produced synergistic interactions when mixed with fungicides. Cationic agents, including dodine, had synergistic action when mixed with fenarimol, a sterol biosynthesis inhibitor fungicide, and tested against Aspergillus nidulans in crossed-paper strip assays. This was true when tested against both wild-type and fenarimol-resistant strains. Interestingly, dodine alone was more toxic to resistant than wild type strains of A. nidulans (De Waard and Nistelrooy, 1982). By contrast, in our experiments the CR6 EC₅₀s for DMI-sensitive isolate were 5.52 ug/ml and 5.59 ug/ml and 10.07 ug/ml for the DMI-resistant isolate but were not statistically different from each other (Table 7 and Figure 10). De Waard and Nistelrooy (1982) also found that increasing concentrations of fenarimol and dodine did not always increase inhibition or synergism (De Waard and Nistelrooy, 1982). Several theories were developed to explain synergism between dodine and fenarimol. First, dodine could accumulate in fungal cell membranes and enhance the uptake of fenarimol. The cationic surfactant could also inhibit energy-dependent efflux of fenarimol, again
allowing the fungicide to accumulate in mycelia (De Waard and Nistelrooy, 1980). The
cationic agent Cu(II) was also shown to inhibit efflux pumps in DMI-resistant and sensitive
isolates of *A. nidulans* (De Waard and Nistelrooy, 1987).

Although synergism may be detected in *in vitro* experiments, it is also crucial to
confirm effects under field conditions. Chemical interactions with plants and environmental
factors could likely affect what components of a mixture are available at the site of action
(De Waard and Nistelroy, 1982). Worthington et. al. (2012) conducted 3 years of field trials
combining a 2-AI with copper and other antibacterial and plant defense activating
compounds between 2008 and 2010. They found that combining the 2-AI with copper
hydroxide (Kocide 3000) and other products resulted in decreased bacterial spot foliar
disease caused by the copper resistant *Xanthomonas euvesicatoria* strain Xcv 611 Cu\(^R\)
(Worthington et. al., 2012). Field testing should be done to confirm the fungicidal effects of
CR6 as well as synergistic and antagonistic interactions between CR6 and propiconazole.

Results from our scanning and transmission electron microscopy studies indicated
changes in gross morphology and ultrastructure in *M. fructicola* conidia treated with CR6.
Scanning electron microscopy images showed that treated conidia had a rougher surface
texture and irregular depressions that were not present in the control spores (Figure 12). This
could be due to changes in membrane permeability, which could result in a loss of cellular
constituents. In addition, the Nucleopore filter in control samples was unobstructed, while
the pores appeared clogged and covered in a substance in treated samples (Figure 12). It is
possible that cellular constituents that were lost from treated conidia formed a film on the
filter. The ultrastructure of CR6-treated conidia also looked similar to *M. fructicola* conidia
exposed to heat treatment by Baker and Smith (1970). In both cases, ribosomal aggregates can be seen (Figure 14) (Baker and Smith, 1970). In addition, lipid bodies were mostly absent in CR6-treated conidia as compared to the control (Figure 13). Lipid bodies act as energy reserves to fungal cells so the loss or dissolution of these structures could prevent germination and growth of *M. fructicola* conidia (Deacon, 2006). Overall cytoplasmic vacuolization, disorganization, and coagulation could be seen in many of the CR6 treated conidia, likely indicating a loss of viability (Madrigal and Melgarejo, 1995).

Our studies indicated that the 2-AI compounds may be effective in the control of agriculturally relevant fungal pathogens. We found that certain analogs in the 2-AI library inhibited growth of *M. fructicola* while others did not, indicating a structure-activity relationship among analogs. The analog CR6 was shown to inhibit conidial germination and mycelial growth in a dose dependent manner when tested against two North Carolina isolates of the fungus. The activity of CR6 was decreased or eliminated when chemical modification was used to neutralize compound charge, suggesting that the positive charge is important for its mechanism of action. CR6 also inhibited growth of several isolates with different fungicide resistance phenotypes and produced synergistic effects when combined with propiconazole. Lastly, scanning and transmission electron microscopy studies indicated changes in gross morphology and ultrastructure of CR6-treated conidia. The 2-AIs have shown potential in our studies and should be investigated further in field trials to determine if they could be integrated into disease management practices.
2.5. Acknowledgements

We would like to thank G. Schnabel (Clemson University) for providing fungal isolates used in this study. We would also like to thank V. Knowlton (North Carolina State University) and the Center for Electron Microscopy for their excellent work on scanning and electron microscopy portions of this study. We would like to thank C. Arellano (North Carolina State University) for her outstanding statistical analysis and consulting.
2.6. Literature Cited


TABLE 1. *Monilinia fructicola* isolates used in experiments.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Year Isolated</th>
<th>Phenotype(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-0</td>
<td>Candor, NC</td>
<td>2008</td>
<td>n/a</td>
</tr>
<tr>
<td>1-4</td>
<td>Candor, NC</td>
<td>2009</td>
<td>n/a</td>
</tr>
<tr>
<td>99.2A3.04</td>
<td>Edgefield, SC</td>
<td>2004</td>
<td>Sensitive</td>
</tr>
<tr>
<td>GADL3-03</td>
<td>Fort Valley, GA</td>
<td>2003</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Bmpc7</td>
<td>Byron, GA</td>
<td>2006</td>
<td>DMI-R</td>
</tr>
<tr>
<td>Dmap2-08</td>
<td>Spartanburg, SC</td>
<td>2008</td>
<td>DMI-R</td>
</tr>
<tr>
<td>Mf.Pdt3-07</td>
<td>Spartanburg, SC</td>
<td>2007</td>
<td>BZI-R</td>
</tr>
<tr>
<td>Mf.Pdt9-07</td>
<td>Spartanburg, SC</td>
<td>2007</td>
<td>BZI-R</td>
</tr>
<tr>
<td>Mf.Bpc15-07</td>
<td>Byron, GA</td>
<td>2007</td>
<td>QoI-RS</td>
</tr>
<tr>
<td>FRf1-08</td>
<td>Forrest, SC</td>
<td>2008</td>
<td>QoI-RS</td>
</tr>
</tbody>
</table>

\(^a\) Sensitive isolates were sensitive to all fungicides tested, DMI-R were resistant to propiconazole, BZI-R were resistant to benzimidazole fungicides, and QoI-RS showed reduced sensitivity to azoxystrobin in experiments done by the laboratory of Dr. Guido Schnabel.
TABLE 2. Results of conidial germination assay performed on *Monilinia fructicola* isolate 10-0 treated with different 2-AI analogs. Analysis of variance followed by Student-Newman-Keuls post hoc test was performed to separate treatments.

<table>
<thead>
<tr>
<th>Analog</th>
<th>Concentration (uM)</th>
<th>% Germination&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SNK Group&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Check</td>
<td>0</td>
<td>95.38</td>
<td>a</td>
</tr>
<tr>
<td>1D3</td>
<td>100</td>
<td>97.07</td>
<td>a</td>
</tr>
<tr>
<td>1C10</td>
<td>100</td>
<td>95.57</td>
<td>a</td>
</tr>
<tr>
<td>1C9</td>
<td>100</td>
<td>95.83</td>
<td>a</td>
</tr>
<tr>
<td>2A5</td>
<td>100</td>
<td>96.20</td>
<td>a</td>
</tr>
</tbody>
</table>

LSD (P = 0.05) = 1.04  
Standard Deviation = 0.37  
CV = 1.95  
Treatment F = 26344.5  
Treatment Prob (F) = 0.0001

<sup>a</sup> Untransformed values  
<sup>b</sup> Arcsine square root percent transformation applied to % germination before ANOVA performed.
TABLE 3. Results of conidial germination assay performed on *Monilinia fructicola* isolate 10-0 treated with different 2-AI analogs. Analysis of variance followed by Student-Newman-Keuls post hoc test was performed to separate treatments.

<table>
<thead>
<tr>
<th>Analog</th>
<th>Concentration (uM)</th>
<th>% Germination$^a$</th>
<th>SNK Group$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>95.75</td>
<td>a</td>
</tr>
<tr>
<td>2B2</td>
<td>100</td>
<td>0</td>
<td>b</td>
</tr>
<tr>
<td>2B3</td>
<td>100</td>
<td>0</td>
<td>b</td>
</tr>
<tr>
<td>CR6</td>
<td>100</td>
<td>0</td>
<td>b</td>
</tr>
<tr>
<td>3H5</td>
<td>100</td>
<td>0</td>
<td>b</td>
</tr>
</tbody>
</table>

LSD (P = 0.05) = 1.47
Standard deviation = 0.53
Treatment F = 8649.4
Treatment Prob (F) = 0.0001

$^a$ Untransformed values

$^b$ Arcsine square root percent transformation applied to % germination before ANOVA performed.
TABLE 4. Results of conidial germination assay with *Monilinia fructicola* isolate 10-0 treated with different 2-AI analogs. Analysis of variance followed by Student-Newman-Keuls post hoc test was performed to separate treatments.

<table>
<thead>
<tr>
<th>Analog</th>
<th>Concentration (uM)</th>
<th>% Germination</th>
<th>SNK Group$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO Check</td>
<td>0</td>
<td>100</td>
<td>a</td>
</tr>
<tr>
<td>2B2</td>
<td>25</td>
<td>90.98</td>
<td>b</td>
</tr>
<tr>
<td>2B2</td>
<td>50</td>
<td>0.84</td>
<td>e</td>
</tr>
<tr>
<td>2B3</td>
<td>25</td>
<td>40.76</td>
<td>d</td>
</tr>
<tr>
<td>2B3</td>
<td>50</td>
<td>0</td>
<td>e</td>
</tr>
<tr>
<td>CR6</td>
<td>25</td>
<td>2.36</td>
<td>e</td>
</tr>
<tr>
<td>CR6</td>
<td>50</td>
<td>0</td>
<td>e</td>
</tr>
<tr>
<td>3H5</td>
<td>25</td>
<td>71.79</td>
<td>c</td>
</tr>
<tr>
<td>3H5</td>
<td>50</td>
<td>96.51</td>
<td>ab</td>
</tr>
</tbody>
</table>

LSD (P = 0.05) = 11.06  
Standard Deviation = 4.79  
CV = 12.14  
Treatment F = 121.7  
Treatment Prob (F) = 0.0001  

$^a$ Untransformed values  
$^b$ Arcsine square root percent transformation applied to % germination before ANOVA performed.
TABLE 5. Results of analysis of variance followed by Student-Newman-Keuls post hoc test for *Monilinia fructicola* conidial germination associated with different rates of CR6, non-charged compound version #1, and non-charged compound version #2.

<table>
<thead>
<tr>
<th>Analog</th>
<th>Concentration (ug/ml)</th>
<th>% Germination</th>
<th>SNK Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>92</td>
<td>a</td>
</tr>
<tr>
<td>CR6</td>
<td>6.9</td>
<td>3</td>
<td>c</td>
</tr>
<tr>
<td>CR6</td>
<td>13.7</td>
<td>0.7</td>
<td>c</td>
</tr>
<tr>
<td>CR6</td>
<td>27.4</td>
<td>0</td>
<td>c</td>
</tr>
<tr>
<td>Non-Charged #1</td>
<td>6.9</td>
<td>90.7</td>
<td>a</td>
</tr>
<tr>
<td>Non-Charged #1</td>
<td>13.7</td>
<td>86</td>
<td>a</td>
</tr>
<tr>
<td>Non-Charged #1</td>
<td>27.4</td>
<td>35.3</td>
<td>b</td>
</tr>
<tr>
<td>Non-Charged #2</td>
<td>6.9</td>
<td>94.3</td>
<td>a</td>
</tr>
<tr>
<td>Non-Charged #2</td>
<td>13.7</td>
<td>91.3</td>
<td>a</td>
</tr>
<tr>
<td>Non-Charged #2</td>
<td>27.4</td>
<td>91.7</td>
<td>a</td>
</tr>
</tbody>
</table>

LSD (P = 0.05) = 8.92  
Standard Deviation = 5.416  
CV = 9.2529  
Treatment F = 380.05  
Treatment Prob (F) = < 0.0001
TABLE 6. Effective concentrations to inhibit mycelial growth by 50% (EC\textsubscript{50}) calculated in confirmation of fungicide resistance phenotypes for isolates of *Monilinia fructicola*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Year</th>
<th>Fungicide</th>
<th>EC\textsubscript{50} (ug/ml)\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>99.2A3.04\textsuperscript{a}</td>
<td>Edgefield, SC</td>
<td>2004</td>
<td>Propiconazole</td>
<td>0.032</td>
</tr>
<tr>
<td>GADL3-03\textsuperscript{a}</td>
<td>Fort Valley, GA</td>
<td>2003</td>
<td>Propiconazole</td>
<td>0.037</td>
</tr>
<tr>
<td>Bmpc7</td>
<td>Byron, GA</td>
<td>2006</td>
<td>Propiconazole</td>
<td>0.624</td>
</tr>
<tr>
<td>Dmap2-08</td>
<td>Spartanburg, SC</td>
<td>2008</td>
<td>Propiconazole</td>
<td>0.468</td>
</tr>
<tr>
<td>99.2A3.04\textsuperscript{a}</td>
<td>Edgefield, SC</td>
<td>2004</td>
<td>Thiophanate-methyl</td>
<td>0.324</td>
</tr>
<tr>
<td>GADL3-03\textsuperscript{a}</td>
<td>Fort Valley, GA</td>
<td>2003</td>
<td>Thiophanate-methyl</td>
<td>0.317</td>
</tr>
<tr>
<td>Mf.Pdt3-07\textsuperscript{a}</td>
<td>Spartanburg, SC</td>
<td>2007</td>
<td>Thiophanate-methyl</td>
<td>2173.945</td>
</tr>
<tr>
<td>Mf.Pdt9-07</td>
<td>Spartanburg, SC</td>
<td>2007</td>
<td>Thiophanate-methyl</td>
<td>0.286</td>
</tr>
<tr>
<td>99.2A3.04\textsuperscript{a}</td>
<td>Edgefield, SC</td>
<td>2004</td>
<td>Azoxystrobin</td>
<td>0.084</td>
</tr>
<tr>
<td>GADL3-03\textsuperscript{a}</td>
<td>Fort Valley, GA</td>
<td>2003</td>
<td>Azoxystrobin</td>
<td>0.079</td>
</tr>
<tr>
<td>Mf.Bpc15-07</td>
<td>Byron, GA</td>
<td>2007</td>
<td>Azoxystrobin</td>
<td>0.275</td>
</tr>
<tr>
<td>FRF1-08</td>
<td>Forrest, SC</td>
<td>2008</td>
<td>Azoxystrobin</td>
<td>0.443</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Isolates 99.2A3.04 and GADL3-03 were considered sensitive to propiconazole, thiophanate-methyl, and azoxystrobin.

\textsuperscript{b} Concentration of propiconazole, thiophanate-methyl, or azoxystrobin in potato dextrose agar required to suppress radial growth of mycelia by 50%.
TABLE 7. Range of mycelial sensitivity to CR6 of *Monilinia fructicola* isolates with different fungicide resistance phenotypes.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Resistance Phenotype</th>
<th>EC$_{50}$ (ug/ml)$^a$</th>
<th>95% C.I. EC$_{50}$ (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mf.Pdt3-07</td>
<td>BZI-R</td>
<td>4.93</td>
<td>3.166 - 7.664</td>
</tr>
<tr>
<td>GADL3-03</td>
<td>Sensitive</td>
<td>5.52</td>
<td>3.489 - 8.735</td>
</tr>
<tr>
<td>99.2A3.04</td>
<td>Sensitive</td>
<td>5.69</td>
<td>3.529 - 9.180</td>
</tr>
<tr>
<td>Bmpe7</td>
<td>DMI-R</td>
<td>10.07</td>
<td>5.804 - 17.465</td>
</tr>
</tbody>
</table>

$^a$ Concentration of CR6 in potato dextrose agar required to suppress radial growth of mycelia by 50%.
TABLE 8. Two-tailed t-test results to assess synergism and antagonism between CR6 and propiconazole in mycelial growth inhibition assays with isolates of *Monilinia fructicola*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Resistance Phenotype</th>
<th>Concentration Propiconazole (ug/ml)</th>
<th>Concentration CR6 (ug/ml)</th>
<th>t-value</th>
<th>Prob(t)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>99.2A3.04</td>
<td>Sensitive</td>
<td>0.01</td>
<td>0.428</td>
<td>5.94</td>
<td>0.0003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Strong Synergism</td>
</tr>
<tr>
<td>99.2A3.04</td>
<td>Sensitive</td>
<td>0.01</td>
<td>0.856</td>
<td>12.64</td>
<td>0.0062&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Strong Synergism</td>
</tr>
<tr>
<td>99.2A3.04</td>
<td>Sensitive</td>
<td>0.1</td>
<td>0.428</td>
<td>1.13</td>
<td>0.2916</td>
<td>Weak Synergism</td>
</tr>
<tr>
<td>99.2A3.04</td>
<td>Sensitive</td>
<td>0.1</td>
<td>0.856</td>
<td>14.04</td>
<td>0.005&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Strong Synergism</td>
</tr>
<tr>
<td>99.2A3.04</td>
<td>Sensitive</td>
<td>0.1</td>
<td>3.426</td>
<td>-26.78</td>
<td>0.0014&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Strong Antagonism</td>
</tr>
<tr>
<td>GADL3-03</td>
<td>Sensitive</td>
<td>0.01</td>
<td>0.428</td>
<td>3.14</td>
<td>0.0139&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Weak Synergism</td>
</tr>
<tr>
<td>GADL3-03</td>
<td>Sensitive</td>
<td>0.01</td>
<td>0.856</td>
<td>0.75</td>
<td>0.4737</td>
<td>ns</td>
</tr>
<tr>
<td>GADL3-03</td>
<td>Sensitive</td>
<td>0.01</td>
<td>3.426</td>
<td>1.3</td>
<td>0.2311</td>
<td>ns</td>
</tr>
<tr>
<td>GADL3-03</td>
<td>Sensitive</td>
<td>0.1</td>
<td>0.428</td>
<td>-1.4</td>
<td>0.199</td>
<td>ns</td>
</tr>
<tr>
<td>GADL3-03</td>
<td>Sensitive</td>
<td>0.1</td>
<td>0.856</td>
<td>-0.43</td>
<td>0.6798</td>
<td>ns</td>
</tr>
<tr>
<td>GADL3-03</td>
<td>Sensitive</td>
<td>0.1</td>
<td>3.426</td>
<td>-7.27</td>
<td>&lt;.0001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Strong Antagonism</td>
</tr>
<tr>
<td>Bmpc7</td>
<td>DMI-R</td>
<td>0.01</td>
<td>0.428</td>
<td>2.52</td>
<td>0.036&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Weak Synergism</td>
</tr>
<tr>
<td>Bmpc7</td>
<td>DMI-R</td>
<td>0.01</td>
<td>0.856</td>
<td>-1.76</td>
<td>0.1163</td>
<td>ns</td>
</tr>
<tr>
<td>Bmpc7</td>
<td>DMI-R</td>
<td>0.01</td>
<td>3.426</td>
<td>0.06</td>
<td>0.9561</td>
<td>ns</td>
</tr>
<tr>
<td>Bmpc7</td>
<td>DMI-R</td>
<td>0.1</td>
<td>0.428</td>
<td>-0.08</td>
<td>0.9384</td>
<td>ns</td>
</tr>
<tr>
<td>Bmpc7</td>
<td>DMI-R</td>
<td>0.1</td>
<td>0.856</td>
<td>-1.59</td>
<td>0.1512</td>
<td>ns</td>
</tr>
<tr>
<td>Bmpc7</td>
<td>DMI-R</td>
<td>0.1</td>
<td>3.426</td>
<td>-2.91</td>
<td>0.0196&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Weak Antagonism</td>
</tr>
<tr>
<td>Dmap2-08</td>
<td>DMI-R</td>
<td>0.01</td>
<td>0.428</td>
<td>1.27</td>
<td>0.2411</td>
<td>ns</td>
</tr>
<tr>
<td>Dmap2-08</td>
<td>DMI-R</td>
<td>0.1</td>
<td>0.428</td>
<td>0.56</td>
<td>0.593</td>
<td>ns</td>
</tr>
</tbody>
</table>

<sup>a</sup> Indicates significance at P(t) = 0.05

<sup>b</sup> Indicates significance at P(t) = 0.01

<sup>c</sup> Synergism detected when difference between C<sub>obs</sub> and C<sub>exp</sub> statistically greater than zero. Antagonism detected when difference between C<sub>obs</sub> and C<sub>exp</sub> statistically smaller than zero. Not significant (ns) indicates the difference between C<sub>obs</sub> and C<sub>exp</sub> was not statistically different than zero.
FIGURE 1. Cubic dose response curve for conidial germination of *Monilinia fructicola* isolate 10-0 exposed to increasing concentrations of CR6.
FIGURE 2. Regression of relative mycelial growth of North Carolina Monilinia fructicola isolates 10-0 and 1-4 on log$_2$ concentration of CR6.
95% confidence intervals (CI) for isolate 10-0 were 8.38 - 11.52 ug/ml.  
95% CIs for isolate 1-4 were 1-4 and 10-0 were 9.66-13.40 ug/ml.

FIGURE 3. Effective concentration required to reduce mycelial growth by 50% (EC₅₀) for North Carolina Monilinia fructicola isolates 10-0 and 1-4. Error bars represent 95% confidence intervals for EC₅₀s.
FIGURE 4. Structures of 2-aminooimidazole CR6, non-charged version #1, and non-charged version #2.
FIGURE 5. Mean % conidial germination associated with different concentrations of CR6, non-charged compound version #1, and non-charged compound version #2. Treatments with the same letter do not differ significantly according to analysis of variance followed by Student-Newman-Keuls post hoc test.
FIGURE 7. Linear and quadratic curves generated to describe the dose response relationship between propiconazole and mycelial growth of *Monilinia fructicola* isolates 99.2A3.04, GADL3-03 (A), Bmpc7, and Dmap2-08 (B). Isolates 99.2A3.04 and GADL3-03 (A) were considered sensitive and Bmpc7 and Dmap2-08 (B) were considered resistant to propiconazole.
FIGURE 8. Linear curves that describe dose response relationship between mycelial growth of *Monilinia fructicola* isolates 99.2A3.04 (sensitive), GADL3-03 (sensitive), FRf1-08 (reduced sensitivity), and Mf.Bpc15-07 (reduced sensitivity) and azoxystrobin.
Isolate 99.2A3.04 sensitive to all fungicides tested.

Isolate GADL3-03 sensitive to all fungicides tested.

Isolate Bmpc7 resistant to propiconazole.

Isolate Mf.Bpc15-07 has reduced sensitivity to azoxystrobin.

Isolate Mf.Pdt3-07 resistant to thiophanate-methyl.

FIGURE 9. Mycelial growth inhibition regressed on log$_2$ CR6 dosage for Monilinia fructicola isolates with different fungicide resistance phenotypes. All regressions were significant at P < 0.001.
FIGURE 10. Effective concentrations of CR6 required to inhibit mycelial growth by 50% (EC$_{50}$) for *Monilinia fructicola* isolates with different fungicide resistance phenotypes. Error bars indicate 95% confidence intervals for EC$_{50}$s.
FIGURE 11. Boxplot synergism results for mycelial growth assays with *Monilinia fructicola* isolates with mixtures of CR6 and propiconazole. Y-axis is the difference between the observed level of control and the expected level of control as calculated by the Gowing Equation/Abbott Method.
FIGURE 12. Images of *Monilinia fructicola* conidia taken with scanning electron microscope. A) Conidia incubated in 6.9 ug/ml DMSO for 3 hours showing relatively smooth surface texture (c) and unobstructed Nucleopore filter (e). B) Conidia incubated in 6.9 ug/ml CR6 solution for 3 hours with irregular depressions (d) and residue clogging the Nucleopore filter (f). Bar = 1 um.
FIGURE 13. Images from transmission electron microscopy. A) Conidia incubated in 6.9 ug/ml DMSO for 3 hours with many black, circular lipid bodies (c). B) Conidia incubated in 6.9 ug/ml CR6 solution for 3 hours with few lipid bodies (d). Bar = 5 um.
FIGURE 14. Images of *Monilinia fructicola* conidia from transmission electron microscopy. A) Conidia incubated in 6.9 ug/ml DMSO for 3 hours showing normal ribosomes (c). B) Conidia incubated in 6.9 ug/ml CR6 solution for 3 hours with ribosomal aggregates (d) and cytoplasmic vacuolization (e), disorganization, and coagulation (f). Bar = 5 um.