

Investigation of Wine Grape Cultivar and Cluster Developmental Stage Susceptibility to Grape Ripe Rot Caused by Two Fungal Species Complexes, *Colletotrichum gloeosporioides*, and *C. acutatum*, and the Evaluation of Potential Controls

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ABSTRACT

Ripe rot of grape is caused by two fungal species complexes: *Colletotrichum gloeosporioides* and *C. acutatum*, both of which cause disease on a variety of crops, such as strawberry and apple. To investigate effect of cultivar and cluster developmental stage on the development of ripe rot, controlled environment and field studies were conducted during 2013-2014. We have identified that a certain level of infection can take place on most cultivars tested from bloom to the near harvest. In most of the cases, significant cultivar and cluster developmental stage interaction effects were observed ($P \leq 0.05$) for the development of disease symptoms in both studies. In general, susceptible cultivar (Cabernet Franc, Cabernet sauvignon, and Chardonnay) demonstrated fluctuations of disease susceptibility among cluster development stages, while resistant cultivars (Merlot) showed consistently low level of the disease throughout the season. To investigate the effect of ten modes of action for control of *C. gloeosporioides* and *C. acutatum*, two methods, alamarBlue® assay and inoculation on fungicide-treated detached fruits, were used. Protective fungicides (mancozeb, captan, and copper) as well as some newer formulations such as azoxystrobin and tetraconazole were identified as excellent products against ripe rot of grape. Four additional materials were identified as potential candidates to investigate further. The information gained from these studies will help growers to determine the critical period for ripe rot management and chemicals to be applied for management. With better control of cluster rot pathogens, Virginia growers can experience an increase in yield and wine quality.

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PUBLIC ABSTRACT

The Virginia wine industry has drastically increased over the past 45 years. In the 1970s, there were only six wineries, but by 2012, the number had increased to over 230 wineries with more than 350 associated vineyards on ~1497.3 bearing hectares statewide (NASS, 2012). Virginia is now tied with Texas for fifth in the nation for annual wine production and was named as one of the top ten wine destinations in the world in 2012 (VDACS, 2015). Ripe rot is a disease of wine grapes caused by two pathogenic fungal species complexes: *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*. Both fungal species complexes are native to Virginia and infect a variety of crops such as apple, blueberry, and strawberry. Ripe rot symptoms begin as a brown spot on the upper part of the berry surface. As the disease progresses, the browning spreads across the berry surface; eventually, numerous fruiting bodies (acervuli) that contain salmon-colored masses of spores (conidia) appear in the brown spot. Generally, these symptoms are more obvious on white-fruited than red-fruited wine grapes; however, red-fruited cultivars are also susceptible. Additionally, Australian studies have shown that an infection of as little as 3% can result in undesirable, tobacco-like off flavors in wine (Meunier and Steel, 2009). In Virginia, the impact of ripe rot was considered minor, partly due to the difficulty of diagnostics (Pearson and Goheen, 1988); however, reports of up to 30% crop loss have been observed in Virginia vineyards in recent years. Therefore, the need for further investigation of these fungal pathogens has arisen.

To investigate the effect of wine grape cultivar and cluster growth stage on the development of ripe rot, controlled environment and field studies were conducted during the 2013-2014 growing seasons. Our results showed that berries were more susceptible at the beginning of the season, became less susceptible around berry touch, and become susceptible again after berries change color (veraison). Some cultivars such as Merlot and Petit Manseng seemed less susceptible than other cultivars such as Chardonnay and Cabernet sauvignon; however, even with less susceptible cultivars, low levels of symptom were observed. In addition, we investigated the efficacy of fungicidal chemicals against both fungal species complexes through two laboratory assays (alamarBlue® and fungicide-applied detached grapes). After examining eleven different modes of action groups, protectant chemicals (captan, copper hydroxide, copper octanoate, and mancozeb) and newer chemistries (azoxystrobin and tetraconazole) were found to be candidates for ripe rot control. Four additional chemicals (*Bacillus subtilis* QST 713, potassium phosphite, pyriofenone, and thiophanate-methyl) also showed some promising results. The better knowledge of infection timing during the season and selection of fungicides will lead to better management of ripe rot so that Virginia growers can experience increased yield and wine quality.

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Literature Review

1.1 *The Virginia Wine Industry*

1.1.1 Introduction to the Virginia Wine Industry – Virginia wine industry has boomed from its original six wineries on 286 acres in the late 1970s to more than 250 wineries and more than 350 associated vineyards with a total of more than 3,700 bearing acres (1497 ha) in 2012 (NASS, 2012). Annual wine production (5.3 million L) (Bureau, 2013) is now tied with Texas for fifth in the nation (VDACS, 2015). In 2010, the Virginia wine industry was estimated to generate \$747 million of annual economic impact in 2010, including revenue from 1.6 million winery visitors. The Virginia wine industry has provided significant impact to the state economy and there is great potential for more growth (VDACS, 2015). Virginia wineries were named in 2012 by Wine Enthusiast magazine as one of the top ten wine destinations in the world (VDACS, 2015).

1.1.2 Impact of Ripe Rot – Ripe rot is a cluster disease of grapes caused by two fungal species complexes: *Colletotrichum gloeosporioides* and *Colletotrichum acutatum* (Damm et al., 2012; Weir et al., 2012). These fungi are endemic in Virginia, and have been reported to cause bitter rot on apples since the late 1800s (Schrenk and Spaulding, 1903; Southworth, 1891). The impact of ripe rot to the wine grape yield has generally been regarded as minor. The disease itself is often misdiagnosed with other late season rots such as bitter rot (caused by a fungus *Greeneria uvicola*) (Pearson and Goheen, 1988) and sour rot (caused by a complex of 70 spp. of fungi and bacteria) (Pearson and Goheen, 1988) due to the similarity of symptoms and appearance time. However, it has been observed in some Virginia vineyards that there may be up to 30% direct

crop loss (Nita, *personal communication*). Moreover, further economic loss can occur during wine production due to the production of unpalatable, tobacco-like, off flavors (Meunier and Steel, 2009) from the infected grapes. Sensory analysis studies showed that this change in flavor in wines occurred with as little as 3% of the total crushed grapes being infected (Meunier and Steel, 2009).

1.2 *Colletotrichum* Epidemiology and Global Impact

1.2.1 Global Importance – Pathogens in the genus *Colletotrichum* have worldwide economic significance because of their ability to infect a wide range of hosts in tropical, sub-tropical, and temperate agricultural areas (Bailey and Jeger, 1992; Hyde et al., 2009; Prusky et al., 2000). This genus contains 40 accepted species (Prusky et al., 2000) that cause disease on hosts ranging from cereals and woody ornamentals to vegetables and tree fruits (Bailey and Jeger, 1992; Peres et al., 2005; Wharton and Dieguez-Uribeondo, 2004). In addition, some species are known to cause some human disease (Hyde et al., 2009). It is one of the most studied plant pathogenic genera (Bailey & Jeger, 1992; Damm et al., 2010; Hyde et al., 2009), and is considered a model organism for infection and host-interaction laboratory studies (Bailey & Jeger, 1992; Prusky et al., 2000).

Colletotrichum acutatum has been described as a cosmopolitan pathogen (Hyde et al., 2009; Peres et al., 2005) with a wide host range that overlaps with *C. gloeosporioides*. Both species are notable pathogens of tropical crops such as avocado (Binyamini and Schiffmann-Nadel, 1971; Dodd et al., 1992; Everett and Timudo-Torrevilla, 2007), mango (Dodd et al., 1991; Dodd et al., 1992; Estrada et al., 2000; Prior et al., 1992), vegetables such as green pepper (Harp et al., 2014) and fruits such as strawberry (King et al., 1997; Leandro et al., 2002; Maas,

1998; Wilson et al., 1990), blueberry (Daykin & Milholland, 1984b; Hartung et al., 1981; Verma et al., 2007), apple (Shane & Sutton, 1981; Southworth, 1891) and grapes (Greer et al., 2014; Kummuang et al., 1996b; Samuelian et al., 2012; Sawant et al., 2012; Simmonds, 1965; Steel et al., 2012). *Colletotrichum* species are considered one of the most important post-harvest pathogens (Prusky et al., 2000) on a variety of fruit crops such as apple (Biggs, 1995; Sutton et al., 2014), blueberry (Caruso and Ramsdell, 1995; Daykin and Milholland, 1984b), and mango (Dodd et al., 1991; Estrada et al., 2000) due to the delay in symptom formation from latent infections.

1.2.2 Taxonomy and Pathogen Description – Historically, *Colletotrichum* species that cause anthracnose diseases on several different fruit crops were classified as *C. gloeosporioides* (Simmonds, 1965; Sutton, 1992) but in the 1960's, *C. acutatum* was described as a separate species (Peres et al., 2005; Simmonds, 1965). This triggered the need for reexamination of the established taxonomy for anthracnose pathogens (Prusky et al., 2000; Sutton, 1992). Several causal agents for diseases, such as almond anthracnose and citrus post bloom fruit drop (Peres et al., 2005), were reclassified as *C. acutatum*.

In general, the colonies of *Colletotrichum gloeosporioides* are flat and gray in culture whereas *C. acutatum* tends to be a darker gray with pink mycelium or pigment in the medium; however, the degree of coloration can vary among strains and culture medium (Peres et al., 2005). Under a microscope, *C. acutatum* conidia are hyaline, with pointed ends, and have the size range of 8.5 - 16.5 x 2.5 - 4 μm (Maas, 1998) (Figure 1.1). However, as the fungus is cultured for a long period in an artificial medium, the conidial features become less apparent due to rounding of at least one end (Weir et al., 2012), making it difficult to distinguish from the slightly larger (12-21 x 3.5-6 μm), rounded conidia of *C. gloeosporioides* (Pearson and Goheen,

1988) (Fig. 1.1). The expansive, overlapping host range of both species has made the species identification process more complicated (Peres et al., 2005; Weir et al., 2012; Wharton and Dieguez-Uribeondo, 2004). Multiple attempts have been made to use morphological data to identify the species (Gunnell and Gubler, 1992; Prusky et al., 2000; Sutton, 1992). The consensus from these attempts is that traditional identifications and classifications of *Colletotrichum* species “must, unfortunately, be consigned to the garbage bin” (Prusky et al., 2000).

Recent advances in molecular techniques have led to multiple attempts to elucidate the phylogenetic relationships in these species (Hyde et al., 2009; Maharaj and Rampersad, 2012; Sreenivasaprasad and Talhinhas, 2005). Despite the issues from incorrectly identified gene fragments in repositories (Hyde et al., 2009), extensive restructurings of the taxonomy of both *C. acutatum* (Damm et al., 2012) and *C. gloeosporioides* (Weir et al., 2012) were conducted. Using a multilocus sequencing technique (MLST) (and traditional morphology in the case of *C. acutatum*), *C. gloeosporioides* and *C. acutatum* have been reorganized into two large species complexes of 22 and 31 individual species, respectively.

1.2.3 Symptomology and Disease Cycle – Both pathogen complexes cause identical symptoms on mature grape berries (Kummuang et al., 1996a; Kummuang et al., 1996b; Southworth, 1891). On white-fruited cultivars, lesions begin as browning of apical epidermal tissues, easily mistaken for sunburn. As the disease progresses, the brown lesion begins to spread over the berry. The fruiting bodies, acervuli, filled with salmon-colored conidial masses, appear in the middle of the lesion in concentric rings (Figure 1.2). Over time, the infected berry will mummify as the lesion covers the berry. Symptoms are less apparent on red-fruited cultivars (Figure 1.2). The formation of acervuli is generally the first observable symptom of infection on red-fruited cultivars.

The disease cycle of ripe rot of grape is not as well understood as that of *Colletotrichum* on strawberry (Maas, 1998), blueberry (Caruso and Ramsdell, 1995; Wharton and Dieguez-Uribeondo, 2004), or apple (Sutton et al., 2014). On strawberry, the initial source of inoculum comes from several places: new transplants with petiole and stolon lesions, infected soil on transplants, infected tissue from the prior season (Maas, 1998), or asymptomatic infections of leaves (Leandro et al., 2002). Blueberry and apple inoculum sources are similar to that of strawberry in that the conidia survive in infected debris and infected fruit mummies that were not removed the previous year (Caruso and Ramsdell, 1995; Sutton et al., 2014). Wharton's study in 2004 also suggested that the largest inoculum source of blueberry anthracnose is inoculum overwintering in the dormant grapevine buds (Wharton & Dieguez-Uribeondo, 2004).

As the season progresses, conidia are spread by water splash to new tissues such as, leaf, blooms, and immature fruit, and to neighboring healthy plants (Caruso and Ramsdell, 1995; Maas, 1998; Sutton et al., 2014; Wharton and Dieguez-Uribeondo, 2004). On strawberry, blueberry, and apple, fruit infection can occur any time from bloom until harvest (Sutton et al., 2014; Wharton and Dieguez-Uribeondo, 2004). As the season continues, acervuli can form in a shriveling lesion on the fruit, allowing for the secondary spread of conidia to new tissues through water splash. Rains also wash conidia onto branches and buds where they may colonize tissue and overwinter (Caruso and Ramsdell, 1995; Maas, 1998; Sutton et al., 2014).

On grape, the disease cycle is thought to have similar elements as to what is observed in the hosts described above. The season starts with resting mycelia and conidia overwintering in several locations; in infected vineyard debris (Pearson and Goheen, 1988), on the woody tissues, or in the dormant grapevine buds (Samuelian et al., 2012). Warm, spring rains wash the conidia and mycelia onto new tissues, such as the blooms (Steel et al., 2012), young clusters and leaves

and favor growth and germination and growth. Once germinated, the pathogen penetrates into plant tissues through appressoria (Daykin and Milholland, 1984a). Often it causes latent infection where symptoms do not appear until late in the season. As the berries enter veraison (= color change), salmon-colored acervuli form on the surface of the berry as described above. Secondary infections can occur on the infected clusters when rain washes the conidia out of these acervuli and down the cluster (Figure 1.3).

1.3 Cultivar and Cluster Developmental Stage Sensitivities

1.3.1 Cultivar Sensitivity Screening – A wide array of grape cultivars from three *Vitis* species (*vinifera*, *labrusca*, *aestivalis*), their hybrids, and *Muscadinia rotundifolia* (syn. *Vitis rotundifolia*, Michx muscadine grape) have been tested for infection sensitivity on fruits to species in both complexes of *Colletotrichum* (Buxton and Sutton, 2006; Jang et al., 2011; Shiraishi et al., 2007, Shiraishi et al., 2006; Steel et al., 2007) (Table 1.1). In a survey by Shirashi (2007) of 235 *Vitis* and 6 *Muscadinia* cultivars, 154 cultivars, including four of the *Muscadinia* were susceptible. In a similar survey in North Carolina, twelve of the 32 tested cultivars, which were susceptible to infection by *C. gloeosporioides* and *C. acutatum*, were inter-specific hybrids and muscadines (Buxton and Sutton, 2006). The 2007 Japanese survey observed that some interspecific hybrids (*V. labrusca* x *V. vinifera*) were more resistant to *C. acutatum* than *V. vinifera* cultivars (Shiraishi et al., 2007); however, the country of vine origin was not a significant factor. Thus, unlike some other diseases of wine grapes such as downy mildew, inter-specific hybrids and muscadine grapes can be as susceptible to ripe rot pathogens as *V. vinifera*.

In addition, susceptibility of a cultivar was not associated to the cultivar fruit color (white or red). ‘Cabernet Sauvignon’, ‘Cabernet Franc’, and ‘Petit Verdot’ were found to be highly

susceptible varieties in studies conducted in Japan and North Carolina (Buxton and Sutton, 2006; Shiraishi et al., 2007). ‘Chardonnay’, which has been used frequently in detached grape lab studies for *Colletotrichum* growth requirements, histology and tissue sensitivity in Australia (Greer et al., 2011; Steel et al., 2012), was identified as one of the most resistant cultivars in the detached grape North Carolina study (Buxton and Sutton, 2006). There is also conflicting results regarding the sensitivity of ‘Merlot’ among testing locations. For example, in Japan, Merlot was found to be highly sensitive (Shiraishi et al., 2007), but in North Carolina, it was one of the most resistant cultivars (Buxton and Sutton, 2006) (Table 1.1).

Anecdotal evidence suggests that the symptom expression relates to the progress of the fruit maturity. One measure of fruit maturity is soluble solids (°Brix), and several comparisons between mature and immature sampled berries were made (Steel et al., 2007). A study by Steel et al., (2007) showed that as the berries matured and °Brix increased to 19.5 - 26.0, cultivars that were resistant prior to veraison (when berries contain 13.7 - 17.5 °Brix) became susceptible and overall quantity of infection increased. However, it was also found that immature berries of Chardonnay, Merlot and ‘Sauvignon blanc’ were susceptible to ripe rot infection, when the °Brix ranged from 13.7 – 15.2 (Steel et al., 2007). In a similar survey of apple susceptibility to *Colletotrichum* species, juice, and fruit attributes were analyzed but no correlation was found between susceptibilities, soluble solids, fruit firmness, or harvest date, which concurs with results on grape (Biggs and Miller, 2001).

1.3.2 Asymptomatic Infections and Cluster Developmental Stage Screening – *Colletotrichum*, as a genus, is known for causing latent infections on host tissue (Peres et al., 2005; Prusky and Plumby, 1992; Verma et al., 2007; Wharton and Dieguez-Urbeondo, 2004). Latent infections occur when there is a time gap between the time of infection and symptom (or reproductive

structure) development. At the time of infection, there is no apparent symptom development or growth of fungal body for a certain period; however, in time, symptoms develop on host tissues, and the pathogen produces conidia and/or conidial structures (Agrios, 2005b; Prusky and Plumb, 1992). Sometimes, symptom development is triggered by events that disturb structure of epidermal cells, such as cell death by freezing (Biggs, 1995; Mertely and Legard, 2004). Both *C. gloeosporioides* and *acutatum* have been observed to have an extended latent period on the fruit of a variety of hosts such as strawberry (Leandro et al., 2002), olive (Moral et al., 2012), apple (Sutton et al., 2014), and blueberry (Caruso and Ramsdell, 1995; Hartung et al., 1981; Verma et al., 2007), where infection can begin during bloom but the symptoms are delayed until harvest or after in storage (Bailey and Jeger, 1992).

The study by Daykin and Milholland (1984) on *Colletotrichum* species infection of grape tissue, *C. gloeosporioides* infected muscadine grape at all tested growth stages from green berry until ripening, and formed appressoria and penetrative hyphae (Daykin and Milholland, 1984a). More recently, *C. acutatum* was found to infect wine grape inflorescences (Greer et al., 2014; Steel et al., 2012), and immature table grapes (Shiraishi et al., 2007), widening the window for the potential grape cluster infection. The berries undergo many chemical and physiological changes throughout the season, such as sugar formation and cuticle thinning that could affect the success of the ripe rot fungi infection (Conde et al., 2007). However, when the susceptibility of cultivars for ripe rot infection was tested, only mature berries were used (Buxton and Sutton, 2006; Shiraishi et al., 2007; Steel et al., 2007). Therefore, the susceptibilities of other cluster developmental stages, from bloom to near harvest, are not well understood.

1.4 Importance of Fungicides

1.4.1 Fungicides in Agriculture – In many agricultural production systems, fungicides have been used as important tools in controlling fungal diseases as a part of Integrated Pest Management (IPM) tactics. Fungicides have been in use in agriculture since the 1600s with the introduction of seed coatings on cereal crops (Morton and Staub, 2008), and specifically in grapes since late 1800s with the introduction of Bordeaux mixture (Agrios, 2005a; Morton and Staub, 2008). In the US, 20% of pesticide applications are fungicides, and agricultural use makes up 57% of the total usage (Ware and Whitacre, 2004b). Applications of fungicides dropped 4.1 million kg between 1999 (Ware and Whitacre, 2004b) and 2007 (Grube et al., 2011), potentially due to the increase in use of newer, more active ingredients such as pyraclostrobin and tebuconazole that are used at lower rates (USGS, 2014).

1.4.2 Modes of Action – Fungicide active ingredients generally function by inhibiting conidial germination or mycelial growth (Agrios, 2005a). The way these chemicals interfere with cellular functions is called the fungicide’s mode of action. Ware and Whitacre (2004a) define mode of action (MOA) as “the sum of anatomical, physiological and biochemical interactions and responses that result in toxic action of a chemical, as well as the physical responses that result in toxic action of a chemical as well as the physical and molecular fate of the chemical in the organism”. An international organization, the Fungicide Resistance Action Committee (FRAC), was formed in the early 1980s (FRAC, 2006) to manage a list of fungicide classifications by MOA. The numbering system, or FRAC group, will be referenced throughout this thesis.

Wine grape production makes use of numerous chemical groups to allow for rotation of FRAC groups for adequate control of fungal pathogens throughout the growing season. There are ten fungicide chemical groups that are investigated in this thesis: methyl-benzimidazole carbamates (MBC), demethylase inhibitors (DMI), succinate dehydrogenase inhibitors (SDHI),

quinone outside inhibitors (QoI), biological agents such as *Bacillus* sp., several multi-site chemical classes such as ethylenebis dithiocarbamates (EBDC) , phthalimides, inorganic coppers and two unknown MOA, phosphonates (FRAC = 33) and aryl-phenyl-ketone (FRAC = U8).

EBDC, phthalimides, and inorganic fungicides are three of the oldest chemical classes still available for agricultural and homeowner use (Morton and Staub, 2008). These classes are non-specific protectant chemicals with modes of actions that are not fully understood, but are assumed to interact with sulfhydryl groups (-SH) in different ways (McCallan, 1949). In the case of copper, the assumed MOA is denaturing of proteins and enzymes by interacting with the sulfur groups (McCallan, 1949). Phthalimide sulfhydryl groups are presumed to interact and interfere with the proper separation of chromosomes during cellular division (McCallan, 1949). There are two proposed MOA for EBDC fungicides; the first involves the formation of isothiocyanate (-N=C=S) radicals that inactivate the same sulfhydryl group as copper and phthalimides (McCallan, 1949). The other group of EBDC forms chelates, organic rings with a metal atom bonded by nitrogen, oxygen, or sulfur, which disrupt protein synthesis in the cell (McCallan, 1949). Although all three chemical groups interact with the same sulfhydryl group, these fall into three different FRAC groups with copper as M1- FRAC, EBDC as M3- FRAC, and phthalimides as M4-FRAC (FRAC, 2014)

In the mid-1960s, over ten years after the introduction of the phthalimides, the first broad-spectrum foliar systemic thiabendazole (Morton and Staub, 2008) was produced, ushering in several new classes of systemic chemicals. Thiabendazole belongs to the MBC (methyl-benzimidazole carbamates) class of chemicals (Morton and Staub, 2008). The original non-toxic form of MBCs is the chemical benzimidazole; however, *in planta*, it forms the active MBC molecule. This secondary molecule disrupts microtubule formation by binding to beta-tubulin

subunits (Hollomon, 2010). This group of chemicals is classified into 1-FRAC group (FRAC, 2014).

Demethylase inhibitors (DMI), which are part of FRAC-3 (FRAC, 2014), are part of the sterol biosynthesis inhibitor (SBI) group and it is currently the largest class of fungicides available (Morton and Staub, 2008). The MOA of DMIs is disruption of sterol formation by inhibiting demethylation of precursors that would normally lead to ergosterol, which causes cells to become unable to manage the permeability of the cell membrane (Montgomery and Paulsrud, 2006).

The succinate dehydrogenase inhibitors (SDHI), part of FRAC-7 (FRAC, 2014), affects the cell's ability to produce energy and are described as nicotinamids (Morton and Staub, 2008), a nicotine derivative. As of 2013, 17 chemicals fell under this classification (Sierotzki and Scalliet, 2013). This class was originally efficacious against basidiomycetes such as bunts, and smuts (Morton and Staub, 2008) but has been expanded to control ascomycetes with newer chemicals. SDHIs achieve this by inhibiting succinate dehydrogenase, which is a part of the citric acid cycle and respiration. The inhibition of succinate dehydrogenase leads to potential buildup of reactive oxygen species (ROS) (Hollomon, 2010). This group is in FRAC group 7 (FRAC, 2014).

The quinone outside inhibitors (QoI), part of the FRAC-11 (FRAC, 2014), is the second largest group of fungicides available. The first products, azoxystrobin and kresoxim-methyl, were introduced in 1992 (Morton and Staub, 2008). The MOA of QoIs is to bind to cytochrome b, and block mitochondrial respiration, thus stopping ATP production (Bartlett et al., 2002). This group of fungicides works particularly well against germinating conidia (Vincelli, 2002). One of

the QoI fungicides is that many can control a wide array of fungal diseases, including diseases caused by water molds, downy mildews, powdery mildews, fruit rots, and even rusts (Morton and Staub, 2008). Thus, this MOA has been used extensively on many different crops.

1.4.3 Fungicide Screening Methods – Since each MOA has a specific target for its activity, the target fungal pathogen may or may not be affected by the chemical. Screening is required to evaluate which MOA has efficacy against each target pathogen. Screening of fungicides is used to calculate the effective concentration where 50% of a population is inhibited (EC50) (Gullino et al., 1985; Greer et al., 2011; Freeman et al.; 1997; Mondal et al., 2005), and to establish a baseline sensitivity to monitor development of fungicide resistance. Using a similar method, screening of biological control agents such as *Streptomyces* can also be achieved (Shahbazi et al., 2014).

Traditionally, fungicide screening is performed *in vitro* using amended growth media (Amiri et al., 2008; Smilanick et al., 2010; Quello, 2009). Sensitivities are often measured by comparing the radial growth of fungal colonies on fungicide-amended media with that of an untreated control. The amount of inhibition is calculated from the ratio, and rates of growth over time are often compared. Tests usually run until the control plate is entirely covered with fungal mycelia, thus, the testing generally takes 6 to 14 days (Amiri et al., 2008; Mondal et al., 2005; Peres et al., 2004; Shephard, 1987; Xu et al., 2014). Variations on media testing methods, such as the chapstick-tube media assay, and the Eppendorf tube media assay, have been made to shorten the testing time to 72 h (Amiri et al., 2008). These assays were considered comparable to the traditional plate assay. Traditional amended media studies have been accomplished with several isolates of both *C. gloeosporioides* and *C. acutatum* from a variety of hosts (Everett & Timudo-Torrevilla, 2007; Greer et al., 2011; Kenny et al., 2012; Mondal et al., 2005).

Other fungicide screening methods include detached fruits (Kenny et al., 2012; Smilanick et al., 2010) or potted plants in controlled environment chambers (Turechek et al., 2006). Some fungicides require a chemical breakdown to become active, such as thiophanate-methyl (Morton and Staub, 2008). In addition, plant structures such as leaf texture and cuticle thickness, affect the ability of the fungicide to persist and incorporate into the plant (Shephard, 1987). Therefore, *in planta* assays may be necessary for completely understanding a chemical's efficacy.

Recently, a liquid media assay using the florescent dye alamarBlue® (Serotec, Raleigh NC), which was developed for cancer and cytotoxicity studies, has been adapted as a fungicide screening method (Rampersad, 2012). Resazurin, the active ingredient in alamarBlue® (O'Brien et al., 2000), is converted from resazurin to resorufin when it is exposed to oxidation-reduction chemical reactions in living cells. This conversion leads to a color change from the original blue to magenta, pink, and clear, as the reaction progresses (Rampersad, 2012). Resazurin fungicide screening produces comparable results to the standard amended media tests (Rampersad, 2011). Recently, these assays have been used to test the fungicide sensitivities of several plant pathogens such as *Verticillium dahliae* (Rampersad, 2011), *Monilinia fructicola* (Cox et al., 2009, Quello, 2009), and several *Colletotrichum* species from avocado, mango, papaya, and bell pepper and strawberry (Rampersad and Teelucksingh, 2012; Smith et al., 2013).

The advantages of this testing method are the shortened period for data collection, ability to test multiple (over three) concentrations at a time, evaluating both species and propagule (mycelia or conidia) in the same plate, and ease of data collection (yes/no per well). However, there are several disadvantages to this testing method due to the sensitive nature of the dye, resazurin. Resazurin only works over a narrow range of pH (Rampersad, 2012) and requires pH adjustment and buffering of fungicide stocks (Rampersad, 2011, Rampersad and Teelucksingh,

2012). In addition, since resazurin is photosensitive, assay plates must be incubated in darkness, which could limit fungi that require light cues. The optimum temperature for the dye to function is higher than most pathogenic fungi prefer (37°C); however, studies can be performed at lower temperatures but the dye will produce a weaker color change (Rampersad, 2012).

1.4.4 Fungicide Sensitivities and Resistance by *Colletotrichum gloeosporioides* and *acutatum* – Establishing the baseline sensitivity of a pathogen to a variety of MOA is part of creating an IPM program that will help reduce the risk of fungicide resistance or if resistance is established, monitor its progress (Pasche et al., 2004; Vega and Dewdney, 2013). Applications of fungicide with ineffective dosages, or at less than ideal application interval (reduction in concentration of active ingredients in the plant surface due to washout of materials and/or light degradation) can promote the formation of resistant populations of pathogens in an agricultural field (Beckerman et al., 2015; Shephard, 1987; van den Bosch et al., 2011).

Quinone outside inhibitors (QoIs), such as pyraclostrobin and trifloxystrobin; phthalimides, such as captafol and captan; and demethylase inhibitors (DMIs), such as tebuconazole, were found to be the most effective chemical control against isolates of *C. gloeosporioides* and *C. acutatum* from hosts such as strawberry, citrus, and muscadine grapes in both lab and field trials (de los Santos García de Paredes & Romero Muñoz, 2002; MacKenzie et al., 2009; Mondal et al., 2005; Samuelian et al., 2014; Silva-Junior et al., 2014; Smith et al., 2013; Wedge et al., 2007). In lab assays using fungicide-amended media, *C. gloeosporioides* and *C. acutatum* were found to be best controlled by different QoI fungicides, especially pyraclostrobin (Everett and Timudo-Torrevilla, 2007; Mondal et al., 2005; Turechek et al., 2006), and the protectants thiram (Kenny et al., 2012) and captan (Smith et al., 2013). *C. acutatum* was not affected by the benzimidazole (MBC) class of fungicides, such as thiophanate-

methyl or benomyl (Chung et al., 2006; Peres et al., 2004). In fields inoculated with *C. gloeosporioides*, thiophanate-methyl mixed with pyraclostrobin provided the best post-inoculation control (MacKenzie et al., 2009). However, a study by Peres revealed that the *C. gloeosporioides* population in Florida was sensitive to MBC fungicides but resistance was quick to develop after repeated exposures (Peres et al., 2004). Due to the rapid development of a resistant population, benzimidazoles had mixed efficacy in the field trial (Wedge et al., 2007). When the efficacy of QoI fungicides against *C. gloeosporioides* and *C. acutatum* was tested (Wedge et al., 2007), mixtures of QoIs and DMIs, such as trifloxystrobin and tebuconazole (Nativo) (Silva-Junior et al., 2014) or mixtures with a SDHI, such as pyraclostrobin and boscalid (Pristine) (Everett and Timudo-Torrevilla, 2007; Silva-Junior et al., 2014), were more efficacious than the QoI alone. Few studies have been conducted with boscalid alone for inhibition of *Colletotrichum* growth. In a study by Everett and Timudo-Torrevilla (2007) on isolates from avocado, they found that there was no inhibition of mycelial growth in the presence of boscalid but there was strong inhibition of conidial germination. Unfortunately, amended-media assays of boscalid alone are not recommended due to the fungi's ability will grow, even in the presence of high concentrations of boscalid in high nutrient conditions such as media (Stammler et al., 2007).

1.5 Potential Chemical Control Tools for Ripe Rot

Basic information on wine grape isolates of *C. gloeosporioides* and *C. acutatum* is limited. For fungicide efficacy, the most recent study was conducted in 2011 using fungicide-amended media (Greer et al., 2011). The efficacies of newly introduced and older chemical compounds have been field tested in the United States in experimental vineyards in Pennsylvania and North Carolina for ripe rot along with other pathogens (Anas & Sutton, 2010; Halbrecht et al.,

2011; Halbrecht et al., 2012; Travis et al., 2005a; Travis et al., 2005b; Travis et al., 2006a; Travis et al., 2006b; Travis et al., 2007). These field tests have provided potential fungicide spray programs for the control of *C. gloeosporioides* and *C. acutatum* in eastern United States vineyards. In Pennsylvania, programs that incorporated protectant fungicides, such as captan, and a quinone outside inhibitor (QoI), such as pyraclostrobin (Pristine) or trifloxystrobin (Flint) provided the best control of ripe rot. The mixture of pyraclostrobin (FRAC-11) and boscalid (FRAC-7) (Pristine) provided the best control in 2007 and 2008. In 2011, separate programs of pyraclostrobin mixed with fluxapyroxad (FRAC-7) (Merivon), and the SBI fenhexamid (Elevate) (FRAC = 17) were the most effective at controlling ripe rot infections. In the following year, trifloxystrobin was the most effective control while, in that same year, the pyraclostrobin mixed with fluxapyroxad program was one of the worst in controlling ripe rot in Pennsylvania. Field tests have been conducted in North Carolina for ripe rot control as well, but all of the spray programs provided no significant control of ripe rot as compared to the untreated control blocks (The spray programs in North Carolina did contain captan, but no QoI fungicides were tested.).

1.6 Project objectives

This thesis encompasses several projects and objectives that are discussed in the main two chapters and the addendum. The objectives of these projects are:

- **Chapter 2:** Effects of cultivar and cluster developmental stage of wine grapes to infection by ripe rot of grape, caused by *Colletotrichum gloeosporioides* and *C. acutatum* species complexes
 - Objective 1: To determine the susceptibilities of wine grape cluster developmental stages against *C. acutatum* and *C. gloeosporioides* infection over the course of the

growing season on several wine grape cultivars using controlled environment and field studies.

- **Chapter 3:** AlamarBlue® and detached-berry assays to determine effective fungicides for management of ripe rot of grape, caused by two fungal species complexes, *Colletotrichum gloeosporioides* and *C. acutatum*.
 - Objective 1: To screen multiple modes of action to evaluate potential field controls for both *C. gloeosporioides* and *C. acutatum* using two methods; alamarBlue® assay (Cox et al., 2009; Rampersad, 2011; Rampersad and Teelucksingh, 2012) and inoculation on fungicide-treated detached fruits.

These studies addresses many important questions for managing ripe rot of grape by understanding infection timing and condition (Chapter 2), as well as examining fungicidal chemicals for control (Chapter 3). The information from our studies will impact not only Virginia vineyards where ripe rot has become a recurring issue, but also many other growing regions, including Australia and New Zealand where ripe rot has become economically important disease (Meunier and Steel, 2009; Steel et al., 2007; Steel et al., 2012).

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demethylation-inhibitor fungicides, prochloraz and tebuconazole. *Australasian Plant Pathology* **43**, 605-13.

Table 1.1: Sensitivity of named and unnamed cultivars in detached grape assays reported in surveys by Shirashi (2007) and Buxton and Sutton (2006) on detached mature grapes

	(Shiraishi et al., 2007) ^Z	(Buxton and Sutton, 2006)
Highly Resistant (≤ 20 %)^Y	Alden, Buffalo, Campbell Early, Hakunan (V), Houman, Katta Kurgan (V), Kyoho, Muscat Bailey A, Nesbit (R), Pierce, Seneca, Shine Muscat, Steuben, Suiho, Tano Red, Urbana, Van Buren, Wayne, Yoho, 72-129, 73-22, 84-12, 384-32, 397-75, 399-1, 626-84, 639-59, 656-29, 658-28, 660-127, 661-61, 662-3, 662-42, 662-68, 662-78, 662-88, 662-93, 662-96, 665-15, 666-24, 667-53, 668-12, 668-31, 668-50, 668-58, 668-61, 668-108, 671-51, 672-6, 676-64	Sunbelt, Petit Sirah, Pride, Merlot, Miss Blanc, Cynthiana Early, Chardonnay, Ark 1271, Nesbit, Barbera
Resistant (21 % - 40 %)	Concord, Delaware, Fry (R), Honey Black, Kuroshio, Morgen Schoen (V), Ontario, Rizamat (V), Takasago, Unicorn (V), 161-32, 335-26, 383-26, 384-25, 399-4, 628-73, 634-70, 643-67, 658-26, 658-67, 660-23, 660-35, 661-23, 662-56, 662-99, 662-154, 664-7, 664-16, 667-9, 668-49, 668-50, 668-53, 668-64, 668-66, 671-112, 682-68, 813-18	Traminette, Charbono, Syrah, Semillon, Rkatsiteli, Summit, Cynthiana Late, Supreme, Touriga Nacional, Chardone1
Susceptible (41 % - 60%)	Aki Seedless, Hakuho, Himrod, Nagano Purple, Niagara, North Black, Oriental Star, Ruby Okuyama (V), Summit (R), Triumph (R), Wallace (R), 86-29, 119-12 (V), 224-22, 224-56, 347-29 (V), 364-30, 365-2, 365-18, 383-35, 392-43, 633-3 (V), 634-12, 634-21, 634-72, 645-67, 658-9, 658-46, 660-125, 662-58, 662-66, 662-163, 664-6, 666-65, 668-8, 668-22, 668-39, 669-15, 671-124, 672-11, 675-103, 676-51, 678-3, 684-11, 685-38, 685-4, 686-3, 817-231	Cayuga White, Seyval, Tempranillo, Tannat, Chambourcin, Sauvignon Blanc, Mataro,
Highly susceptible (≥ 61 %)	Aki Queen, Azumashizuku, Benizuiho, Black Hamburg (V), Cabernet Sauvignon (V), Carlos (R), Dark Ridge, Flame Muscat (V), Fuefuki, Fujiminori, Golden Muscat, Gorby, Gros Colman (V), Hiro Hamburg (V), Honey Seedless, Honey Venus, Italia (V), Kai Noir, Kaiji (V), Kosu (V), Merlot (V), Muscat of Alexandria (V), Muscat of Alexandria 4X (V), Neo Muscat (V), North Red, Pione, Pizzutello Bianco (V), Portland, Rosaki (V), Rosario Bianco (V), Shinanosmile, Sunny Rouge, Tamayutaka, Yamanashi No.38 (V), Yamanashi No.42, Yamanashi No. 44 (V), 85-62, 165-12, 168-39, 301-1, 350-19, 380-4, 381-14, 384-26, 384-60, 389-1, 390-76, 390-84, 400-57, 617-14, 643-25, 645-39, 658-21, 658-34, 658-95, 659-17, 659-23, 659-25, 659-30, 659-45, 660-136, 661-110, 661-113, 662-7, 662-10, 662-16, 662-39, 662-46, 662-98, 662-164, 666-17, 666-29, 667-51, 668-6, 668-23, 668-35, 668-56, 668-60, 668-68, 668-111, 668-115, 668-124, 668-131, 668-133, 669-5, 669-17, 671-17, 671-108, 671-120, 671-122, 674-14, 675-125, 675-147, 675-153, 676-57, 676-75, 683-3, 684-23, 685-2, 686-53, 806-3, 808-3, 811-13, 813-24, 817-18, 817-587	Cabernet Franc, Vidal Blanc, Carlos, Petit Verdot, Cabernet Sauvignon

^Z Numbers (xxx-xx) denote the parentage of unnamed cultivar crosses located at the Grape and Persimmon Research Station, National Institute of Fruit Tree Science in Japan

^Y Represents the percentage of diseased grapes in a detached grape assay

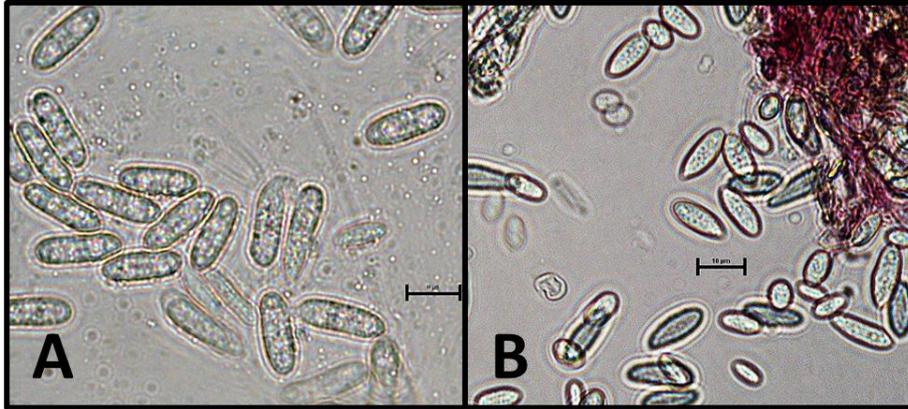


Figure 1.1: Conidial morphology of *Colletotrichum gloeosporioides* (A) and *C. acutatum* (B) from isolates found in Virginia vineyards. The black line represents 10 μm .



Figure 1.2: Salmon-colored ripe rot acervuli on symptomatic Chardonnay (A) and Merlot (B) grapes from clusters found Virginia vineyards.

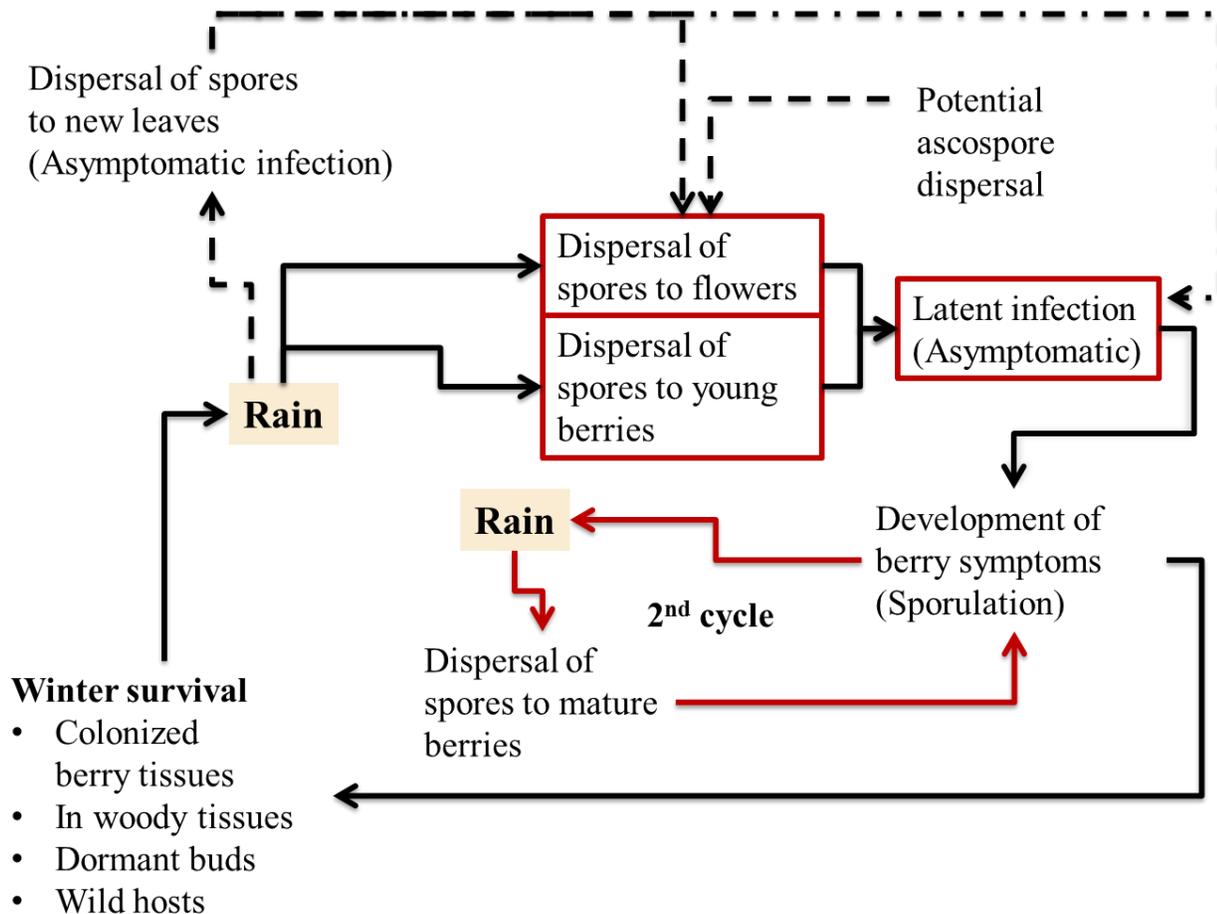


Figure 1.3: Disease cycle of ripe rot. The resting mycelia and conidia overwinter in several ways: In colonized grape tissues, in woody tissues, in dormant grapevine buds or on wild hosts. During warm, spring rains, the conidia and mycelia are washed onto new tissues, such as the blooms, young clusters, or leaves. The conidia germinate and form latent infections on the tissues. When the berries begin to change color (veraison), salmon-colored acervuli form on the surface of the berry. As the season progresses, the berry shrivels as the acervuli form in concentric rings around the berry. Secondary infection occurs when rains wash the conidia out of these acervuli and down the ripening cluster. Rain also washes mycelia and conidia into the woody tissues and dormant buds to colonize and overwinter

Chapter 2.

Effects of cultivar and cluster developmental stage of wine grapes to infection by ripe rot of grape, caused by *Colletotrichum gloeosporioides* and *C. acutatum* species complexes

Abstract

Ripe rot is a cluster rot of wine grapes caused by two fungal species complexes: *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*. To investigate the effect of cultivar and cluster developmental stage to ripe rot infection controlled environment and field inoculation studies were conducted during 2013-2014. Clusters were inoculated with either *C. gloeosporioides* or *C. acutatum* conidia (1×10^5 conidia/ml) at six different cluster developmental stages: bloom, BB-size, pea-size, berry touch, veraison (berry color change), and pre-harvest. On the mean percentage of infected berries, significant effects ($P \leq 0.05$) of cultivar, cluster developmental stage, and their interaction were observed with the controlled environment study in 2013. In 2014, there were significant effects ($P \leq 0.05$) of cultivar, *Colletotrichum* species, the cultivar and *Colletotrichum* species interaction, and the cultivar and cluster developmental stage interaction on the mean percentage of infected berries. In the field inoculation study, there were significant effects ($P \leq 0.05$) of cultivar, cluster developmental stage, and their interaction on the mean percentage of infected berries per cluster in both years. These two studies illustrated that wine grape clusters were more susceptible at the beginning of the season, became less susceptible around berry touch, and become susceptible again after veraison. Overall, susceptible cultivars (e.g. Chardonnay, Cabernet sauvignon, Cabernet Franc) showed more pronounced differences between cluster developmental stages than less susceptible cultivars (e.g. Merlot), which resulted in a constant, low level of symptom development over the course of the season.

2.1 Introduction

Ripe rot is a cluster disease of grapes caused by the *Colletotrichum gloeosporioides* and *C. acutatum* species complexes (Damm et al., 2012; Weir et al., 2012,), which favor warm and wet environmental conditions (Greer et al., 2011; Melksham et al., 2002; Steel et al., 2007).

These pathogenic fungal species are endemic in the commonwealth of Virginia, USA

(Southworth, 1891), and were assumed to have a minor presence. However, up to 30% direct

crop losses have been observed in several Virginia vineyards in recent years (Nita, *personal communication*).

Latent infection of grapes is an inactive infection early in the season (inflorescences or young fruit) than changes to an active infection later in the season to produce symptom and acervuli development on mature fruit (Prusky & Plumbly, 1992). A long latent phase has been observed with *C. gloeosporioides* and *C. acutatum* on a variety of hosts, including grape (Leandro et al., 2002; Moral et al., 2012; Sutton, 1992; Verma et al., 2007). Several studies documented that *C. acutatum* caused infection on inflorescence tissues; however, symptoms did not appear until close to harvest, over 3 months after the initial infection (Daykin and Milholland, 1984; Greer et al., 2014; Steel et al., 2012). On muscadine berries (*Vitis rotundifolia*), *C. gloeosporioides* produced appressoria and penetrative hyphae on all cluster developmental stages (small green fruit, large green fruit, color change, ripe fruit) under the warm, humid, summer conditions in North Carolina, USA (Daykin and Milholland, 1984). In addition, immature table grape berries (Shiraishi et al., 2007) were shown to be susceptible to *C. acutatum* infection.

Grape berries could have variable sensitivity during different developmental stages. For example, with black rot of grape, caused by *Guignardia bidwellii*, clusters become resistant to infection as they mature, which happens three to five weeks after bloom, depending on the cultivar (Hoffman et al., 2002). Thus, timing of fungicide application can be adjusted to target the period when grape tissues are susceptible to infection by the target pathogen. For ripe rot, a study in Australia found that applications of pyraclostrobin (Cabrio, BASF, Research Triangle Park, NC) at bloom and veraison helped to reduce the severity of ripe rot at harvest (Samuelian

et al., 2014). However, information is lacking on the tissue susceptibility of the other cluster developmental stages.

Information on cultivar susceptibility to pathogen infection is also generally lacking, and, in addition, studies have shown conflicting results. For example, both red- and white-fruited wine grape cultivars, such as ‘Cabernet sauvignon’ and ‘Chardonnay’ (*Vitis vinifera*), as well as some intra-specific hybrid cultivars such as ‘Traminette’ and ‘Vidal blanc’, were found to be susceptible to infection (Buxton and Sutton, 2006; Shiraishi et al., 2006). Additionally, the ‘Merlot’ cultivar tested in Japan was found to be highly susceptible (Shiraishi et al., 2007) whereas a North Carolina study observed Merlot to be highly resistant (Buxton and Sutton, 2006).

The Virginia wine industry has increased from six wineries in the 1970s to over 250 wineries and 350 vineyards across the state in 2014 (VDACS, 2015). Virginia was recognized as one of the top ten wine destinations by Wine Enthusiast magazine in 2012 (VDACS, 2015). As the industry expands, the importance of ripe rot in wine grape production has increased, especially for growers in central and southern Virginia where warmer temperatures favor ripe rot development.

The objective of this research is to determine the susceptibilities of wine grape berries at different cluster developmental stages to either *C. gloeosporioides* or *C. acutatum* infection over the course of the growing season. Several wine grape cultivars were examined in the controlled environment and field inoculation studies. Information on vulnerable cluster developmental stage and cultivar susceptibility will be used for development of sound management strategies.

2.2 Materials and Methods

2.2.1 Preparation of fungal isolates – The isolate of *Colletotrichum gloeosporioides* (CTCH1A11A) used in these studies was isolated from a wine grape cluster from a commercial VA vineyard in 2011. The isolate of *C. acutatum* (ACAP1A11A) was isolated from an apple grown at Virginia Tech’s AHS Jr. Agricultural Research and Extension Center (AHS AREC) in Winchester, VA (39°N 6’ 33.55”, -78°W 16’ 56.08) in 2011. The isolates were tested for the ability to infect grapes by inoculating detached table grapes with a conidial suspension of 1×10^5 conidia/ml. Isolates were identified using traditional morphological characteristics and internal transcribed spacer (ITS) region sequencing. Based on the BLAST search (Bonants et al., 2013), the *C. gloeosporioides* isolate had a similarity score of 98.9% to *C. siamense* within the *C. gloeosporioides* complex (Weir et al., 2012). The *C. acutatum* isolate matched *C. fioriniae*, with a similarity score of 100.0% (Damm et al., 2012).

Single-spore cultures of both species were maintained on potato dextrose agar (PDA) (Acumedia®, Neogen Company, Lansing, MI) then transferred onto quarter strength PDA amended with streptomycin and chloramphenicol (100 mg/ml each) (amended ¼ PDA) for conidial production. These amended ¼ PDA plates were placed in an incubator (Precision™ 818, Thermo Scientific Inc, Waltham, MA) at 25 °C with a diurnal light cycle (12 h day /12 h night) for seven to 14 days prior to conidial harvest. In order to maintain the level of pathogenicity, both isolates were inoculated into table grape or apple tissues after every two mycelial transfers onto amended ¼ PDA plates. Seven days after inoculation, these fungi were re-isolated from the table grape or apple tissues. Clean hyphal tips were obtained after 4- to 7-days of growth on amended ¼ PDA plates, then transferred and cultured on amended ¼ PDA plates for conidial harvest, as described above.

For conidial harvest, the surfaces of 7- to 14-day old cultures were flooded with 2-3 ml of sterile distilled water, and gently rubbed with a sterilized bent needle or a glass rod to loosen conidia. The suspension was filtered through two layers of sterilized Miracloth (EMD Millipore, Germany) to remove mycelia. The concentration of the conidial suspension was determined by counting conidia on a hemocytometer (Neubauer Bright-line™, Hausser Scientific, Horsham, PA) and adjusted to 1×10^5 conidia/ml. An addition of 0.1% of Tween 20 (Sigma-Aldrich, St. Louis, MI) was added to the suspension to reduce the surface tension.

2.2.2 Controlled-environment inoculation study – Potted grapevines were utilized for the inoculation study in 2013 and 2014. Grafted Chardonnay, Merlot, and Cabernet sauvignon vines were potted to five-gallon pots (27.9 cm in both top and bottom diameter and 25.4 cm in depth) in 2008, and additional Chardonnay vines were potted to one-gallon pots (16.6 cm in top diameter, 12.4 cm in bottom diameter, and 16.5 cm in depth) in 2013. Pots were filled with a mix of topsoil, peat moss, and Perlite, with a layer of Styrofoam packing peanuts placed at the bottom for drainage. The vines were pruned to produce four shoots that bore two to four clusters per plant. These shoots were trained to a 120-cm bamboo stake such that the whole plant could be placed into environmental growth chambers. Vines were maintained in a high tunnel structure at AHS AREC, which was covered with a polyethylene film (Sun Master®, Farmtek, Dyersville, IA). In order to reduce light intensity and heat accumulation, the structure was covered with a 30% reflective shade cloth (Farmtek, Dyersville, IA), from early June (12 June 2013, and 16 June 2014) through the end of the season.

The experimental design of the controlled environment inoculation study was a three-factor factorial design where cultivar, *Colletotrichum* species, and timing of inoculation (= cluster developmental stage) were examined. Inoculations were conducted at six different cluster

developmental stages: bloom, BB-size, pea-size, berry touch, veraison, and two weeks before harvest (= pre-harvest). At each cluster developmental stage in 2013, fourteen clusters from five randomly selected pots of Cabernet sauvignon, and five clusters from two randomly selected Chardonnay pots were inoculated with each *Colletotrichum* species complexes [*C. gloeosporioides* (Cg) or *C. acutatum* (Ca)]. In addition, five clusters from two randomly selected Merlot pots were inoculated with Cg. At each cluster developmental stage in 2014, three clusters from two randomly selected Cabernet sauvignon pots and two clusters from one Chardonnay pot were inoculated with each *Colletotrichum* species complex. In 2014, only one cluster was available per cluster developmental stage for Merlot for inoculation with Cg.

Four hours prior to the inoculation, vines were placed inside a controlled-environment chamber (E-75L Percival, Perry, IA) to bring the vine to the target temperature of 30 °C (2013) or 25 °C (2014) with a diurnal light cycle (12 h day /12 h night). The temperature was reduced in 2014 to prevent damage to inflorescences observed in 2013 with some cultivars. Conidial suspensions of 10^5 conidia/ml of Cg or Ca were prepared as described above, and then the entire cluster was inoculated by spraying the conidial suspension with a hand atomizer. The berry surfaces were wetted until uniformly covered with droplets, but not until run-off. Each cluster was then covered with a quart bag (0.96 L Ziploc, SC Johnson, Racine, WI) containing a wet paper towel to maintain high relative humidity during the inoculation period (Greer et al., 2011; Samuelian et al., 2012; Steel et al., 2012). Bags were removed after 23.5 h, then allowed 30 min for the clusters to air dry to complete the 24 h inoculation period (Turecek et al., 2006).

At the end of the growing season, clusters were hand-harvested and individually bagged (Table 2.1). Clusters were visually assessed and numbers of diseased berries were counted for each inoculated cluster. All berries within a cluster were cut from the rachis and placed in

individual wells of quail egg containers (EggCartons.com, Machung, MA), then incubated at 25 °C with a diurnal light cycle (12 h day /12 h night) to promote ripe rot symptom development. During the incubation, containers were kept at low relative humidity (< 50% RH) to minimize the risk of saprophytic microorganism growth. After 14 days of incubation, each berry was visually assessed for the presence or absence of disease symptoms. The percentage of infected berries per cluster was calculated by dividing the total number of symptomatic berries by the total number of berries in the cluster.

2.2.3 Field inoculation study – This study was conducted in 2013 and 2014 on Chardonnay, Merlot, Cabernet sauvignon, and ‘Cabernet Franc’ vines in AHS AREC at Winchester, VA. The vines were planted in 2009, spur-pruned, and trained in the vertical shoot positioning system. Spacing between vines within a row was 1.5 m and between two rows was 3 m. Ten to 30 vines of each cultivar were used for the study. Locations of cultivars were physically separate from each other, but within a small planting area (approximately 0.16 ha).

A fungicide spray program was maintained over the course of the season to reduce the occurrence of natural ripe rot and other fruit rot and foliar diseases. In 2013, a tank mixture of mancozeb (Dithane 75DF Rainshield, Dow AgroSciences, Indianapolis, IN), sulfur (Microthiol Disperss, United Phosphorus, Inc, King of Prussia, PA), potassium phosphite (ProPhyt, Helena Chemical Company, Collierville, TN), and quinoxyfen (Quintec, Dow AgroSciences, Indianapolis, IN), which was alternated with cyflufenamid (Torino, Gowan Company, LLC, Yuma, AZ), were applied on a 14-day schedule by a backpack sprayer (SP3, Jacto Inc, Tualatin, OR). In 2014, a mixture of mancozeb, sulfur and quinoxyfen, was alternated with a mixture of captan (Captan Gold, Makhteshim Agan, Raleigh, NC), sulfur and cyflufenamid, and applied on a 7-day schedule. Two applications of potassium bicarbonate (Armicarb, Helena Chemical

Company, Collierville, TN) were applied on 31 June and 7 August 2014, to the clusters to manage powdery mildew.

The experimental design of the field inoculation study was a split-split-plot design where main-plot factor was cultivar and sub-plot factors were *Colletotrichum* species and cluster developmental stages. As with the controlled-environment study, inoculations were made at six cluster developmental stages: bloom, BB-size, pea-size, berry touch, veraison, and two weeks before harvest (pre-harvest). For both years, inoculations were carried out on Chardonnay, Merlot, Cabernet sauvignon, and Cabernet Franc vines, thus, the selection of cultivar was different from the controlled environment study. In 2013, during each cluster developmental stage, six to eight randomly selected clusters were inoculated per *Colletotrichum* species complex (Cg or Ca) per cultivar. The experimental setting was the same for Cabernet sauvignon and Cabernet Franc in 2014, but the number of clusters inoculated at each cluster developmental stage was increased to 40 for Merlot and Chardonnay, to ensure cluster availability at harvest.

Conidial suspensions of 10^5 conidia/ml of Cg or Ca were prepared as described above, and the entire cluster was inoculated and then bagged in a similar manner as in the controlled environment inoculation study. In both years, two additional clusters per cultivar per cluster developmental stage were sprayed with sterile distilled water to measure naturally occurring ripe rot (= non-inoculated control). Inoculations began at approximately 3:30 pm and bags were left on clusters for 22-24 h. Bags were removed early (1-2 pm) on very hot, sunny days, to prevent excessive heat accumulation in the bags.

After the inoculation, clusters were left on the vines until harvest (Table 2.2). Clusters were harvested at 23-24 °Brix (percent soluble sugar content) for white-fruited cultivars and 20-22 °Brix for red-fruited cultivars. The °Brix thresholds were based on typical criteria for wine

grape harvest in Virginia. Clusters were harvested by hand and individually bagged. A very limited expression of ripe rot symptoms was observed with a cursory evaluation conducted at harvest; therefore, clusters were incubated for 14 days (Table 2.2) in an environmental growth chamber at 25 °C with a diurnal light cycle (12 h day /12 h night) to promote symptom development. Each berry was individually evaluated for presence or absence of disease symptoms. Since there was a consistently low level of ripe rot symptoms observed with the non-inoculated check treatment, an adjusted mean percentage of infected berries per cluster for each treatment was estimated as: adjusted mean percentage of infected berries per cluster = [(observed percentage of infected berries per cluster) – (mean percentage of infected berries per cluster from non-inoculated check)]. When the value was at or below zero, it was considered as an absence of infected berries. The cluster disease incidence (presence or absence of a diseased berry per cluster) was also estimated using this adjusted value.

2.2.4 Statistical analysis –The effect of *Colletotrichum* species complex, cluster developmental stage, cultivar, and their interactions on the mean cluster disease incidence and on the mean percentage of infected berries per cluster were analyzed using a generalized linear mixed model (PROC GLIMMIX, SAS, ver. 9.4, SAS institute, Cary, NC) for both studies. The GLIMMIX model utilized the logit link function for mean cluster disease incidence and identity for the mean percentage. When the effect of a factor or their interaction was found to be significant, the mean cluster disease incidence or the mean percentage of infected berries among treatments was compared using Fisher’s least significant difference (LSD) method.

In addition, for the controlled-environment study, a pair-wise comparison of the mean cluster disease incidence and the mean percentages of infected berries per cluster between the visual assessment at and after the 14-day incubation was conducted. The effect of cultivar,

assessment date, and their interaction on the mean cluster disease incidence, and the mean percentage of infected berries, were examined using a longitudinal linear mixed model (GLIMMIX). Cultivar, assessment date, and their interactions were considered as fixed factors, and each cluster was defined as a subject for a repeated measurement. Compound symmetry covariance structure was selected for both analyses, based on comparisons of several covariance structures with Akaike's Information Criterion. Once a significant effect of the assessment date was observed, the difference in the mean cluster disease incidence and the mean percentage of infected berries per cluster were compared between assessment dates for each cultivar using Student's *t*-test.

2.3 Results

2.3.1 Controlled environment inoculation study – Inoculated berries were rated twice, at harvest and 14 days after harvest in both 2013 and 2014. Using the combined data from the six cluster developmental stages, a longitudinal analysis was conducted to examine the effect of the incubation on the mean disease incidence and the mean percentage of infected berries per cluster. Consistent increases were observed in the mean cluster disease incidence and the mean percentage of infected berries per cluster after 14 days of incubation across cultivars in both years (Table 2.3). The difference was significantly larger ($P \leq 0.05$) for many of the cultivar and *Colletotrichum* species combinations in 2013 (Table 2.3). Moreover, even if a very low level of symptom development was observed at harvest, the actual level of infection was often higher. For example, the mean cluster disease incidence was 0% at the time of harvest for *C. gloeosporioides* (Cg-) inoculated Merlot in 2013 and *C. acutatum* (Ca-) inoculated Cabernet sauvignon in 2014; however, Cg inoculations on Merlot resulted in 16% and Ca inoculations on

Cabernet sauvignon resulted in 27% mean cluster disease incidence after the incubation (Table 2.3). Therefore, analyses hereafter are based on the data collected after the incubation period.

The mean cluster disease incidence among the four cultivars varied according to the developmental stage from 0.0% to 100.0% for both Cg-inoculated clusters and Ca-inoculated clusters (Fig. 2.1) in 2013. Higher overall mean cluster disease incidences were observed with Cg- and Ca-inoculated Chardonnay clusters (Fig 2.1, A and D). Inoculation of Merlot with Cg resulted in sustained levels of mean cluster disease incidence across cluster developmental stages, and only a period between BB-size and berry touch showed development of disease.

When differences among cluster developmental stages were examined, relatively lower mean cluster disease incidences were observed at berry touch with both Cg- and Ca-inoculated clusters among the three tested cultivars (Fig 2.1, A-E). However, the ANOVA results showed that there was no significant effect ($P > 0.05$) of pathogen species, cluster developmental stage, cultivar, and their interactions on the mean cluster disease incidence (Table 2.4). The lack of significant difference among cluster developmental stages was probably due to the high variability among samples by (Fig. 2.1).

The mean percentage of infected berries per cluster across the three cultivars for each cluster developmental stage varied from 0.0% to 65.0% for Cg-inoculated clusters (Fig. 2.1, F-H) and 0.0% to 54.8% for Ca-inoculated clusters (Fig. 2.1, I and J) in 2013. Significant effects ($P \leq 0.05$) of cultivar, cluster developmental stage, and their interaction on the mean percentage of infected berries were observed (Table 2.4). The significant interaction resulted from the differences in the susceptible cluster developmental stages among cultivars (Fig. 2.1). For example, two peaks in the mean percentage of infected berries per cluster were observed with Chardonnay (at pea-size and pre-harvest) and Cabernet sauvignon (at pea-size and veraison)

(Fig. 2.1, F and I) while Merlot (Fig. 2.1, G) resulted in very low percentage of infected berries per cluster across all six cluster developmental stages.

In 2014, mean cluster disease incidence among the four cultivars for each cluster developmental stage varied from 0.0% to 100.0% for both *Colletotrichum* species (Fig. 2.2). As in 2013, Chardonnay clusters exhibited higher overall mean cluster disease incidence, and all cluster developmental stages resulted in some level of disease development (Fig 2.2, A and D). As with 2013, Merlot sustained low levels of mean cluster disease incidence throughout the season, with the exception of pea-size where it was 100% (Fig. 2.2, B). However, since only one cluster per cluster developmental stage was available for inoculation, Merlot was not included in the ANOVA. Similar to 2013, the ANOVA results showed that there were no significant effects ($P > 0.05$) of *Colletotrichum* species, cluster developmental stage, cultivar, and their interaction on the mean cluster disease incidence (Table 2.4). With Cabernet sauvignon, inoculation at veraison resulted in a relatively higher mean cluster disease incidence for both Cg and Ca (Fig 2.2, C and E), which was also observed in 2013.

Overall, the mean percentages of infected berries for Ca-inoculated clusters in the controlled environment were lower in 2014 than in 2013. The mean percentage of infected berries per cluster among four cultivars for each cluster developmental stage varied from 0.0% to 66.7% for Cg-inoculated clusters (Fig. 2.2, F-H) and 0.0% to 23.8% Ca-inoculated clusters (Fig. 2.2, I and J) in 2014. Unlike 2013, there were significant effects ($P \leq 0.05$) of cultivar, *Colletotrichum* species complexes, the cultivar and *Colletotrichum* species complexes interaction, and the cultivar and cluster developmental stage interaction (Table 2.4). As with 2013, Chardonnay sustained higher mean percentages of infected berries per cluster (Fig. 2.2, H and I). The results from Chardonnay clusters clearly showed the interaction between

Colletotrichum species complexes and cultivar. For instance, on Cg-inoculated Chardonnay clusters, an increase in the mean percentage of infected berries was observed toward the middle of the season (pea-size to veraison) then a decrease as the season progressed (Fig. 2.2, F). In contrast, the overall level of disease development was very low with Ca-inoculated Chardonnay clusters with higher mean percentages observed at berry-touch and pre-harvest (Fig. 2.2, I). The interaction between cultivar and cluster developmental stage is best observed in the comparison of Ca-inoculated Chardonnay and Cabernet sauvignon (Fig. 2.2, I and J). On Chardonnay, the mean percentage of infected berries per cluster increased through the season to the highest percentage at the end during preharvest (Fig. 2.2, I). However, on Cabernet sauvignon, the mean percentage of infected berries per cluster was the highest during bloom and BB-size, and then decreased to a very low level for the rest of the growing season (Fig. 2.2, J).

2.3.2 Field inoculation study – In 2013, the mean cluster disease incidence across six cluster developmental stages across the four cultivars varied from 25.0% to 100.0% for Cg-inoculated clusters (Fig. 2.3, A-D) and 11.1% to 100.0% for Ca-inoculated clusters (Fig. 2.3, E-H). There were no data available for clusters of Cabernet sauvignon that were inoculated at bloom, BB-size, berry touch, and veraison; thus, data from Cabernet sauvignon in 2013 were not included in the ANOVA. As in the controlled environment study, many clusters had 100% mean cluster disease incidence regardless of pathogen species, cluster developmental stages, and cultivar (Fig. 2.3, A-H); therefore, no significant effects ($P > 0.05$) of cultivar, *Colletotrichum* species complexes, and cluster developmental stage and their interactions on the mean cluster disease incidence were observed (Table 2.5). There were several cases where numerically lower mean cluster disease incidences were observed: Cg-inoculated clusters of Merlot from bloom to berry

touch (Fig. 2.3, B); Ca-inoculated clusters of Chardonnay at bloom, Merlot at berry touch; and Cabernet Franc at pea-size and pre-harvest (Fig. 2.3, A, F, and H).

In 2013, the mean percentage of infected berries per cluster for each cluster developmental stage across the four cultivars varied from 0.0% to 35.2% for Cg-inoculated clusters (Fig. 2.3, I-L), and 0% to 58.6% Ca-inoculated clusters (Fig. 2.3, M-P). There were significant effects ($P \leq 0.05$) of cultivar, cluster developmental stage, and their interaction (Table 2.5). The significance of cultivar and cluster developmental stage was well illustrated in the differences between the results from Chardonnay and Cabernet Franc (Fig. 2.3, I and M for Chardonnay, and L and P for Cabernet Franc). With Chardonnay, all cluster developmental stages resulted in similar degrees (15-20%) of infection with the exception that Ca-inoculation resulted in relatively higher mean percentage at pea-size inoculation. In contrast, there were higher mean percentages of infected berries observed at earlier stages (bloom and BB-size) on Cabernet Franc, then decreased as the season progressed. As in the controlled environment study, Merlot resulted in relatively lower mean percentages of infected clusters (Fig. 2.3, J and N).

The mean cluster disease incidences among pathogen species, cultivars, and cluster developmental stages in the field were much lower in 2014 than in 2013. It varied from 50.0% to 100.0% for Cg-inoculated clusters (Fig. 2.4, A-D), and 40% to 100% for Ca-inoculated clusters (Fig. 2.4, E-H). Similar to 2013, the majority of observed mean cluster disease incidence per cluster developmental stage was close to 100%, with a few exceptions (Fig. 2.3, A-H). Numerically lower mean cluster disease incidences were observed: both Cg- and Ca-inoculated Merlot at pre-harvest (Fig. 2.4, B and F); Cg-inoculated clusters of Cabernet Franc at berry touch (Fig. 2.4, D); Ca-inoculated clusters of Chardonnay at berry touch; Cabernet sauvignon at BB-size, berry touch, and veraison; and Cabernet Franc at pea-size (Fig. 2.3, E, G, and H).

Overall, the mean percentage of infected berries per cluster among the tested cultivars and six cluster developmental stages in the field was lower in 2014 than in 2013. In 2014, mean percentage of infected berries per cluster among four cultivars varied from 1.3% to 14.4% for Cg-inoculated clusters (Fig. 2.4, I-L), and 1.0% to 25.1% Ca-inoculated clusters (Fig. 2.4, M-P), with smaller differences among the cluster developmental stages than in 2013 (Fig. 2.3). There were significant effects ($P \leq 0.05$) of cultivar, cluster developmental stage, and their interaction on the mean percentage of infected berries (Table 2.5). In 2014, that interaction was obvious with Cabernet sauvignon, which was not included in the analysis in 2013. Higher mean percentages of infected berries were observed at bloom, BB-size, and pre-harvest with Ca-inoculated Cabernet sauvignon (Fig. 2.4, O), while with the other cultivars, differences among cluster developmental stages were not as large (Fig. 2.4). It should be noted that, as in 2013 and the controlled environment study, even clusters with low mean percentages of infected berries, resulted in high cluster disease incidences (Figs 2.1-2.4).

A common attribute between the two studies was the significant effect of cluster development stage on the mean percentage of infected berries (Tables 2.4 and 2.5). When the mean percentage of infected berries per cluster was compared across the six cluster developmental stages, consistently low development of disease was observed at berry touch. At berry touch, mean percentage of infected berries was often significantly lower ($P \leq 0.05$) than other cluster developmental stages (Fig. 2.5) with the one exception of the controlled environment study in 2014 (Fig. 2.5).

2.4 Discussion

As shown in previous studies (Daykin and Milholland, 1984; Greer et al., 2014; Steel et al., 2012), our two studies illustrated that wine grape clusters are susceptible to ripe rot latent infection during bloom, especially with more susceptible cultivars such as Chardonnay, Cabernet Franc and Cabernet sauvignon. In addition, our studies demonstrate that other cluster developmental stages are also susceptible to latent infection by both *C. gloeosporioides* and *C. acutatum*. The exception was the cluster developmental stage berry touch, where overall latent infection development was lowest with many tested cultivars.

These changes in susceptibility based on host tissue developmental stages have been shown in studies of *Colletotrichum* species on other hosts. For example, with olive and strawberry, latent infection susceptibility increases as the fruits mature and change color (Moral et al., 2008; Wilson et al., 1990). In contrast, studies on prune (Luo and Michailides, 2001), peach (Biggs and Northover, 1988) and nectarines Ibbotson-Darhower et al., 1998) demonstrated that the latent infection susceptibility of fruit to *Monilinia fructicola* was moderate at bloom, increases in the middle of the season at pit hardening, and decreases at embryo growth stage (fruit maturation), and increases again toward harvest. Wine grapes shift from berry formation to berry maturation between berry touch and veraison, which occurs approximately 55-60 days after bloom (Coombe and McCarthy, 2000; Keller, 2010). The stone fruit susceptibility trend is similar to the one observed in our wine grape studies where the cluster sensitivity to latent infections is higher during bloom to pea-size, decreases during berry touch, and increases again during veraison and harvest. As with stone fruits, wine grapes shift from berry formation to berry ripening during berry touch and veraison, which occurs approximately 55-60 days after bloom (Coombe and McCarthy, 2000; Keller, 2010).

In the previous studies, *C. acutatum* isolates were utilized to examine latent infection on flowers (Daykin and Milholland, 1984; Greer et al., 2014; Steel et al., 2012); however, our results showed that *C. gloeosporioides* is also capable of infecting flowers. To our knowledge, this is the first study to demonstrate the capability of *C. gloeosporioides* to cause latent infection on wine grape inflorescences. Moreover, the consistent lack of difference between two *Colletotrichum* species complexes in our studies demonstrated that *C. gloeosporioides* was as virulent as *C. acutatum* on wine grapes, despite the fact that the *C. acutatum* isolate was obtained from apple. *C. gloeosporioides* and *C. acutatum* complexes include 22 and 31 species, respectively (Damm et al., 2012; Weir et al., 2012). In South Carolina studies with peaches, the species identified as belonging to the *C. gloeosporioides* species complex were found to have variable levels of fungicide sensitivity (Hu et al., 2015a; Hu et al., 2015b).

Differences in cultivar susceptibility were observed among the cultivars tested in our studies. In the controlled environment inoculation study, Chardonnay was the most susceptible, followed by Cabernet sauvignon for both *Colletotrichum* species. Merlot exhibited limited ripe rot symptoms in both 2013 and 2014. In the field inoculation study, Cabernet sauvignon was the most susceptible, followed by Cabernet Franc, Chardonnay, and Merlot. Although the overall level of mean percentage of infected berries observed on Merlot was low, consistent successful infection was shown as high disease incidence across all cluster developmental stages in both years. Our results on Merlot agreed with the results from a study conducted in North Carolina (Buxton and Sutton, 2006) where they also documented low susceptibility with Merlot. However, our results are also in agreement with a study from Japan that showed Merlot to be one of the more susceptible cultivars because they only measured disease incidence (Shiraishi et al., 2007). Susceptibility may also vary based on the clone of Merlot (Mullins, 1992), or specific

species of *Colletotrichum* species within the complex that caused the infection (Biggs and Miller, 2001; Damm et al.; 2012, Weir et al., 2012).

There are two major disparities observed in the results between the controlled environment inoculation study and the field inoculation study. The first is relatively low percentages of infected berries at bloom to BB-size in the controlled environment study observed on all four cultivars and with both *Colletotrichum* species. Since the mean cluster disease incidence at the bloom inoculation was high on Chardonnay in both years, the latent infection did occur, however, the percentage of infected berries was not as high as in the field inoculation study. We encountered very high temperatures during bloom even with a shade cloth; thus, the infection process after incubation might have been compromised. The second is high percentages of infected berries with Cg-inoculated Chardonnay at berry touch with the controlled environment study in 2014. Due to the high temperatures under the high tunnel, the phenological progression was accelerated, causing each stage to occur faster than under the field conditions, and shortened the time span of each stage (Mullins, 1992). Since we had to rely on visual changes on berries, by the time we conducted berry touch inoculation, berries might have been moved biochemically into the veraison stage.

Another notable inconsistency was very low level of ripe rot disease development observed in 2014, especially with the field inoculation study. From the weather station located in the Chardonnay vineyard at the AHS AREC, the average temperature in 2013 between Apr. and Sept. was 19.0 °C, which was similar to 2014 when the average temperature was 18.7 °C. However, frequent rain events that occurred in the beginning of the 2013 season resulting in 109.2 mm of rain falling during bloom, and a total of 406.5 mm between bud-break and pre-harvest. On the other hand, 2014 was drier with 96.3 mm during bloom with a total of 374.4 mm

between bud-break and pre-harvest. Warm and humid conditions are known to promote high conidial survival rates of *Colletotrichum* species (Diéguez-Uribeondo et al., 2011; Dodd et al., 1992; Estrada et al., 2000; Wilson et al., 1990). Thus, conidia would have more opportunities to initiate infection in the field in 2013, even after the artificial humid environment (i.e. the bag placed at the time of inoculation) was removed.

Overall, our studies demonstrated that the susceptibility of wine grape berries to latent infection by ripe rot pathogens was high during bloom to pea-size, lowered at berry touch, and became high again from veraison to harvest. In addition, the variation among cluster developmental stages was more apparent with the more susceptible cultivars such as Chardonnay and Cabernet sauvignon. Moreover, the results from the more resistant cultivar, Merlot, in 2013 and all cultivars from the field inoculation study in 2014 showed that low mean percentages of infected berries per cluster could result in very high the mean cluster disease incidences. Results from the controlled environmental study also demonstrate that even if little to no symptom development is observed at the time of harvest, latent infections may be present. These are particularly important findings for this disease because even a small amount of infection, as low as 3%, the quality of wine can be negatively affected (Meunier and Steel, 2009). Therefore, it is important to have good protection of grape clusters from early bloom to reduce the risk of latent infection by ripe rot pathogens. As far as we know, there is only one study, which was conducted by Samuelian et al. (2014), to address the application of fungicide treatments at different cluster developmental stages. Future studies should focus on field tests of fungicide programs that utilize applications during bloom to BB-size, or after veraison in order to provide effective management of ripe rot.

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Table 2.1: Dates for the inoculation, harvest and assessment of clusters from the controlled environment inoculation study for both isolates, *C. gloeosporioides* and *C. acutatum*, in 2013 and 2014

Cultivar	Cluster Developmental Stage	Inoculation		Assessment	
		Date	Harvest	Date	Date
Chardonnay	Bloom	6/1/2013		10/25/2013	11/21/2013
	BB-size	6/20/2013		10/25/2013	11/21/2013
	Pea-size	7/2/2013		10/25/2013	11/21/2013
	Berry touch	7/25/2013		10/25/2013	11/21/2013
	Veraison	8/6/2013		10/25/2013	11/21/2013
	Pre-harvest	10/1/2013		10/25/2013	11/21/2013
Merlot	Bloom	6/4/2013		10/25/2013	11/20/2013
	BB-size	6/20/2013		10/25/2013	11/20/2013
	Pea-size	7/2/2013		10/25/2013	11/20/2013
	Berry touch	7/25/2013		10/25/2013	11/20/2013
	Veraison	8/6/2013		10/25/2013	11/20/2013
	Pre-harvest	10/1/2013		10/25/2013	11/20/2013
Cabernet sauvignon	Bloom	6/4/2013		10/26/2013	11/18/2013
	BB-size	6/20/2013		10/26/2013	11/18/2013
	Pea-size	7/2/2013		10/26/2013	11/18/2013
	Berry touch	7/25/2013		10/26/2013	11/18/2013
	Veraison	8/6/2013		10/26/2013	11/20/2013
	Pre-harvest	10/1/2013		10/26/2013	11/20/2013
Chardonnay	Bloom	5/28/2014		9/3/2014	9/15/2014
	BB-size	6/10/2014		9/3/2014	9/22/2014
	Pea-size	6/18/2014		9/3/2014	9/15/2014
	Berry touch	7/8/2014		9/3/2014	9/15/2014
	Veraison	7/23/2014		9/3/2014	9/15/2014
	Pre-harvest	8/27/2014		9/3/2014	9/15/2014
Merlot	Bloom	5/28/2014		9/9/2014	9/25/2014
	BB-size	6/18/2014		9/9/2014	9/25/2014
	Pea-size	7/8/2014		9/9/2014	9/25/2014
	Berry touch	7/23/2014		9/9/2014	9/25/2014
	Veraison	7/23/2014		9/9/2014	9/25/2014
	Pre-harvest	8/27/2014		9/9/2014	9/25/2014
Cabernet sauvignon	Bloom	5/28/2014		9/9/2014	9/29/2014
	BB-size	6/10/2014		9/9/2014	9/29/2014
	Pea-size	7/8/2014		9/9/2014	9/29/2014
	Berry touch	7/23/2014		9/9/2014	9/29/2014
	Veraison	7/23/2014		9/9/2014	9/29/2014

Table 2.2: Dates for the inoculation, harvest and assessment of clusters from the field inoculation study for both isolates, *C. gloeosporioides* and *C. acutatum*, in 2013 and 2014

Cultivar	Cluster Development Stage	Inoculation Date	Harvest Date	Assessment Date
Chardonnay	Bloom	6/1/2013	10/3/2013	10/21/2013
	BB-size	6/16/2013	10/3/2013	10/22/2013
	Pea-size	6/25/2013	10/3/2013	10/23/2013
	Berry touch	7/2/2013	10/3/2013	10/24/2013
	Veraison	8/6/2013	10/3/2013	10/25/2013
Merlot	Bloom	6/12/2013	10/4/2013	10/28/2013
	BB-size	6/25/2013	10/4/2013	10/29/2013
	Pea-size	7/2/2013	10/4/2013	10/30/2013
	Berry touch	7/17/2013	10/4/2013	11/1/2013
	Veraison	8/16/2013	10/4/2013	11/4/2013
Cabernet sauvignon	Pre-harvest	9/20/2013	10/4/2013	11/5/2013
	Pea-size	7/2/2013	10/4/2013	11/16/2013
Cabernet Franc	Pre-harvest	9/20/2013	10/4/2013	11/16/2013
	Bloom	6/12/2013	10/4/2013	11/7/2013
	BB-size	6/25/2013	10/4/2013	11/8/2013
	Pea-size	7/2/2013	10/4/2013	11/12/2013
	Berry touch	7/17/2013	10/4/2013	11/13/2013
	Veraison	8/16/2013	10/4/2013	11/14/2013
	Pre-harvest	9/20/2013	10/4/2013	11/15/2013
Chardonnay	Bloom	6/10/2014	9/10/2014	10/7/2014
	BB-size	6/23/2014	9/10/2014	10/14/2014
	Pea-size	6/23/2014	9/10/2014	10/16/2014
	Berry touch	7/8/2014	9/10/2014	10/22/2014
	Veraison	8/13/2014	9/10/2014	10/23/2014
Merlot	Pre-harvest	8/27/2014	9/10/2014	10/29/2014
	Bloom	6/10/2014	9/29/2014	10/27/2014
	BB-size	6/23/2014	9/29/2014	10/28/2014
	Pea-size	7/1/2014	9/29/2014	10/30/2014
	Berry touch	7/23/2014	9/29/2014	10/31/2014
Cabernet sauvignon	Veraison	8/13/2014	9/29/2014	11/3/2014
	Pre-harvest	9/3/2014	9/29/2014	11/4/2014
	Bloom	6/10/2014	9/29/2014	11/11/2014
	BB-size	6/23/2014	9/29/2014	11/11/2014
	Pea-size	7/1/2014	9/29/2014	11/11/2014
Cabernet Franc	Berry touch	7/23/2014	9/29/2014	11/12/2014
	Veraison	8/13/2014	9/29/2014	11/12/2014
	Pre-harvest	9/3/2014	9/29/2014	11/12/2014
	Bloom	6/10/2014	9/29/2014	11/13/2014
	BB-size	6/23/2014	9/29/2014	11/13/2014
Cabernet Franc	Pea-size	7/1/2014	9/29/2014	11/14/2014
	Berry touch	7/23/2014	9/29/2014	11/15/2014
	Veraison	8/13/2014	9/29/2014	11/15/2014
	Pre-harvest	9/3/2014	9/29/2014	11/15/2014

Table 2.3 Effect of a 14-day incubation period after harvest on grape ripe rot mean cluster disease incidence and infected berries per cluster in percentages from the controlled environment inoculation study conducted with two *Colletotrichum* species complexes and four different cultivars in 2013 and 2014.

Year	Species ^Y	Cultivar	Cluster disease incidence (%) ^Z		Percentage of infected berries per cluster ^Z			
			Harvest	Day 14		Harvest	Day 14	
2013	Cg	Cabernet sauvignon	5.2	15.5	*	0.5	2.1	*
		Chardonnay	28.6	81.0	**	2.0	20.5	**
		Merlot	0.0	15.8		0.1	1.2	
	Ca	Cabernet sauvignon	12.3	36.8	**	0.6	6.4	**
		Chardonnay	47.4	78.9	*	9.5	22.9	*
2014	Cg	Cabernet sauvignon	7.7	15.4		0.6	0.9	
		Chardonnay	61.5	84.6		18.1	30.7	**
		Merlot	0.0	11.1		0.1	1.8	
	Ca	Cabernet sauvignon	0.0	27.3		0.1	4.3	
		Chardonnay	23.1	38.5		2.7	7.6	

^Z Results from a generalized linear mixed model is shown with asterisk. Asterisk next to the number indicates significant difference between two assessments at the 95% level (one asterisk), and at the 99% level (two asterisks)

^Y Cg = *Colletotrichum gloeosporioides* complex, Ca = *Colletotrichum acutatum* complex

Table 2.4: ANOVA table for the effects of cluster developmental stage, cultivar, *Colletotrichum* species complexes, and their interactions on the mean cluster disease incidence and the mean percentage of infected berries per clusters from the controlled environment inoculation study in 2013 and 2014

Mean cluster disease incidence		2013		2014	
Effect^z	F-value	P-value^y	F-value	P-value^y	
Cultivar	0.0	1.00	0.0	0.95	
<i>Colletotrichum</i> species complexes	0.8	0.37	0.0	0.94	
Cluster developmental stage	0.0	1.00	0.0	1.00	
Cultivar x <i>Colletotrichum</i> species complexes	2.3	0.13	0.0	0.93	
Cultivar x Cluster developmental stage	0.5	0.90	0.0	1.00	

Mean percentage of infected berries per cluster		2013		2014	
Effect^z	F-value	P-value^y	F-value	P-value^y	
Cultivar	26.4	< 0.01 **	16.5	< 0.01 **	
<i>Colletotrichum</i> species complexes	1.3	0.26	6.4	0.02 *	
Cluster developmental stage	4.7	< 0.01 **	1.4	0.26	
Cultivar x <i>Colletotrichum</i> species complexes	0.6	0.46	11.0	< 0.01 **	
Cultivar x Cluster developmental stage	4.8	< 0.01 **	2.8	< 0.01 **	

^z Generalized linear mixed model (PROC GLIMMIX) was used for analysis of the effects on the mean disease incidence per cluster and the mean percentage of infected berries per cluster

^y Asterisk next to the number indicates significant difference between two assessments at the 95% level (one asterisk), and at the 99% level (two asterisks)

Table 2.5: ANOVA table for the effects of cluster developmental stage, cultivar, *Colletotrichum* species complexes, and their interactions on the mean cluster disease incidence and the mean percentage of infected berries per clusters from the field inoculation study in 2013 and 2014

Mean cluster disease incidence		2013		2014	
Effect^z	F-value	P-value^y	F-value	P-value^y	
Cultivar	0.0	0.99	2.2	0.09	*
<i>Colletotrichum</i> species complexes	2.9	0.09	4.8	0.03	*
Cluster developmental stage	0.1	0.99	0.3	0.94	
Cultivar x <i>Colletotrichum</i> species complexes	2.7	0.07	2.9	0.04	*
Cultivar x Cluster developmental stage	1.1	0.38	1.0	0.42	

Mean percentage of infected berries per cluster		2013		2014		
Effect^z	F-value	P-value^y	F-value	P-value^y		
Cultivar	9.1	< 0.01	**	14.41	< 0.01	**
<i>Colletotrichum</i> species complexes	1.0	0.33		0.86	0.35	
Cluster developmental stage	5.5	< 0.01	**	3.19	< 0.01	**
Cultivar x <i>Colletotrichum</i> species complexes	1.6	0.20		0.6	0.61	
Cultivar x Cluster developmental stage	6.3	< 0.01	**	2.31	< 0.01	**

^z Generalized linear mixed model (PROC GLIMMIX) was used for analysis of the effects on the mean disease incidence per cluster and the mean percentage of infected berries per cluster

^y One asterisk next to the number indicates significance at the 95% level, two asterisks next to the number indicates significance at the 99% level

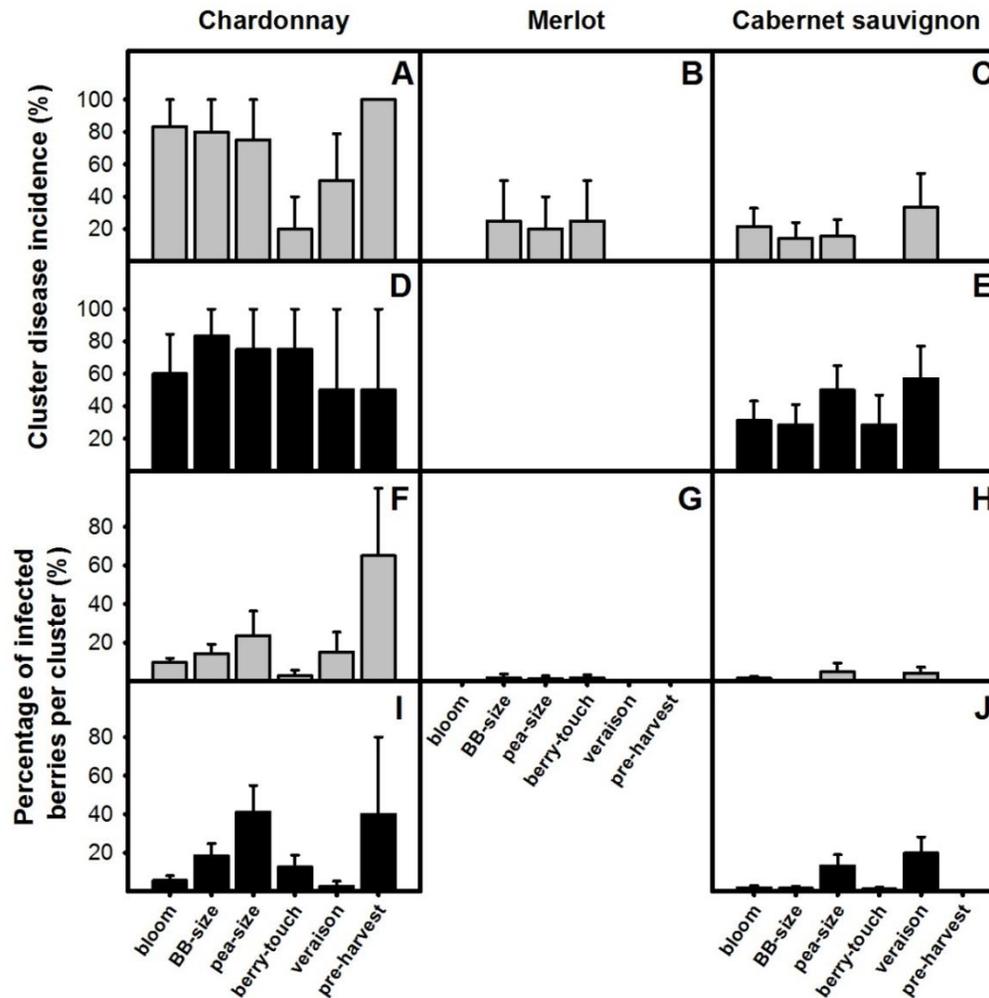


Figure 2.1: The mean cluster disease incidence (A-F) [presence or absence of infection per cluster] and the mean percentage of infected berries per cluster (G-L) from the controlled environment inoculation study in 2013. Four wine grape cultivars were shown vertically [Chardonnay (A, D, F, and I), Merlot (B and G), and Cabernet sauvignon (C, E, H, and J)]. Gray bars represent clusters inoculated with *C. gloeosporioides* and black bars represent clusters inoculated with *C. acutatum*, with standard errors as error bars.

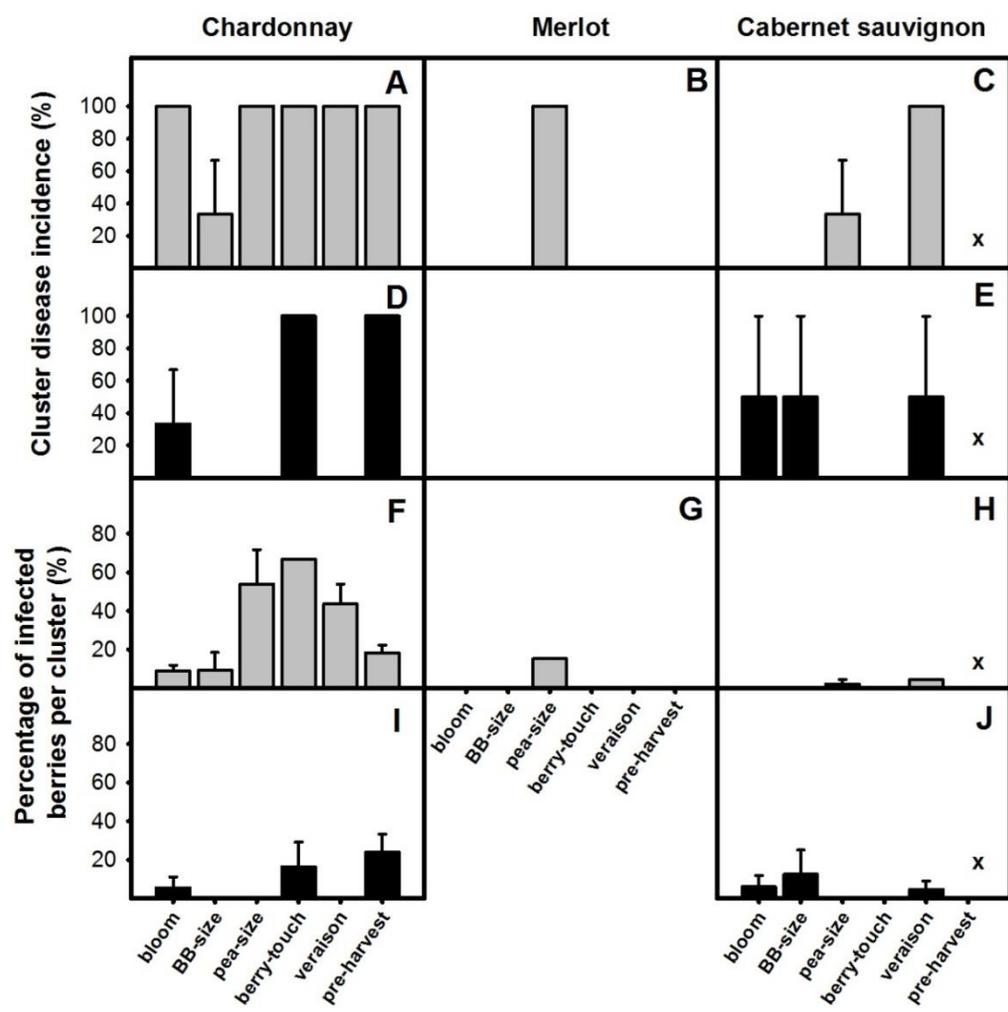


Figure 2.2: The mean cluster disease incidence (A-F) [presence or absence of infection per cluster] and the mean percentage of infected berries per cluster (G-L) from the controlled environment inoculation study in 2014. Four wine grape cultivars were shown vertically [Chardonnay (A, D, F, and I), Merlot (B and G), and Cabernet sauvignon (C, E, H, and J)]. Gray bars represent clusters inoculated with *C. gloeosporioides* and black bars represent clusters inoculated with *C. acutatum*, with standard errors as error bars. Data were not collected at certain cluster developmental stages due to a lack of clusters (x).

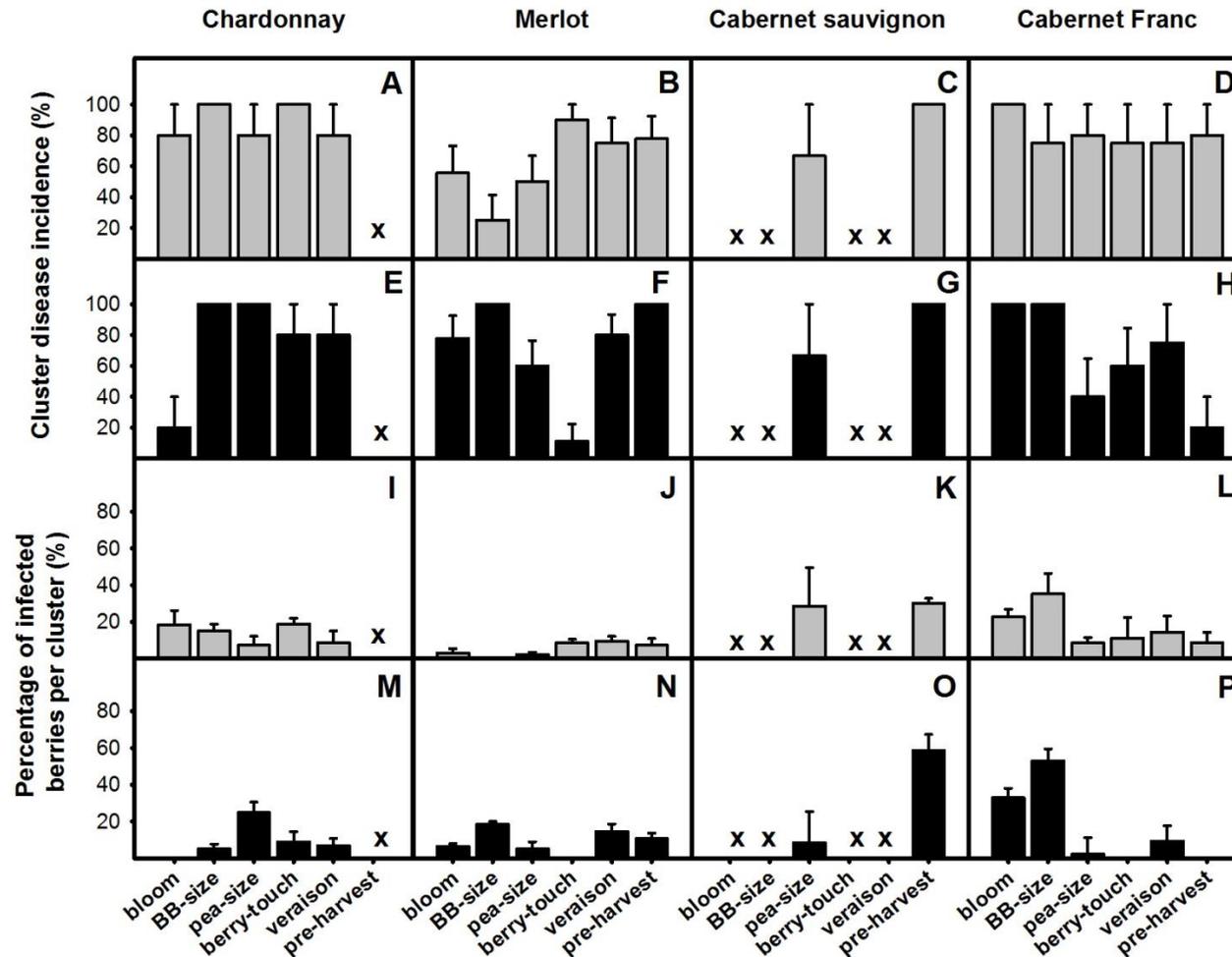


Figure 2.3: The mean cluster disease incidence (A-H) [presence or absence of infection per cluster] and the mean percentage of infected berries per cluster (I-P) from the field inoculation study in 2013. Four wine grape cultivars were shown vertically [Chardonnay (A, E, I, and M), Merlot (B, F, J, and N), Cabernet sauvignon (C, G, K, and O), and Cabernet Franc (D, H, L, and P)]. Gray bars represent clusters inoculated with *C. gloeosporioides* and black bars represent clusters inoculated with *C. acutatum*, with standard errors as error bars. Data were not collected at certain cluster developmental stages due to a lack of clusters (x).

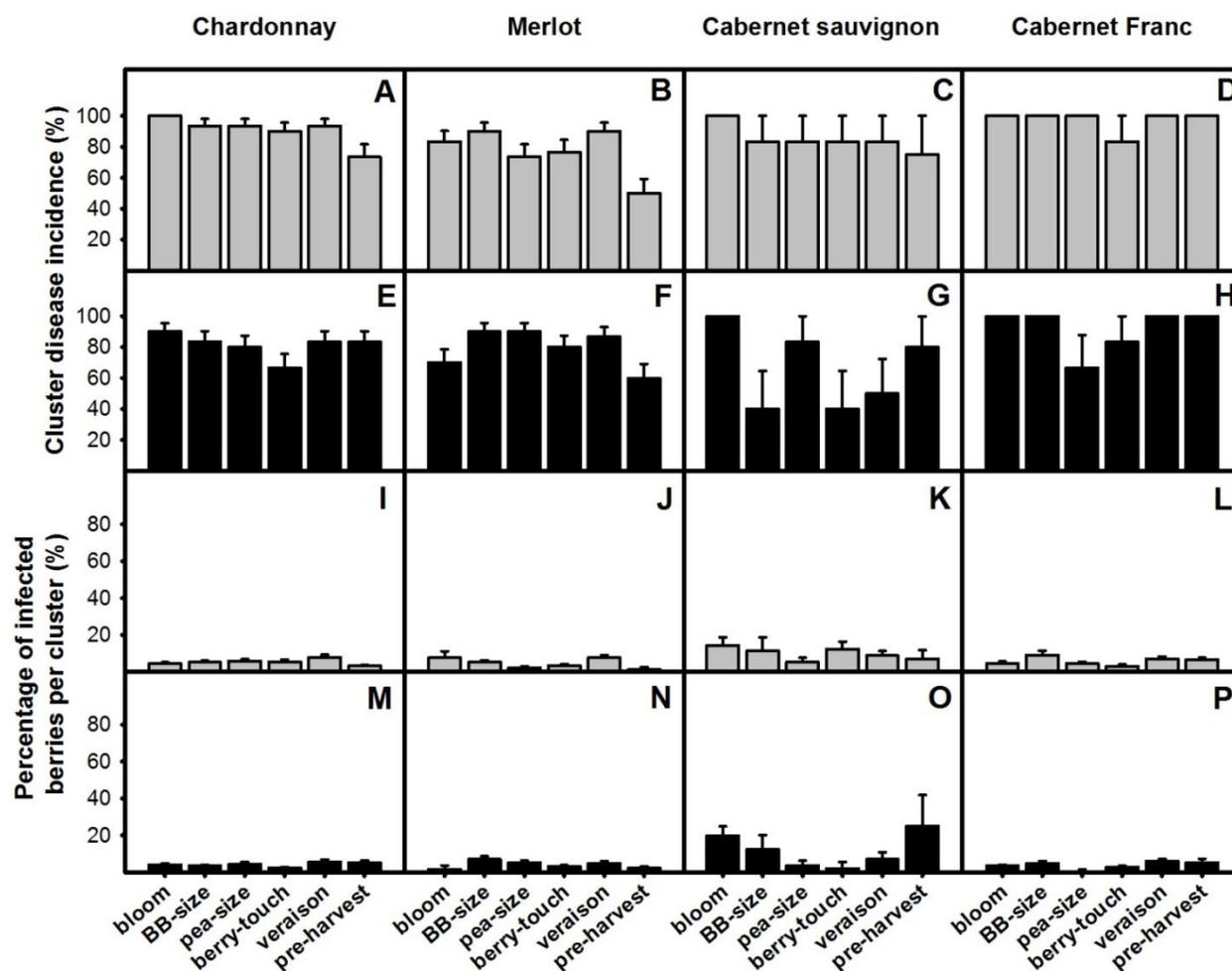


Figure 2.4: The mean cluster disease incidence (A-H) [presence or absence of infection per cluster] and the mean percentage of infected berries per cluster (I-P) from the field inoculation study in 2014. Four wine grape cultivars were shown vertically [Chardonnay (A, E, I, and M), Merlot (B, F, J, and N), Cabernet sauvignon (C, G, K, and O), and Cabernet Franc (D, H, L, and P)]. Gray bars represent clusters inoculated with *C. gloeosporioides* and black bars represent clusters inoculated with *C. acutatum*, with standard errors as error bars.

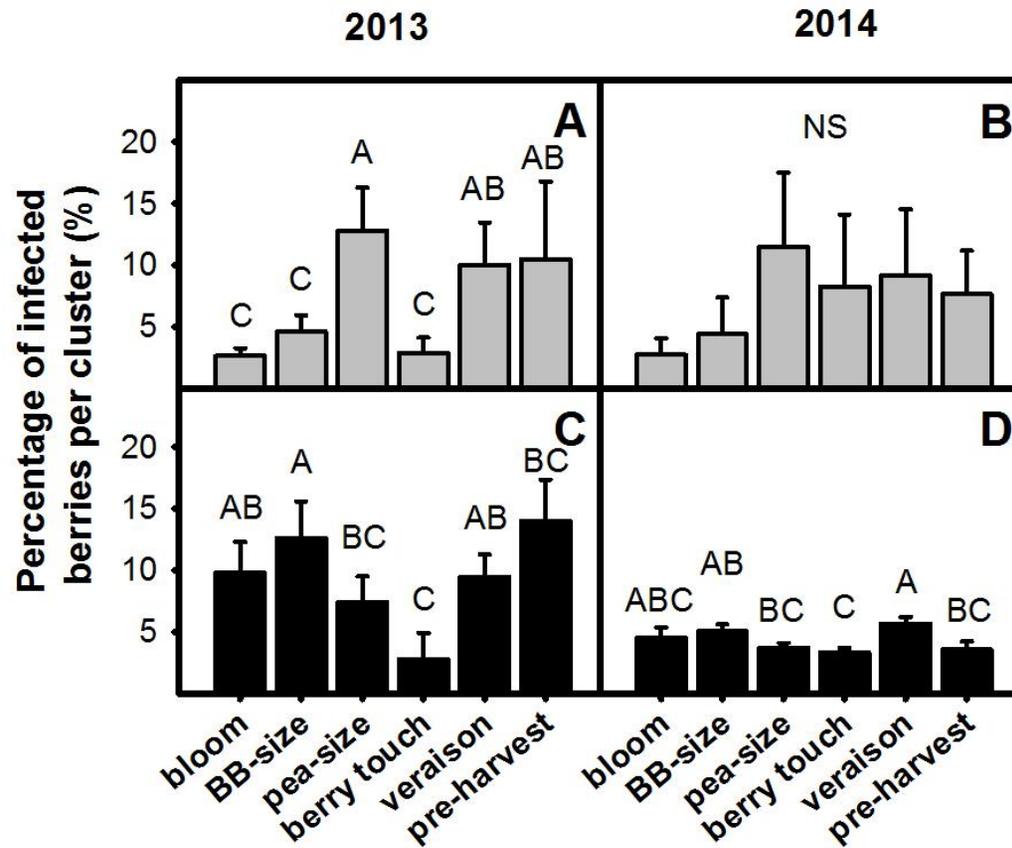


Figure 2.5: Effect of cluster developmental stage on the mean percentage of infected berries across four cultivars among six cluster developmental stages in 2013-2014. Gray bars represent clusters inoculated in the controlled environment study (A and B) and black bars represent clusters inoculated in the field inoculation study (C and D). The error bar represents the standard error. Significant mean separations based on LSD ($P \leq 0.05$) between cluster developmental stages are denoted by non-overlapping lettering.

Chapter 3.

AlamarBlue® and detached-berry assays to determine effective fungicides for management of ripe rot of grape, caused by two fungal species complexes, *Colletotrichum gloeosporioides* and *C. acutatum*.

Abstract

Colletotrichum gloeosporioides and *C. acutatum* fungal species complexes, which are the causal agents of ripe rot of grape, are endemic pathogens in Virginia. Partially due to lack of information, only a few fungicides are currently listed for control of ripe rot on wine grape. The efficacy of eleven modes of action for inhibition of *C. gloeosporioides* and *C. acutatum*, were investigated with two laboratory methods, alamarBlue® assay and fungicide-treated detached berry assay. AlamarBlue® is a dye that changes color in the presence of REDOX reactions. A liquid media assay using alamarBlue® was constructed to test active ingredient dilution series from 0 to 1000 µg/ml on both mycelia and conidia (1×10^5 conidia/ml). After 48 hours of incubation (25 °C), results were visually assessed as a color change. Results were analyzed to determine the effective concentration with 50% inhibition. In the detached berry assay, field-rate concentrations of fungicides were sprayed onto berries. After a 24-hour drying period, 5 µl conidial suspension (5×10^5 conidia/ml) was placed onto the berry surface, and incubated under a high humidity condition at 25 °C for 24 hours. Berries were then incubated under a dry condition, and visual disease assessments were conducted at the day 17. Protective fungicides (mancozeb, captan, and copper) as well as several newer chemistries (azoxystrobin and tetraconazole) were identified as products for use against ripe rot of wine grape. Other materials (*Bacillus subtilis* QST 713, potassium phosphite, and pyriofenone) were identified as potential candidates to be investigated further.

3.1 Introduction

According to the Fungicide Resistance Action Committee (FRAC), there are currently twelve major characterized fungicide modes of action (MOA) (FRAC, 2014). When all the subgroups under major MOAs are considered, there are approximately 220 specific active ingredients currently available for management of fungal pathogens. Despite the large number of active ingredients, what matters is the number of MOA when it comes to management of fungicide resistance because in many cases, there will be cross-resistance among different active ingredients within the same MOA (Leroux et al., 1999; Pfeufer and Ngugi, 2011). Insensitivity to

a MOA can be caused by one or several specialized mechanisms such as cellular chemical pumps (e.g., increased quantities of ATP binding cassette transporters and major facilitators), or modification of the MOA target site (e.g., mutation in ERG3 gene found in triazole resistance) (White et al., 1998). Even if there is no evidence of fungicide resistance or lack of efficacy, the list of known MOAs effective against a particular pathogen can be limited due to a lack of information or lack of registration. This is especially common for newly emerging (or re-emerging) pathogens.

Ripe rot of grape is caused by *Colletotrichum gloeosporioides* and *C. acutatum* species complexes (Damm et al., 2012; Weir et al., 2012), which is favored by warm and humid environmental conditions (Greer et al., 2011; Melksham et al., 2002; Steel et al., 2007). These endemic fungal species were considered minor pathogens by wine grape growers in the state of Virginia, USA; however, several recent ripe rot outbreaks have resulted in as high as 30% crop loss in the field (Nita, *personal communication*). However, there is very limited information on fungicide efficacy against ripe rot pathogens available.

Currently in the US, product labels of mancozeb, ziram, captan, and quinone outside inhibitors (QoI) list ripe rot of grape as a target disease. The pre-harvest interval (PHI) is 66-day and 44-day for mancozeb and ziram products, respectively. This creates limits on late-season fungicide selection, when ripe rot symptoms appear. In addition, Pennsylvania and North Carolina field studies have indicated that the QoI products are inconsistent in protection against ripe rot of grape (Halbrent et al., 2011; Hu et al., 2015; Travis et al., 2007). Furthermore, reports of QoI resistance among *C. gloeosporioides* have been observed on other host crops such as blueberries and peaches in South Carolina (FRAC, 2013; Hu et al., 2015). There is also a concerning Australian study that demonstrated a potential case of insensitivity to captan by a *C.*

gloeosporioides isolate (Greer, 2011). With these limitations, there is a strong need for an investigation of additional MOAs to provide grape growers more options for ripe rot chemical management.

The most common laboratory practice for fungicide efficacy screening utilizes comparisons of mycelial radial growth on fungicide-amended and fungicide-free media (Amiri et al., 2008; Smilanick et al., 2010; Quello, 2009). The quantity of fungal inhibition is calculated over a range of six to 14 days (Amiri et al., 2008; Mondal et al., 2005; Peres et al., 2004; Shephard, 1987; Xu et al., 2014). Recently, a liquid media-based fungicide screening method utilizing the non-toxic fluorescent dye alamarBlue® (AbD Serotec, Raleigh, NC), was adapted from cancer and cytotoxicity studies to fungicide sensitivity assays (Rampersad, 2012). The active ingredient of alamarBlue®, resazurin (O'Brien et al., 2000), undergoes a color change as it is converted to resorufin in living cells, under oxidation-reduction (REDOX) conditions (Rampersad, 2012). Rampersad (2011) demonstrated that the results from the alamarBlue® (AB) assay were comparable to those of a traditional amended media assay. Several advantages of the AB assay are; reduction from 7 to 14 days until data collection to two days and instead of radial mycelial growth comparisons, presence/absence of color change was collected. AlamarBlue®, or resazurin, assays have been successfully used for several plant pathogens, such as *Verticillium dahliae*, *Colletotrichum gloeosporioides*, and *Monilinia fructicola* (Cox et al., 2009; Quello, 2009; Rampersad and Teelucksingh, 2012; Vega et al., 2012).

The objective of this study is to screen rapidly multiple MOAs for candidate materials against both *C. gloeosporioides* and *C. acutatum* using two methods: an alamarBlue® (AB) assay (Cox et al., 2009; Rampersad, 2011; Rampersad and Teelucksingh, 2012) and inoculation on fungicide-treated detached berries.

3.2 Materials and Methods

3.2.1 Preparation of fungal isolates – The isolates of *Colletotrichum gloeosporioides* (CTCH1A11A) and *C. acutatum* (ACAP1A11A) were isolated from a wine grape cluster grown in a VA vineyard and an apple grown at the Alson H. Smith Jr. Agricultural Research and Extension Center (AHS AREC) in Winchester, VA (39°N 6' 33.55", -78°W 16' 56.08), respectively, in 2011. Isolates were identified based on traditional morphological characteristics as well as internal transcribed spacer (ITS) region sequencing and BLAST comparison (Bonants et al., 2013). The *C. gloeosporioides* isolate matched *C. siamense* within the *C. gloeosporioides* complex with 98.9% similarity score (Weir et al., 2012). The *C. acutatum* isolate matched *C. fioriniae* within the *C. acutatum* complex with a similarity score of 100.0% (Damm et al., 2012).

Single-spore cultures of both species were maintained on plates of potato dextrose agar (PDA) (Acumedia®, Neogen Company Lansing, MI) then transferred onto quarter strength antibiotic-amended PDA [streptomycin and chloramphenicol (100 mg/ml each)] (amended ¼ PDA) for conidial production. To maintain pathogenicity, both isolates were inoculated into apple or table grape after two mycelial transfers onto the amended ¼ PDA. The fungi were then re-isolated from the apple or table grape tissues at day 7 after inoculation. Hyphal tips were obtained after four to seven days of growth on amended ¼ PDA plates, then transferred and cultured on amended ¼ PDA plates for conidial harvest. Prior to harvest, plates were stored in an incubator (Precision™ 818, Thermo Scientific Inc, Waltham, MA) at 25 °C with a diurnal light cycle (12 h day /12 h night) for 7 to 14 days.

3.2.2 Mycelial and conidial harvest – To obtain a conidial suspension, the surface of seven- to 14-day old cultures were flooded with 2.5 ml of 2% potato dextrose broth (PDB) (Acumedia®,

Neogen Company, Lansing, MI) or sterile distilled water for the alamarBlue® (AB) and detached berry assays, respectively. The culture surface was gently rubbed with a sterilized glass rod to suspend conidia. The fungal suspension was filtered through two layers of autoclaved miracloth (EMD Millipore, Germany) to remove mycelium. The conidial suspension was then adjusted to the target concentration for each assay by counting on a hemocytometer (Neubauer Bright-line™, Hausser Scientific, Horsham, PA). For the mycelial harvest in the AB assay, aerial mycelia were scraped from the colony surface using sterilized forceps from seven- to 14-day old cultures grown on amended ¼ PDA plates.

3.2.3 AlamarBlue (AB) assay: The experimental design of the AB assay was a three-factor factorial design where *Colletotrichum* species, inoculum type, and active ingredient concentration were examined for their effect on the inhibition of fungal growth. The efficacies of ten active ingredients formulations (azoxystrobin, boscalid, captan, copper hydroxide, copper octanoate, mancozeb, potassium phosphite, pyriofenone, tetraconazole, and thiophanate-methyl) (Table 3.1) were compared with the positive (mycelia or conidia without a fungicide) and negative (no mycelium or conidia without a fungicide) controls. The commercial fungicide formulations of the tested active ingredients s were suspended in 17 ml of 95% ethanol for 24 h prior to use to kill potential biological contaminants. The concentrated stocks were then diluted to 2,000 µg/ml (= ppm) with the addition of 23 ml 2% PDB to reduce the ethanol concentration to 40%. Since AB dye is the most effective in neutral or mildly basic (pH 7-8) solutions (O'Brien et al., 2000), the acidic fungicide stocks (captan and tetraconazole) were adjusted to 7.0 by titration with 1 M sodium bicarbonate (Sigma-Aldrich, St. Louis, MO). Also, in order to inhibit the alternative oxidase pathway (Wood & Hollomon, 2003), salicylhydroxamic acid (SHAM)

(Alfa Aesar, Ward Hill, MA) was added to the azoxystrobin fungicide stock solution (2,000 µg/ml) to achieve a concentration of 10 µg/ml.

Sterile, 48-well culture plates with 1 ml wells (Celltreat Scientific Products, Shirley, MA) were used for the AB assay. Each well contained 220 µl of solution that was comprised of: 22 µl (10%) AB dye; 100 µl of diluted fungicide suspended in 2% PDB. Either 100 µl 1×10^5 conidial suspension in 2% PDB or 100 µl of 2% PDB containing mycelia was added to each well. Two dilution series of active ingredient were tested with this assay: 1000, 100, 10, 1, 0.1, 0.01, and 0 µg/ml and 500, 300, 200, 150, 50, 30, and 0 µg/ml. The culture plates were incubated in a moist chamber for 48 hours at 25 °C in darkness. At 24 and 48 h, color change (= evidence of fungal inhibition) was visually assessed. Fungal inhibition (no germination of conidia or growth of mycelium) resulted in blue (i.e., no color change) and unsuccessful fungal inhibition resulted in purple, pink, or clear wells as the fluorescent dye became quenched. The content of wells was arranged to allow the side-by-side observation of fungal inhibition for mycelial growth and conidial germination across the range of tested fungicide concentrations (Figure 3.1). The assay was repeated five times with each active ingredient dilution series.

3.2.4 Detached berry assay: The experimental design of the detached berry assay was a two-factor factorial design where commercial fungicides and *Colletotrichum* species were examined for their effect on development of ripe rot symptoms. White table grapes (cultivar ‘Autumn King’) from a grocery store and mature wine grapes harvested from AHS AREC in Winchester, VA (cultivars Petit Manseng and Chardonnay) were used for these experiments. Both table and wine grapes were prepared in the same manner. Berries without wounds or disease symptoms were cut from the rachis with the pedicel still attached. Berries were disinfected by soaking in 10% bleach solution for 1.5 min, then triple rinsed with sterile distilled water to remove bleach

residue and dried in a laminar flow hood. Then, the pedicel end of each berry was dipped twice in liquid wax to seal the natural opening. Once waxed, berries were stored in airtight containers at 4 °C for less than 24 h until use.

For the table and wine grape assays, ten commercial fungicide formulations at recommended field application rates for wine grape (azoxystrobin, *Bacillus subtilis* strain QST 713, boscalid, captan, copper hydroxide, mancozeb, potassium phosphite, pyriofenone, tetraconazole, and thiophanate-methyl), positive control (non-treated and inoculated), and negative control (non-treated and non-inoculated) were tested (Table 3.1). Fungicide suspension was applied to berries using an airbrush (model E91, TCP Global, San Diego, CA) at 30 psi (206.8 kPa) until run-off. Fungicide-treated berries were dried in the trays for 4-6 h in a laminar flow hood, and then placed in quail egg trays (EggCartons.com, Machung, MA), which were surface sterilized with a citric acid solution (35.0 L of distilled water, 75.0 ml citric acid, 12.1 g of potassium metabisulfite), and 70% isopropyl alcohol. Each tray contained 12 berries, which were comprised of four groups of three berries with the same treatment (a.i, positive or negative control). There were four (table grape) or six (wine grape) replicates of the tray per species for each experimental run, and four experimental runs were conducted for both table and wine grape. Thus, 48 and 72 berries were assessed per treatment for table and wine grape, respectively.

On the surface of the treated and positive control berries, 5 µl of 5×10^5 conidia/ml suspension of either *C. gloeosporioides* or *C. acutatum* were placed with a pipet. The negative control received 5 µl of sterilized distilled water. Trays were placed into moist chambers for an incubation of 23.5 h at 25 °C with a diurnal light cycle (12 h day /12 h night) to keep the berry surface wet. Berries in trays were then dried for 30 min in a laminar flow hood. Once the surface of the treated berries was dry, trays were incubated in a controlled environment chamber (E-75L,

Percival, Perry, IA) adjusted to RH < 50% to keep the berry surface dry. Incidence of berry symptom development (yes or no) was recorded on days 3, 5, 7, 10, and 14 (table grape) or 5, 7, 10, 12, 15, and 17 (wine grape). Berries were frozen for 20 min at -20 °C on day 10 to accelerate symptom development by breakdown of berry epidermal cells (Biggs, 1995), and data from day 14 (table grape) or day 17 (wine grape) were used for the analyses. The mean percentage of infected berries per experimental run was calculated for each fungicide treatment. In the wine grape assays, symptoms were observed on a limited quantity of the negative controls due to naturally occurring infections in the AHS AREC vineyard. Therefore, prior to data analysis, an adjusted mean percentage of infected berries for each fungicide treatment was estimated as: mean percentage of infected berries = [(observed percentage of infected berries) – (mean percentage of infected berries from negative control)].

3.2.5 Statistical analysis – For the AB assay, the intercept and slope of reaction of the *C. gloeosporioides* (Cg) or *C. acutatum* (Ca) isolate against the active ingredient concentration were estimated using a generalized linear mixed model in SAS (PROC GLIMMIX, ver. 9.4, SAS institute, Cary, NC). Since the response variable is a binomial variable (yes or no on fungal inhibition), three common different link functions for binomial data (logit, probit, and complementary log-log (CLL)) were used in the generalized linear mixed model (Bolker et al., 2009). The best link function for each *Colletotrichum* species complexes, inoculum type, and active ingredients combination was selected based on the comparison of Akaike's Index of Criterion and deviance (Pearson's chi-square divided by degrees of freedom) (SAS Institute, 2012). Using the intercept and slope from the best-fit model, a nonlinear regression mixed model (PROC NLMIXED in SAS) was fitted to the actual data (Littell et al., 2006). The resulting

model was used to calculate the effective concentration for which 50% of samples are inhibited (EC50) for each combination.

For the detached-berry assays, the mean percentage of infected berries of each treatment was analyzed using a generalized linear mixed model (PROC GLIMMIX in SAS) where treatment effect was considered as a fixed factor, and replicate effect was treated as a random factor. When the effects of treatments were found to be significant, treatment means were compared using Fisher's LSD with a 95% confidence interval.

3.3 Results

3.3.1 AlamarBlue® assay –ANOVA, which was based on a generalized linear mixed model with a logit as a link function, was utilized to examine the effect of *Colletotrichum* species, inoculum type, active ingredient concentration, and their interactions (Table 3.2). The effect of inoculum type or the interactions of inoculum type and active ingredient concentration were often significant ($P \leq 0.05$) (Table 3.2). This indicated that there were differences in the efficacy of active ingredient between mycelia and conidia. In some cases, the effect of inoculum type on active ingredient a.i. efficacy was also affected by the concentration of the active ingredient. This interaction was significant with azoxystrobin, captan, copper hydroxide, and potassium phosphite, and the interaction of inoculum type and active ingredient concentration was significant with mancozeb (Table 3.2). In addition, a significant effect of the two *Colletotrichum* species complexes ($P \leq 0.05$) was observed with tetraconazole, and an interaction between active ingredient concentration and *Colletotrichum* species complexes was observed with copper hydroxide and mancozeb (Table 3.2). Therefore, models were developed for each *Colletotrichum* species complexes, inoculum type, and active ingredient concentration combination to describe

the effect of active ingredient concentration on fungal inhibition (= no color changes in the AB dye) so that EC50 can be estimated (Table 3.3).

The intercept and slope of each combination were calculated and were found to be significant ($P \leq 0.05$) for mycelia and conidia for most of the active ingredients (Table 3.3). Thus, both the intercept and slope were significant predictors of the resulting EC50. On the other hand, neither the intercept nor slope were significant ($P > 0.05$) with some of fungicides, such as boscalid, pyriofenone, and two of the supplemental chemicals (Table 3.3), suggesting a lack of fungal inhibition, even at the high concentrations (Fig. 3.2, E-H, Fig. 3.3, I-L, Fig. 3.4, A-H).

The effect of inoculum type and its interaction with active ingredient concentration on fungal inhibition could result in differences in the selection of the link function (Table 3.3). For example, for *C. gloeosporioides* complex (Cg) treated with copper hydroxide, the mycelium model was better fit with a logit link function (Fig. 3.2, M and O) while the conidial model fit better with a probit (Fig. 3.2, N and P). The difference in link functions resulted in an EC50 of 85 $\mu\text{g/ml}$ for mycelium, and 48 $\mu\text{g/ml}$ for conidia. The interaction effect was also shown as different intercept and slope when the link function was the same. For instance, for azoxystrobin, the intercept was 1.9 and 3.7 and the slope was -1.0 and -1.6, for mycelium and conidial models, respectively. Thus, EC50 resulted in 210 $\mu\text{g/ml}$ for mycelia, and 473 $\mu\text{g/ml}$ for conidia (Table 3.3).

The effect of *Colletotrichum* species complexes on fungal inhibition was often not significant ($P > 0.05$) in the initial screening of factors with a generalized linear mixed model (Table 3.2). However, when the mycelial and conidial models between *C. gloeosporioides* (Cg) and *C. acutatum* (Ca) were compared, 50% of the cases resulted in selections of different link

functions between Cg and Ca. Very large differences in EC50 values were observed between the species complexes (Table 3.3).

In spite of these differences between *Colletotrichum* species complexes, when the resulting EC50 was compared with the active ingredient concentration applied in the field, the results between two species were similar. For both species complexes, EC50 of azoxystrobin, boscalid, tetraconazole, pyriofenone, and thiophanate-methyl exceeded that of the concentration applied in the field (Table 3.3), indicating that these materials did not inhibit the activity of Cg or Ca even at very high rates. When the two inoculum types were compared for all five fungicides listed above, both inoculum types resulted in higher EC50 values than the field rates (Table 3.3) with an exception of a conidial model for thiophanate-methyl.

3.3.2 Detached berry assays – With the table grape assay, the mean percentage of infected berries per treatment ranged from 0% to 43.3% for *C. gloeosporioides* (Cg) and from 0% to 32.5% for *C. acutatum* (Ca) where higher means were observed with the positive controls (Fig 3.5, A and C). There was no significant difference between the two species ($F = 0.5$, $P = 0.49$) or the interaction between the *Colletotrichum* species complexes and the fungicides ($F = 0.9$, $P = 0.51$) on the mean percentage of infected berries. Thus, although *Colletotrichum* species complexes are displayed in two separate panels in Fig 3.5 (A and C), both *Colletotrichum* species complexes resulted in the same mean separation on the significant treatment effect ($F = 8.1$, $P < 0.01$). All tested active ingredients resulted in significantly lower mean percentage of infected berries than that of the positive control (Fig. 3.5, A and C). Tetraconazole resulted in the lowest mean percentage of infected berries. Although the other active ingredients resulted in numerically higher mean percentages of infected berries, there was no significant difference ($P > 0.05$) between the other active ingredients and tetraconazole (Figure 3.5, A and C). When the

mean percentage of infected berries of each a.i was compared to the negative control, only copper hydroxide and mancozeb sustained significantly higher ($P \leq 0.05$) mean percentage of infected berries (Fig. 3.5, A and C).

The effect of the *Colletotrichum* species complexes was significant ($P = 0.03$) in the wine grape assay; therefore individual mean separations of the treatment effect are shown in Fig. 3.5 (B and D). The mean percentage of infected berries ranged from 0.0% to 19.5% on Cg-inoculated berries and 0.0% to 16.7% on Ca-inoculated berries, where the highest means were observed on the positive controls (Fig. 3.5, B and D). Although there were numerical differences among tested active ingredients, there was no significant treatment effect ($F = 1.3$, $P = 0.24$) with Ca-inoculated berries.

With Cg-inoculated berries, treatment effect was significant ($F = 2.2$, $P = 0.01$). As with the table grape assay, tetraconazole resulted in the lowest mean percentage of infected berries. No significant difference ($P > 0.05$) was observed between tetraconazole and the negative control (Fig. 3.5, B). Results from azoxystrobin, boscalid, captan, and copper hydroxide were significantly lower ($P > 0.05$) than the positive control and not significantly higher ($P > 0.05$) than the negative control (Fig. 3.5, B). On the other hand, the mean percentage of infected berries for *Bacillus subtilis*, mancozeb, potassium phosphite, pyriofenone, and thiophanate-methyl did not differ significantly ($P > 0.05$) from the positive control (Fig. 3.5 B)

Inoculation on Petit Manseng berries harvested from the AHS AREC resulted in very low development of ripe rot symptoms, even after artificial inoculations. There were only 14 infected berries out of 720 inoculated berries. The late-season fungicide program at the Petit Manseng vineyard was minimal (no fungicide applications between 20 July and 16 Sept. in 2013), thus, Petit Manseng appears to be very resistant to ripe rot infection.

3.4 Discussion

In the alamarBlue® assay, protectant chemicals in the M-FRAC groups, such as captan (phthalimides), copper hydroxide (copper), copper octanoate (copper) and mancozeb (EBDC), resulted in EC50s that were much lower than their recommended field application rate regardless of inoculum type (mycelia or conidia). Currently, both captan and mancozeb list ripe rot as a target grape disease. Both captan and mancozeb resulted in significantly lower percentages of infected berries ($P \leq 0.05$) with the table grape assay; however, to our surprise, mancozeb did not result in significantly lower ($P > 0.05$) percentage of infected berries in the wine grape assay. The copper hydroxide treatment also resulted in numerically lower but not significantly lower mean percentage of infected berries in both table and wine grape assays with *C. gloeosporioides*. Although field studies are still necessary to verify the efficacy of copper, copper formulations tested in both assays showed some promising results for ripe rot management.

Azoxystrobin, a quinone outside inhibitor (QoI) fungicide, is another fungicide that is registered for ripe rot control in grape. Results from detached table and wine grape assay showed that percentages of infected berries were significantly lower ($P \leq 0.05$) with both *C. gloeosporioides* and *C. acutatum*; however, in the AB assay, the azoxystrobin treatment resulted in higher EC50 value than its field application rate. QoI resistant isolates of *C. gloeosporioides* and *C. acutatum* have been discussed in several studies (Ishii, 2009; Pasche et al., 2004; Vega et al., 2012; Vega and Dewdney, 2013). Our lab completed a bioassay where mycelial growth inhibition was measured on media amended with 100 µg/ml of azoxystrobin and 1,000 µg/ml of SHAM where our isolates were found to be sensitive to QoI fungicides (Nita, *data not shown*). SHAM has been used for various QoI fungicide-amended medium assays in order to suppress an

alternative-oxidase pathway that the target fungal species can utilize when a QoI fungicide affects the target cytochrome b site (Hollomon et al., 2005; Joseph-Horne et al., 2001). However, in our AB assay, we used a much lower concentration of SHAM in the testing wells than the radial growth bioassay; therefore, it is most likely that the low quantity of SHAM was not adequate to suppress the alternative pathway (Pasche et al., 2004).

Results from both wine and table grape assays showed that the tetraconazole treatment resulted in significantly lower percentages of infected berries ($P \leq 0.05$) than the positive control. The exception to this was the results of *C. acutatum* in wine grape detached-berry assay where none of the treatments was significantly different from the positive control. On the other hand, the AB assay results showed that EC50 values of tetraconazole were 40.5 and 65.8 $\mu\text{g/ml}$ for Cg- and Ca-mycelia, respectively, and 39.5 and 22.8 $\mu\text{g/ml}$ for Cg- and Ca-conidia, respectively. Field application rate of tetraconazole is 13.7 $\mu\text{g/ml}$, which is lower than the calculated EC50 for control of both mycelial and conidial development. Demethylase inhibitor (DMI) fungicides function by inhibiting the production of ergosterol, a cell membrane component (Hollomon, 2010; Wong and Midland, 2007), and generally achieve higher efficacy when applied post infection (Turechek et al., 2006; Wong and Midland, 2007). However, our results from the AB assay indicated lower degree of inhibition on mycelial growth. Other studies suggested that there is variation in efficacy among DMI fungicide against *C. gloeosporioides*, such as sensitivity to prochloraz but not to tebuconazole (Xu et al., 2014).

Considering how well tetraconazole worked in our table grape detached berry assays, it was surprising to see the relatively high EC50 values in the AB assay. One possible cause of this discrepancy is the required adjustment of the pH level for the latter. When tetraconazole was suspended as a stock in ethanol and 2% PDB, its pH was 4.3. In order for alamarBlue® to work

correctly, the pH of the solution has to be adjusted to 7.0 through the addition of sodium bicarbonate. However, tetraconazole is considered to be stable in a pH range of 5-9 pH (Meylan and Howard, 1993). Thus, the change in pH should not have affected the efficacy of tetraconazole. Captan also required sodium bicarbonate to raise the pH of the stock (from pH 5.3). Captan is known to degrade faster as pH increases from pH 5 (32 hr) to pH7 (8 hr) (Schilder, 2008). Our assay indicated that sodium bicarbonate itself could have efficacy against *C. gloeosporioides* and *C. acutatum*; however, the amount of sodium bicarbonate in the stock was also diluted along with the active ingredient. The concentration of sodium bicarbonate ranged from 600 µg/ml to 0.006 µg/ml in the tetraconazole wells, and 300 µg/ml to 0.003 µg/ml in the captan wells, resulting in a much lower concentrations than the original stock solution. The diluted sodium bicarbonate in the lower concentrations should not affect either the growth of mycelia or germination of conidia.

Potassium phosphite is the *in planta* active molecule of the U33-FRAC group products (FRAC, 2014; Smillie et al., 1989). The AB assay results showed potassium phosphite to have EC50 of 48% (conidia) and 15% (mycelia) of the field recommended rate. The potassium phosphite treatment also resulted in a significant reduction ($P \leq 0.05$) in the mean percentage of infected berries than the positive control in the table grape assay; however, the results from the detached wine grape assay did not support the table grape results. While it was numerically lower, the mean percentage of infected berries in the potassium phosphite treatment was not significantly lower ($P > 0.05$) than those of the positive control.

Information is limited on the effects of succinate dehydrogenase inhibitor (SDHI) fungicides on the inhibition of *Colletotrichum* species (Wedge et al., 2007). In our assays, boscalid, a SDHI fungicide, showed mixed results. In the detached berry assay, boscalid was an

effective control of both *Colletotrichum* species complexes; however, in the AB assay, the EC50 value was above the field application rate. Boscalid is an older SDHI that was introduced in 2003 but newer active ingredients have been introduced more recently with the potential to more effectively control *Colletotrichum* species (Glattli et al., 2005).

Thiophanate-methyl, a methyl benzimidazole carbamate (MBC) fungicide, also provided inconsistent fungal inhibition in the AB and detached berry assays. In the table grape assay, thiophanate-methyl was effective but in the other two assays, the AB and wine grape assay, thiophanate-methyl was ineffective. Thus, the efficacy of thiophanate-methyl may not be strong. Several studies (Kim et al., 2007; Peres et al., 2004; Peres et al., 2005; Zhan & Huang, 2007) suggested the existence of MBC resistance among *Colletotrichum* isolates, such as benomyl insensitivity in *C. acutatum* isolates, benomyl resistance in *C. gloeosporioides* (Peres et al., 2004) and *C. gloeosporioides* carbendazim resistant isolates (Zhan and Huang, 2007).

As of 2015, pyriofenone has not been introduced as a commercial product in the US. Currently, we do not know the exact MOA of pyriofenone, but it inhibits formation of appressoria of powdery mildews, according to the product description. In the AB assay, pyriofenone provided no inhibition of conidial germination or mycelial growth with EC50 values well over the field application rate. However, since pyriofenone inhibits appressorium formation, germinated conidia probably provided enough respiration activity to change the color of the AB dye, or germinating conidia suspended in a liquid may not form appressoria due to lack of a hard substrate. Thus, the current format of the AB assay may not be the best method for testing this MOA. In the wine grape assays, the percentage of infected berries of pyriofenone was similar to the positive control. However, the detached table grape assays indicated that the mean

percentage of infected berries for pyriofenone was significantly lower than that of the inoculated control. Further study on this MOA is needed.

Bacillus subtilis strain QST 713 (Serenade Optimum) produced one of the lowest percentage of infected berries of on table grapes among tested active ingredients, and was significantly lower than the positive control ($P \leq 0.05$). On the other hand, on wine grapes, the percentage of infected berries of the *Bacillus subtilis* treatment was not significantly different ($P > 0.05$) from the positive control. Since this is a biological agent, differences between the table and wine grape assays could be due to the physical berry attributes of Chardonnay and Autumn King (e.g. pH, firmness, °Brix level, skin attributes, such as thickness (Rolle et al., 2010; Smillie et al., 1989), the effect of fungicide applied prior to the harvest, or the age of the product. The table and wine grape assays were conducted almost one year apart; however, the same stock of *Bacillus subtilis* was used in both assays. Since *Bacillus subtilis* is a living organism, the length and condition of the storage might have affected its efficacy.

Differences between inhibition of mycelium and conidia between active ingredients in the AB assay were congruent with the differences in the cellular site of action for each MOA (Morton and Straub, 2008). Since active ingredients interact with different cellular components of pathogens, a chemical's efficacy can differ accordingly. For example, phthalimide fungicides, such as captan and copper compounds (copper hydroxide, or copper octanoate), inhibit conidial germination (Morton and Straub, 2008) whereas MBCs such as thiophanate-methyl inhibit nuclear division (Hu, et al., 2015). The EC50 values from the AB assay coincide with the cellular effect of each active ingredient with lower EC50 values for conidia than mycelia. In general, EC50s for mycelium were higher than the EC50s of conidial germination, and this may partially be due to the sensitivity of the dye and inconsistent quantities rather than inoculum sensitivity

differences. Since we placed aerial mycelium directly to the well containing the chemical solution, the time lag between the chemical contact and the time of inhibition may have been a factor, allowing enough respiration for the dye to change color. A potential improvement may be to add the dye a few hours after placing mycelia in the well and we plan to test this approach.

This study demonstrated that the AB assay could be used as a quick and reliable fungicide screening method. We were able to examine the effects of ten active ingredients and their dilution series on both mycelial growth and conidial germination of two *Colletotrichum* species. However, understanding of MOA and other chemical and physical properties of fungicides is important to utilize this assay in a proper manner. With a combination of the AB and two detached berry assays, we have identified azoxystrobin, captan, copper hydroxide, copper octanoate, mancozeb, and tetraconazole as excellent candidates for ripe rot control. With further investigation, *Bacillus subtilis*, potassium phosphite, pyriofenone, and thiophanate-methyl could be considered potential fungicide choices.

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Table 3.1: List of fungicide active ingredients tested in alamarBlue® and detached grape assays, including trade name, active ingredient (a.i.), company, and FRAC group

Active ingredient (a.i)	Trade Name	a.i.%	Company	FRAC ^Z	Rate per Acre	µg/ml ^Y in field
Azoxystrobin	Abound®	22.9	Syngenta Crop Protection	11	355 ml	81.3
<i>Bacillus subtilis</i> strain QST 713	Serenade® Optimum	26.2	Bayer CropScience	44	454 g	314.8
Boscalid	Endura®	70.0	BASF Corporation	7	237 ml	165.6
Captan	Captan Gold™ 80 WDG	78.2	Makhteshim Agan of North America	M4	907 g	709.2
Copper hydroxide	Champ® Dry Prill	57.6	NuFarm Americas	M1	1361 g	782.2
Copper octanoate	Cueva®	10.0	Certis USA	M1	18927 ml	378.5
Mancozeb	Dithane® 75DF Rainshield	75.0	Dow AgroScience, LLC	M3	1361 g	1020.2
Potassium phosphite	ProPhyt®	54.5	Helena Chemical Company	33	2366 ml	773.6
Pyriofenone	Property® F	18.0	ISK Biosciences	U8	148 ml	26.6
Tetraconazole	Mettle® 125 ME	11.6	Gowan Company	3	118 ml	13.7
Thiophanate-methyl	Topsin® M 70 WDG	70.0	United Phosphorus, Inc	1	454 g	317.4

Supplemental chemicals used in alamarBlue® assay

Ethanol			Fisher Scientific
Salicylhydroxamic acid	(SHAM)		Fisher Scientific
Sodium bicarbonate			Fisher Scientific

^Z Fungicide Resistance Action Committee (FRAC) codes

^Y µg/ml = parts per million of active ingredient applied recommended by the label in a field application in Virginia vineyards based on application volume of 100 gallons per acre (or 935 liters per ha)

Table 3.2 ANOVA table for the effect of *Colletotrichum* species complex (Cg vs Ca), inoculum type (mycelia vs conidia), active ingredient (a.i.) concentration, and their interactions on fungal inhibition based on the alamarBlue® assay results.

Effects	Azoxystrobin		Boscalid		Captan		Copper hydroxide		Copper octanoate	
	F-value	P-value ^Z	F-value	P-value ^Z	F-value	P-value ^Z	F-value	P-value ^Z	F-value	P-value ^Z
<i>Colletotrichum</i> species complex	1.9	0.16	0.0	1.00	2.5	0.11	0.9	0.34	0.3	0.61
Inoculum type	15.8	< 0.01 **	0.0	1.00	0.0	0.94	1.4	0.23	630.7	< 0.01 **
A.i. concentration	24.8	< 0.01 **	0.0	0.98	34.2	< 0.01 **	46.4	< 0.01 **	376.9	< 0.01 **
<i>Colletotrichum</i> x a.i. concentration	2.1	0.15	0.0	0.99	0.1	0.74	5.3	0.02 *	1.4	0.24
Inoculum type x a.i. concentration	8.2	< 0.01 **	0.0	0.99	11.1	< 0.01 **	2.3	0.13	Infinity	< 0.01 **

Effects	Mancozeb		Potassium phosphite		Pyriofenone		Tetraconazole		Thiophanate-methyl	
	F-value	P-value ^Z	F-value	P-value ^Z	F-value	P-value ^Z	F-value	P-value ^Z	F-value	P-value ^Z
<i>Colletotrichum</i> species complex	0.0	0.99	0.5	0.47	0.2	0.63	4.6	0.03 *	1.1	0.29
Inoculum type	2.6	0.11	2.0	0.16	0.9	0.33	3.5	0.06	0.3	0.56
A.i. concentration	28.2	< 0.01 **	50.2	< 0.01 **	51.9	< 0.01 **	12.0	< 0.01 **	38.9	< 0.01 **
<i>Colletotrichum</i> x a.i. concentration	0.8	0.37	0.4	0.51	0.2	0.64	3.4	0.07	0.9	0.33
Inoculum type x a.i. concentration	15.3	< 0.01 **	9.0	< 0.01 **	0.3	0.60	1.9	0.16	2.2	0.14

^Z One asterisk and two asterisks next to the number indicate significance at the 95% and 99% level, respectively.

Table 3.3: Estimated effective concentrations (EC50) of ten fungicide active ingredients and three assay additives for the control of *C. gloeosporioides* complex(CTCH1A11A) and *C. acutatum* complex(ACAP1A11A) mycelia and conidia ,determined by the alamarBlue® *in vitro* assay.

Inoculum type	Active ingredient	Link ^Y	Cg ^Z			Ca ^Z			
			Intercept ^X	Slope ^X	EC50 ^W	Intercept ^X	Slope ^X	EC50 ^W	
Mycelia	Azoxystrobin	CLL	1.9*	-1.0 *	210.5+	logit	1.3 *	-0.3	5216.7 +
	Boscalid	logit	231.3	-81.2	710.3+	logit	9.0	-2.3	NC +
	Captan	probit	1.4**	-1.0 **	23.6	CLL	5.2 **	-2.9**	103.3
	Copper hydroxide	probit	3.9*	-2.1 **	85.3	probit	5.3 *	-2.6 *	166.8
	Copper octanoate	logit	127.7	-68.8	97.6	logit	145.2 **	-73.0**	96.6
	Mancozeb	CLL	2.6	-2.4 **	27.1	CLL	2.0 *	-1.9**	26.7
	Potassium phosphite	probit	5.1**	-2.4 *	331.1	CLL	5.3 *	-2.4 *	505.1
	Pyriofenone	logit	21.3	-7.3	1000.0+	CLL	7.6	-2.8	1116.9 +
	Tetraconazole	probit	3.4**	-2.1 **	40.5+	probit	5.6 **	-3.3**	65.8 +
	Thiophanate-methyl	logit	12.2*	-4.6 *	414.3+	CLL	4.1 *	-1.6 *	552.5 +
Conidia	Azoxystrobin	CLL	3.7*	-1.6	472.8+	probit	3.6 **	-1.4**	385.3 +
	Boscalid	logit	16.5	0.0	NC+	logit	11.3	-3.4	NC +
	Captan	probit	1.8**	-1.8 **	8.9	probit	2.7 **	-2.2 **	16.6
	Copper hydroxide	probit	5.6**	-3.3 **	48.3	logit	4.0 **	-2.9**	35.5
	Copper octanoate	probit	2.7*	-1.7 **	53.7	probit	3.1 *	-2.0**	43.6
	Mancozeb	logit	21.6	-32.0	3.5	CLL	2.2	-3.1 *	11.1
	Potassium phosphite	probit	5.1**	-2.8 **	118.9	CLL	18.0 **	-10.2**	87.7
	Pyriofenone	CLL	4.8	-1.6	1140.9+	CLL	1.1 **	-0.2	7.1x10 ⁹ +
	Tetraconazole	probit	3.8**	-2.4 **	39.5+	probit	2.5 **	-2.0**	22.8 +
	Thiophanate-methyl	CLL	6.2**	-2.7 *	281.4	logit	7.5 **	-3.2**	238.3
Supplemental chemicals used in the alamarBlue® assay									
Mycelia	Ethanol	logit	124.0	-41.3	2322.9	logit	15.0	-0.1	-1.0
	SHAM	logit	208.6	-73.3	706.2	logit	14.7	0.0	NC
	Sodium bicarbonate	logit	173.7	-70.1	300.0	logit	120.7	-65.0	70.6
Conidia	Ethanol	logit	15.0	-0.1	NC	logit	15.0	-0.1	NC
	SHAM	logit	14.7	0.0	NC	logit	14.7	0.0	NC
	Sodium bicarbonate	probit	4.8	-2.4 *	167.9	logit	152.2	-95.1	38.7

^Z Cg = *Colletotrichum gloeosporioides* complex, Ca = *Colletotrichum acutatum* complex

^Y Link function in a generalized linear mixed model, CLL = complementary log-log

^X Intercept and slope parameters of the generalized linear mixed model. One asterisk and two asterisks next to the number indicate significance at the 95% and 99% level, respectively.

^W The effective concentration that suppresses 50% of growth of mycelia or germination of conidia (EC50) in µg/ml. A ‘+’ denotes an EC50 that is higher than the labeled concentration for field applications. A ‘NC’ denotes an EC50 could not be calculated for the chemical

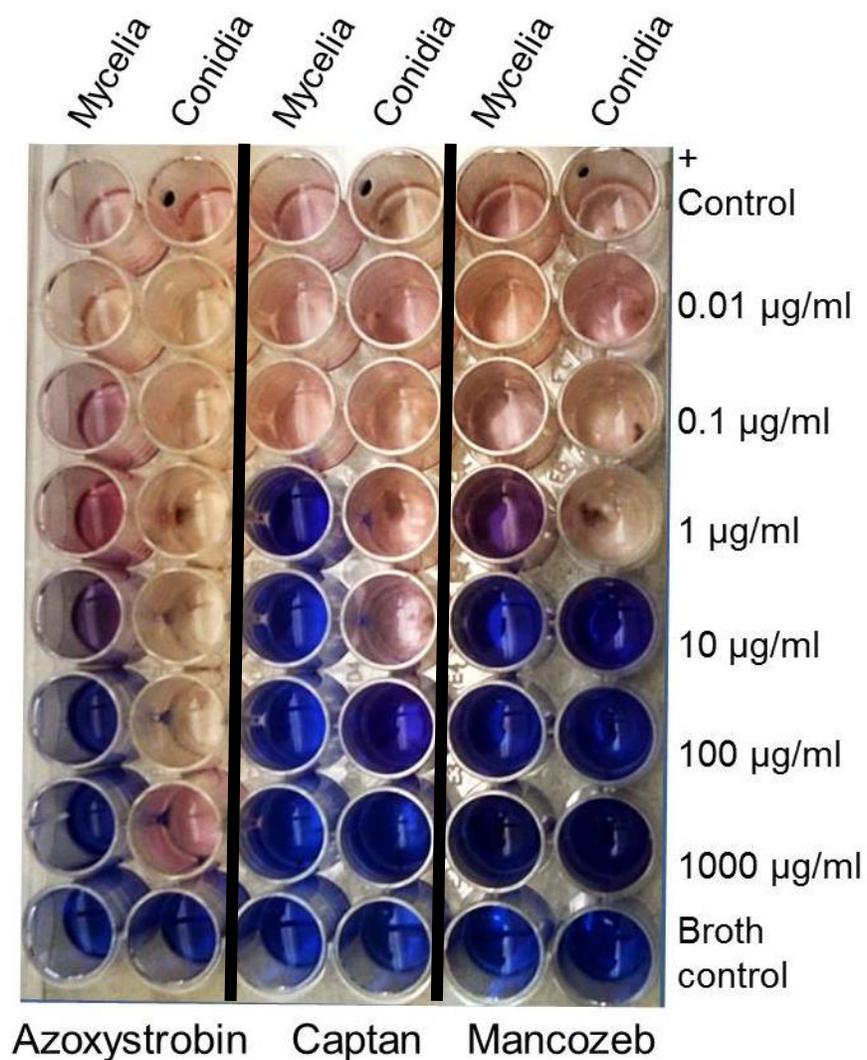


Figure 3.1: Sample layout of alamarBlue® 48-well plates for the serial dilution series (0.01-1000 µg/ml). A similar layout was also used for the second dilution series (0-500 µg/ml).

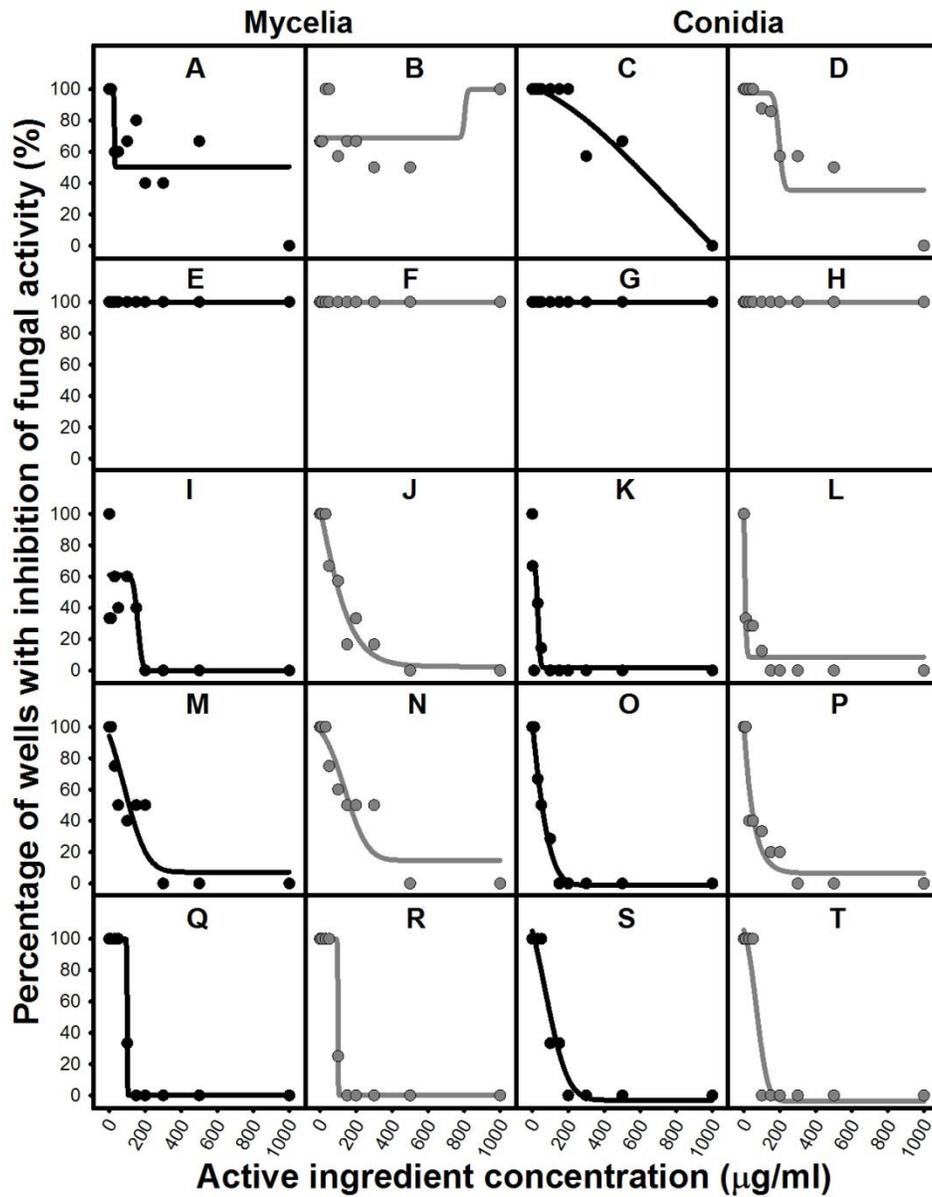


Figure 3.2: Percentage of wells with fungal inhibition by five active ingredients at 0.01 to 1000 $\mu\text{g/ml}$ concentrations in the alamarBlue® assay. The five active ingredients are shown horizontally [azoxystrobin (A-D), boscalid (E-H), captan (I-L), copper hydroxide (M-P), and copper octanoate (Q-T)]. The black dots represent the mean proportion of wells with inhibition of *C. gloeosporioides* complex and the gray dots represent wells with inhibition of *C. acutatum* complex. The black and gray lines represent the curvature of the concentration that effectively suppresses 50% of growth (EC50) using the intercept and slope using the best-fit link function obtained from PROC NL MIXED (SAS, ver. 9.4).

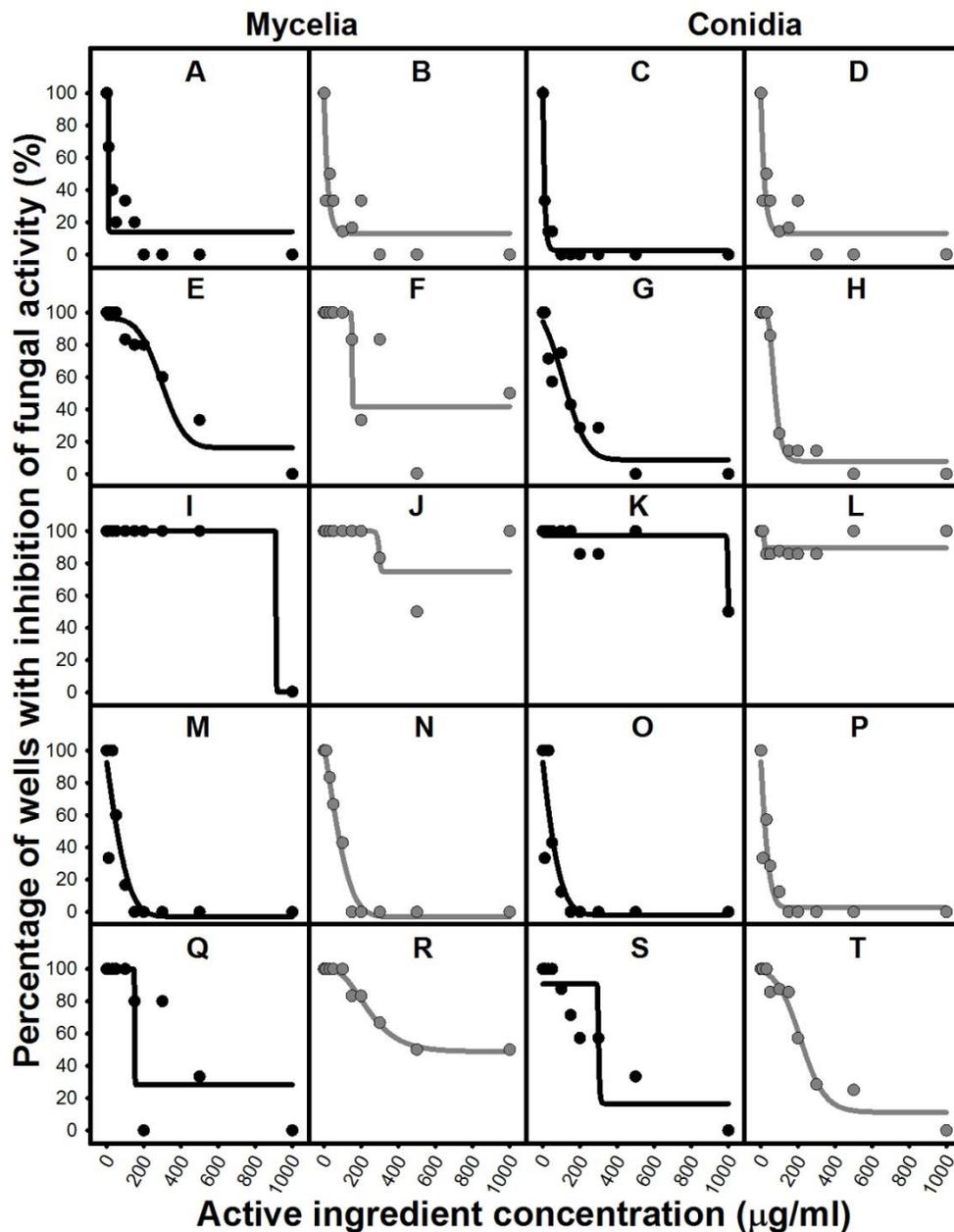


Figure 3.3: Percentage of wells with fungal inhibition by five active ingredients at 0.01 to 1000 µg/ml concentrations in the alamarBlue® assay. The five active ingredients are shown horizontally [mancozeb (A-D), potassium phosphite (E-H), pyriofenone (I-L), tetraconazole (M-P), and thiophanate methyl (Q-T)]. The black dots represent the mean proportion of wells with inhibition of *C. gloeosporioides* complex and the gray dots represent wells with inhibition of *C. acutatum* complex. The black and gray lines represent the curvature of the concentration that effectively suppresses 50% of growth (EC50) using the intercept and slope using the best-fit link function obtained from PROC NLMIXED (SAS, ver. 9.4).

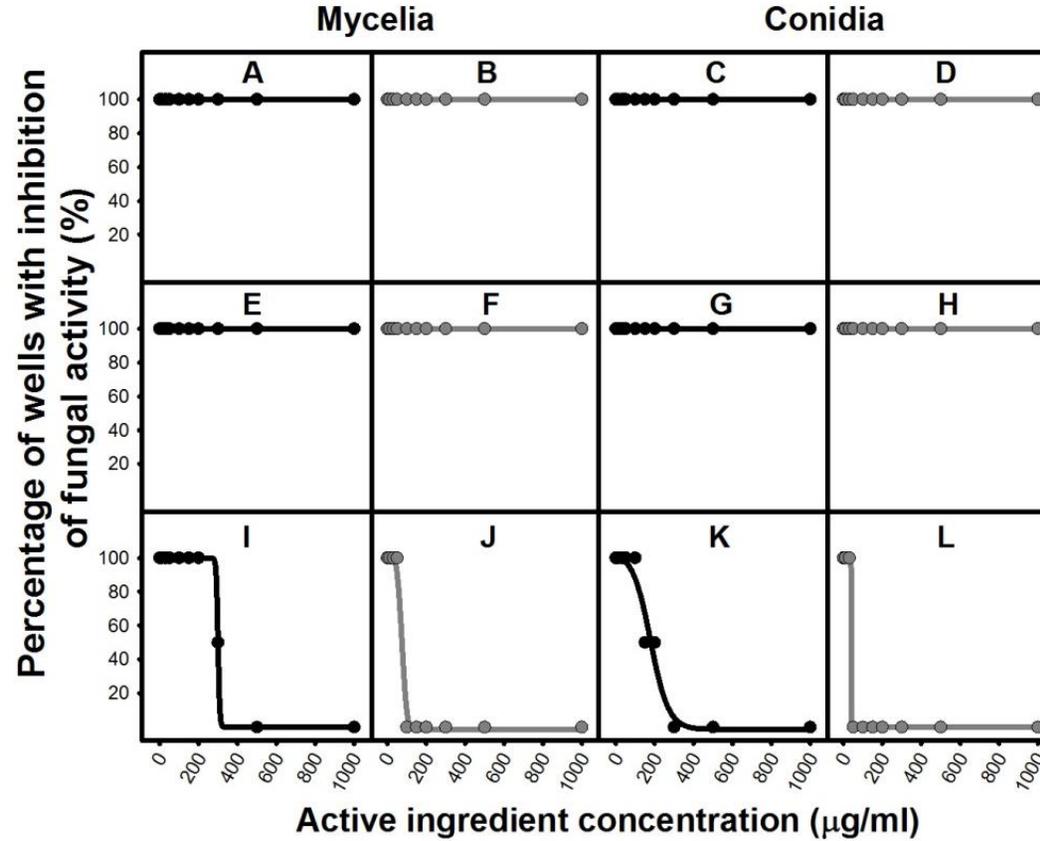


Figure 3.4: Percentage of wells with fungal inhibition by three supplemental chemicals at 0.01 to 1000 $\mu\text{g/ml}$ concentrations in the alamarBlue® assay. The five active ingredients are shown horizontally [ethanol (A-D), SHAM (E-H), and sodium bicarbonate (I-L)]. The black dots represent results for *C. gloeosporioides* complex and the gray dots for *C. acutatum* complex. The black and gray lines represent the curvature of the concentration that effectively suppresses 50% of growth (EC50) using the intercept and slope using the best-fit link function obtained from PROC NLMIXED (SAS, ver. 9.4).

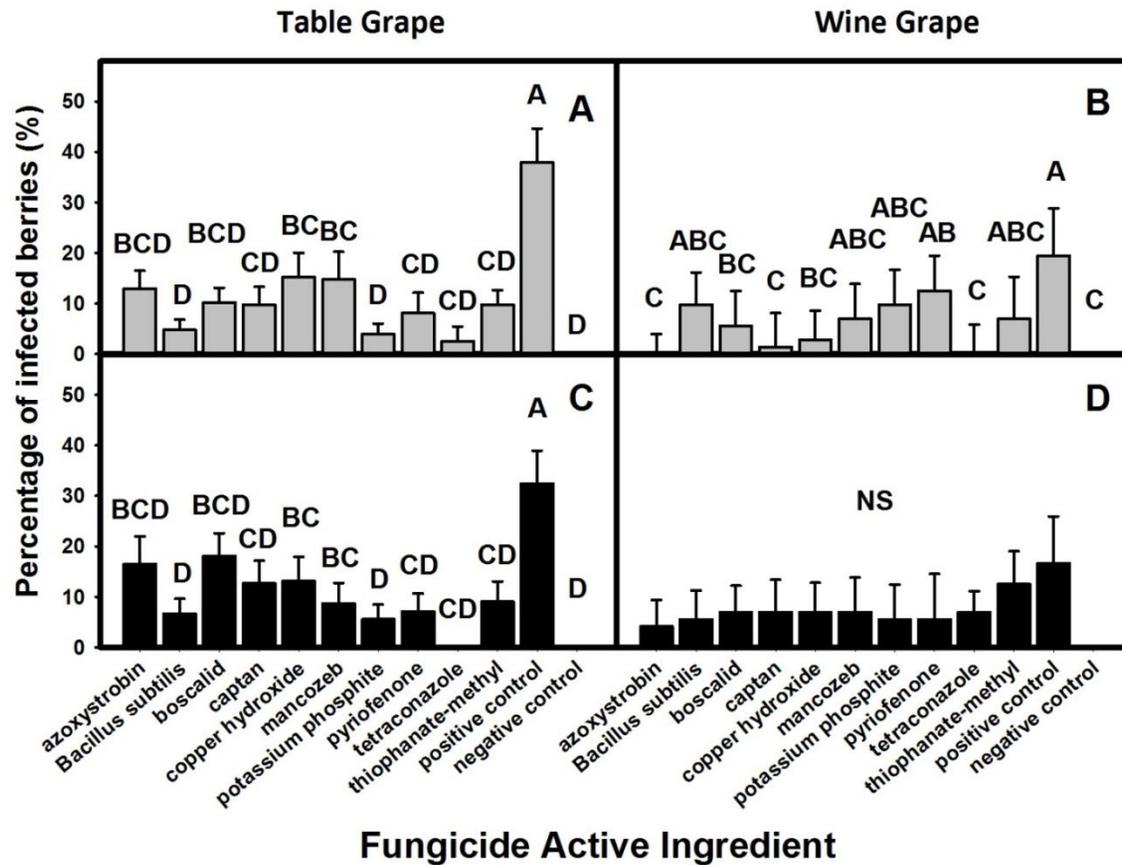


Figure 3.5: Mean percentage of infected berries from detached wine and table grape assays. Berries were sprayed with field formulations of ten commercial fungicides and then incubated for twelve days (table grapes) and seventeen days (wine grapes) prior to the visual estimation of berry disease incidence. Gray bars represent berries inoculated with *C. gloeosporioides* complex (A and B) and black bars represent berries inoculated with *C. acutatum* complex (C and D). The error bars represent one standard error of the mean. Significant mean separations based on LSD ($P \leq 0.05$) between *Colletotrichum* species complexes and fungicide active ingredient are denoted by non-overlapping lettering or NS for no significant separations