BLUEBERRY FLOWER INFECTION BY *MONILINIA VACINII-CORYMBOSI*: SYSTEMIC ACTIVITY OF FUNGICIDES AND DEVELOPMENT OF A cDNA SYNTHESIS PROTOCOL FROM INOCULATED AND POLLINATED PISTILS

by

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(Under the Direction of Harald Scherm)

ABSTRACT

The fungus *Monilinia vaccinii-corymbosi* infects open blueberry flowers through the gynoecial pathway, causing mummy berry disease. The activity of the fungicides fenbuconazole and azoxystrobin sprayed at different flower developmental stages was investigated. In the greenhouse, the two fungicides performed similarly: only flowers sprayed at anthesis were protected from infection, while those treated between 1and 15 days before anthesis were not. In the field, azoxystrobin failed to decrease the incidence of fruit mummification regardless of flower stage, while fenbuconazole significantly decreased disease incidence at all stages. Thus, while neither fungicide is able to prevent hyphal ingress into styles of flowers treated before anthesis, other factors such as reduction of inoculum or residual activity in the ovary may explain the pre-anthesis activity of fenbuconazole in the field.

To lay the groundwork for comparing gene expression patterns in pollinated vs. infected styles, mRNA extraction and cDNA synthesis procedures were developed.

INDEX WORDS: Mummy berry, *Monilinia vaccinii-corymbosi*, rabbiteye blueberry, *Vaccinium ashei*, flower infection, host-pathogen interaction, fungicide activity, fenbuconazole, azoxystrobin.

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DEDICATION

To David, who gave unending physical and emotional support so that I could get through this whole thing in one piece. Thank you for putting up with me when I was stressed out and grouchy, and for giving up your weekends to work in the field tying thread around blueberry flowers. We became superheroes and got the job done. Your love has been appreciated.

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CHAPTER 1

INTRODUCTION

Since the 1980s, blueberry production in Georgia has increased rapidly, with production area increasing from less than 500 hectares in the 1980s to over 3,200 in 2003 (Scherm and Krewer, 2003; Boatright and McKissick, 2004). Due to recent findings regarding the health benefits of the fruit (McCord, 1999; Mazza et al., 2002; Zheng and Wang, 2003; Rimando et al., 2004), the industry is expected to continue growing as consumer demand increases. Ninety percent of the Georgia blueberry production area is planted to the native rabbiteye blueberry (*Vaccinium ashei*), with the rest comprised of southern highbush cultivars (*V. corymbosum* interspecific hybrids) (Scherm et al., 2001). These southern highbush cultivars have increased in popularity because their earlier maturation date in late April/ early May puts their harvest period into a higher price window (Scherm and Krewer, 2003).

In a producer survey published in 2001, the most important blueberry disease was mummy berry disease, with over 75% of growers considering it to be a moderate to major problem (Scherm et al., 2001). The disease is an important concern not only in Georgia, but also in other blueberry growing regions in North America (Honey, 1936; Lockhart, 1961; Pepin and Toms, 1969; Wallace et al., 1976; Batra, 1983; Lambert, 1990; Cline and Milholland, 1995). It is caused by the fungus *Monilinia vaccinii-corymbosi* (Reade) Honey. The *Monilinia* genus is characterized by having moniloid macroconidia and forming a pseudosclerotium on which apothecia are produced (Honey, 1936). *Monilinia vaccinii-corymbosi* first causes a leaf and shoot blight in late winter/ early spring (the result of infection by ascospores), followed by conidial

infection of the ovary via the open flower during bloom to convert the developing fruit into a pseudosclerotium (mummy). Mummies present in harvested fruit loads lower the grade of the fruit and thus the economic return to the grower (Scherm and Copes, 1999).

Conidial infection of blueberry flowers by *M. vaccinii-corymbosi* follows the same pathway as the pollen tube (Batra and Batra, 1985; Shinners and Olson, 1996; Ngugi et al., 2002a; Ngugi and Scherm, 2004). Conidia are deposited on the stigmatic surface (primarily by pollinating insects such as bees), and the hyphae produced by the germinating conidia grow into the ovary via the stylar canal. This infection pathway is highly specialized, requiring close synchronization of the pathogen's life cycle with the phenology of the host (Lehman and Oudemans, 1997).

Management of mummy berry typically follows one of three approaches. First, mummies on the ground can be buried mechanically to decrease apothecium production in the spring (Milholland, 1974; Ngugi et al., 2002b). Second, biological control agents may be applied to blossoms to decrease secondary infection (Scherm and Stanaland, 2001; Dedej et al., 2004; Scherm et al., 2004). Third, repeated fungicide sprays from leaf bud swell to the end of bloom provide control of both primary and secondary infection (Ramsdell et al., 1975; Hildebrand and McRae, 1995; Scherm and Stanaland, 2001; Stanaland et al., 2004).

To minimize the number of sprays needed for mummy berry control in Georgia, Scherm and Stanaland (2001) investigated spray schedules linked more closely to host phenology. Two to three spray applications during bloom proved effective when utilizing a benomyl/captan mix (traditionally used for mummy berry control) or fenbuconazole (Indar). The latter active ingredient (a demethylation inhibitor, DMI) is currently registered under a Section 18 emergency use permit in Georgia. Other active ingredients currently labeled against mummy berry disease

include the Quinone outside inhibitors (Q_oI) azoxystrobin (Abound) and pyraclostrobin + boscalid (Pristine).

A problem with timing bloom sprays against mummy berry disease is that a large proportion of flowers can open within just a few days if the weather is suitable for bloom progression. Thus, numerous new infection courts (stigmatic surfaces) can become exposed to inoculum within a relatively short time. It is unknown to what degree a flower stigma is protected from infection when sprays are made prior to the opening of that flower, even if the fungicide application was made just a few days prior to anthesis. This raises the question as to the systemic activity of fungicides in flowers, i.e., the translocation of the active ingredient from the ovary (the only part of the pistil exposed to the spray in pre-anthesis flowers) to the style through which *M. vaccinii-corymbosi* infects. While considerable translocation of DMI fungicides has been documented in leaves (Garland et al., 1999; Tsuda et al., 2004), there is no information about their activity in blossoms. Strobilurins such as azoxystrobin have been shown to move quasi-systemically through leaves (Ypema and Gold, 1999; Bartlett et al., 2002), but as is the case with the DMI fungicides, there is no data on translocation in flowers. A more limited movement of azoxystrobin from the ovary to the style could explain the lower efficacy of this active ingredient compared with fenbuconazole which is commonly observed in field trials (Scherm and Stanaland, 2001; Stanaland et al., 2004).

On a more basic level, the nature of the host-pathogen interaction during conidial infection of flowers by *M. vaccinii-corymbosi* is a fascinating research topic. The fungus is hypothesized to engage in pollen mimicry to secure its infection success (Ngugi and Scherm, 2004). As stated above, conidia of the pathogen are deposited on blueberry stigmas by insect pollinators (Batra and Batra, 1985), and their germ tubes follow an identical pathway as pollen

tubes to reach the ovaries. Similar to pollen tubes, the advancing hyphae of *M. vaccinii-corymbosi* adhere to the extracellular matrix produced within the stylar canal and grow unidirectionally in close proximity to the adaxial epidermis of the stylar canal with minimal branching. *Monilinia fructicola*, a related species that is not pathogenic on blueberry, entered the stylar canal through the stigma but failed to grow through the transmitting tract and exhibited extensive branching, indicating that it lacked the recognition and/or guidance mechanisms necessary for directed growth into the ovary (Ngugi and Scherm, 2004). To support the theory of pollen mimicry during secondary infection by *M. vaccinii-corymbosi*, molecular-level data comparing blueberry gene expression in pollinated versus infected flowers is needed. If the expression profiles are similar, blueberry flowers respond similarly to pollen and conidial germ tubes of *M. vaccinii-corymbosi*, suggesting that they are unable to distinguish between congruent pollen and hyphae of this specialized pathogen. This would be the strongest, most direct evidence to date for the pollen mimicry hypothesis.

Based on the above considerations, the overall goal of this study was twofold: 1) to determine the efficacy of fenbuconazole and azoxystrobin in preventing ingress of *M. vaccinii-corymbosi* into blueberry pistils when applied at different intervals prior to anthesis; and 2) to optimize mRNA isolation and cDNA synthesis from blueberry styles to lay the groundwork for comparing host gene expression in infected and pollinated styles to determine how similar these two processes are from the host's perspective.

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CHAPTER 2

LITERATURE REVIEW

Blueberry production and mummy berry disease. The number of hectares in the United States devoted to commercial blueberry production has increased steadily over the past 10 years, from around 13,500 in 1992 to over 16,000 in 2003 (Anonymous, 1995; 2004). The value of the 2003 crop nationwide was more than $220 million (Anonymous, 2004). The recent reports of the multiple health benefits of the fruit have contributed to its increased popularity (McCord, 1999; Mazza et al, 2002; Zheng and Wang, 2003; Rimando et al., 2004).

Blueberries are native to North America, where four species are grown in several regions (Austin, 1994). The lowbush blueberry (*Vaccinium angustifolium)* grows in natural stands primarily in Maine and Canada. There are no improved cultivars of this species. In contrast, the northern highbush blueberry (*V. corymbosum*) is grown widely in several states, with different cultivars adapted to different regions. The rabbiteye blueberry (*V. ashei*) is native to the Southeast and is well-adapted to growing conditions in this region. Southern highbush blueberries consist of interspecific hybrids between *V. corymbosum* and other cultivated or non-cultivated blueberry species. They are bred in Florida and Georgia to incorporate characteristics of the northern highbush variety (especially its early fruit maturation) and the blueberry species found in the Southeast (Eck, 1988).

In Georgia, blueberry production increased from 500 hectares to over 3,200 hectares in the 23-year period from 1980 to 2003 (Scherm and Krewer, 2003; Boatright and McKissick, 2004). Blueberries brought almost $19 million in revenue to Georgia producers in 2003

(Anonymous, 2004). Most of the hectares are devoted to the production of rabbiteye cultivars (Scherm et al., 2001a), although southern highbush varieties are gaining in popularity because of their earlier harvest dates and associated higher fruit prices. However, southern highbush varieties present certain production challenges over rabbiteye blueberries because of their greater disease susceptibility and the need for high organic matter levels in the soil (Scherm and Krewer, 2003), which limits their geographical expansion. Southern highbush cultivars also yield less fruit than rabbiteye cultivars. Over half of the blueberry volume produced in Georgia is sold as fresh fruit, with most of the remainder sold to be processed (Scherm et al., 2001a).

Over 75 percent of Georgia blueberry producers reported mummy berry disease (caused by the fungus *Monilinia vaccinii-corymbosi*) to be a moderate to major problem, making it the most important blueberry disease in Georgia (Scherm et al., 2001a). Other diseases reported to be moderate or major problems by 50% or more of growers included Botrytis flower blight (caused by *Botrytis cinerea*), leaf spots (caused by a complex of fungi), Botryosphaeria stem blight (*Botryosphaeria dothidea*), and Phytophthora root rot (Scherm et al., 2001a).

Mummy berry disease is not only a problem for Georgia growers. It is considered a major disease on all species of cultivated blueberry in every growing region in North America (Honey, 1936; Lockhart, 1961; Pepin and Toms, 1969; Wallace et al., 1976; Batra, 1983; Lambert, 1990; Cline and Milholland, 1995). A recent report confirmed the presence of mummy berry in Austria (Gosch, 2003). Before this report, Europe was considered free of the disease, although there are similar *Monilinia* species on other *Vaccinium* hosts (Batra, 1991). The impact of mummy berry on blueberry production in Europe remains to be seen.

***Monilinia vaccinii-corymbosi* -- biology and epidemiology.** The genus *Monilinia* was erected by Honey (1936) for fungal species that possess a moniloid macroconidial stage and

produce a pseudosclerotium. All known species of *Monilinia* are plant pathogens. *Monilinia vaccinii-corymbosi* is a pathogen of several blueberry (*Vaccinium*) species (Batra, 1983), and can cause severe economic losses due to the formation of pseudosclerotia, i.e., mummified fruit. The phenology of the pathogen is synchronous with that of its host and comprises three main life stages: a sexual stage (responsible for primary infection of vegetative tissues in late winter/ early spring), an asexual stage (responsible for secondary infection of flowers in the spring), and an oversummering and overwintering stage (the pseudosclerotium on the ground).

Primary infection by ascospores. Pseudosclerotia will only germinate and produce apothecia (their sexual fruiting bodies) after accumulating a sufficient number of chill-hours during the winter. The number of hours necessary varies by latitude, with 900 to 1200 hours needed for maximum germination in North Carolina and New Jersey (Milholland, 1977; Lehman and Oudemans, 2000), and a lower optimum reported in Georgia (at around 400 to 800 hours) (Scherm et al., 2001b). The study from Georgia (Scherm et al., 2001b) also found an inverse relationship between the number of chill-hours and number of heat units (degree-days) needed for apothecium production. This relationship implies that after a colder winter, fewer degree-days were needed for apothecium production, whereas after a milder winter, more degree-days were needed. Thus, *M. vaccinii-corymbosi* populations in Georgia are adapted to produce apothecia even in the mild winters often encountered in the South. Lehman and Oudemans (1997, 2000) found that apothecial development is adapted to coincide with cultivar-specific leaf bud break of the host. Populations of pseudosclerotia taken from early-season cultivars developed earlier than populations taken from late-season cultivars. These studies suggest that pathogen phenology may adapt to blueberry host phenology, as synchronous life cycles enable more successful infection.

Apart from sufficient chilling, germination of pseudosclerotia depends on several other environmental factors. The most important factor is soil moisture (Milholland, 1974; Cox and Scherm, 2001c). Apothecia developed at temperatures as low as 5°C, but development was optimal at 16°C. Burial of sclerotia more than 2.5 cm under the soil reduced or eliminated apothecial emergence (Milholland, 1974; Ngugi et al., 2002b), suggesting the mummies must be in close proximity to the soil surface to germinate. Ascospores are produced by the fungus for a period of one to several weeks, corresponding to the period of vegetative bud break and expansion in the host (Ramsdell, 1975b; Batra, 1983). They are most likely wind-dispersed to expanding leaves and shoots (Cox and Scherm, 2001b). The infection of leaves by ascospores results in the primary disease symptom, the development of leaf and shoot blight. Ascospores infect developing leaves either by direct penetration of leaf tissue or indirect penetration through the stomata (Milholland, 1977). Hildebrand and Braun (1991) found that frost damage in lowbush blueberries increased susceptibility to ascospore infection. As hyphae invade, the leaf tissue collapses. The first symptom seen is a drooping of infected leaves and shoots. A browning of the upper side of the shoots and midribs or lateral veins of leaves follows, with young tissue often appearing reddish or pinkish. The shoot is killed within 1 to 3 days after discoloration, and conidia (secondary inoculum) are produced (Milholland, 1977; Batra, 1983). Production of ascospores and conidia can overlap during the spring (Batra, 1983).

Secondary infection of flowers by conidia. Macroconidia produced on blighted leaves and shoots are dispersed primarily by insect pollinators to blueberry flowers (Batra and Batra, 1985). Shinners and Olsen (1996) investigated the histopathology of infection by *M. vaccinii-corymbosi* and found that the hyphae developing from conidia on the stigmatic surface grew through the stylar canal to the ovary, following the same path as pollen tubes. Such hyphae have

been reported to require 4 to 7 days to reach the ovary (Milholland, 1977; Shinners and Olson, 1996; Ngugi et al., 2002a). The age of the flower is an important factor in the success of infection by conidia. Hyphal growth into the style is greatest when flowers are inoculated on the day of anthesis, and decreases as the flower ages (Ngugi et al., 2002a). While simultaneous pollination does not affect hyphal growth, pollination before inoculation can decrease growth of the pathogen (Ngugi et al., 2002a).

The spread of inoculum by pollinators and the growth of germ tubes through the transmitting tract to the ovary suggest that *M. vaccinii-corymbosi* engages in a type of pollen mimicry to infect the ovaries of its host. Ngugi and Scherm (2004) compared the behavior of blueberry pollen, *M. vaccinii-corymbosi* conidia, and conidia of *Monilinia fructicola* (a non-pathogen on blueberry) in blueberry styles. Both blueberry pollen and conidia of *M. vaccinii-corymbosi* adhered to imprints of stylar transmitting tract tissue *in vitro*. Similar to blueberry pollen tubes, the conidial germ tubes of the pathogen grow unidirectionally through the style toward the ovary. In contrast, germ tubes of *M. fructicola,* a species non-pathogenic on blueberry, showed no directional growth in the style. This study supports pollen-mimicry as a mechanism of infection, but molecular data (e.g., a comparison of host gene expression during pollination vs. infection) is needed to confirm or refute this hypothesis.

Once the fungal mycelium has entered the ovary, it covers the seeds and invades the locules of the developing fruit. In the berry, the fungus colonizes the pericarp both inter- and intracellularly (Milholland, 1977; Shinners and Olson, 1996). The host tissue is eventually ramified by the fungal mycelium, which hardens and eventually replaces the entire pericarp. The host cell debris covering the resulting pseudosclerotium gives the mummy its wrinkled appearance.

Oversummering and overwintering as pseudosclerotium. As the healthy blueberry fruit mature, the infected berries first turn cream and then salmon, become wrinkled, and drop to the ground just prior to or during fruit harvest to become the sole source of primary inoculum for the next season. A mature mummy is light brown or grayish white and irregularly wrinkled (Milholland, 1977; Batra, 1983). Ngugi et al. (2002b) found that most mummies dropped within 1 m from the crown of the host plant. The survival of the mummy through the summer is greatest for more mature pseudosclerotia (in a more advanced state of fruit colonization at the time of abscission), while higher soil surface temperatures and higher soil moisture decrease survival (Cox and Scherm, 2001a).

Transcript profiling to investigate pollen mimicry theory. As mentioned earlier, molecular studies measuring host gene expression during pollination versus infection could provide compelling evidence in favor or against pollen mimicry in the mummy berry pathosystem. Several molecular methods exist to characterize gene expression. cDNA microarrays and cDNA-AFLP (amplified fragment length polymorphisms) both have been used widely in gene expression studies (Reijans et al., 2003). As a hybridization-based analysis, microarrays require knowledge of the genome of the organism being studied. This poses a challenge to scientists working with non-model organisms (such as blueberry or *M. vaccinii-corymbosi*) whose genomes have not been characterized. An alternative is cDNA-AFLP, a PCR-based transcript profiling approach that has been shown to possess similar specificity, sensitivity, and reproducibility as microarrays (Reijans et al., 2003). This method requires no previous knowledge of an organism's genome. mRNA is extracted from the tissue of interest, and double-stranded cDNA synthesized. The cDNA is digested by two different restriction enzymes, then ligated to specific adapters (Bachem et al., 1998). Primers corresponding to these adapters are

used in PCR to amplify the different fragments of cDNA. After the PCR products are run on an electrophoresis gel, a transcript profile is generated by identifying the resulting bands. A gene sequencer can also produce electropherograms showing peaks where bands occur, thus automating the acquisition of the transcript profile. By comparing profiles of cDNA from an organism under different conditions, similarities and differences of gene expression under those conditions can be characterized. Because little of the blueberry genome has been sequenced, cDNA-AFLP is an appropriate method for gene expression studies.

cDNA-AFLP has been used to characterize gene expression during pollination in some plant species, and in the plant host and pathogen during infection in several pathosystems. A study comparing gene expression in unpollinated and pollinated rice pistils showed a large degree of down-regulation of genes during pollination (Chen et al., 2001). Gene expression profiles in plant hosts when challenged with a pathogen have been characterized using cDNA-AFLP in sugar cane (Carmona et al., 2004), barley (Eckey et al., 2004), and *Arabidopsis* (Tao et al., 2003). Expression in cell types of *Phytophthora infestans* shortly before and during the early stages of potato infection was profiled using cDNA-AFLP (Avrova et al., 2003), as was gene expression in germinating conidia of the barley net blotch pathogen, *Pyrenophora teres* (Dilger et al., 2003). Many previous studies using cDNA-AFLP went on to identify the genes and functions of many of the cDNA transcripts that were identified in the AFLP analysis as being up- or down-regulated. This is accomplished by sequencing transcripts of interest and finding homologous genes in a sequence database such as GenBank.

Management of mummy berry disease. The two infection stages of *M. vaccinii-corymbosi* are managed differently. Primary infection by ascospores is controlled by targeting the pseudosclerotia on the ground as well as their ascospore inoculum. Mechanical and chemical

control methods decrease pseudosclerotial germination and ascospore production. Because burying pseudosclerotia below 2.5 cm diminishes their ability to germinate (Milholland, 1974; Ngugi et al., 2002b), tilling the orchard soil deep and close enough to bushes to reach the majority of mummies can reduce the risk of apothecial emergence by an average of 50% (Ngugi et al., 2002b). Combining several tillage methods can improve this effect, but cultivation alone will not likely control primary inoculum.

In laboratory studies, several soil-applied chemicals (e.g., the herbicides diuron and simazine) decrease pseudosclerotial germination and apothecium production. Diuron inhibits apothecial development, while simazine results in malformed apothecia and near absence of ascospore production (Wallace et al., 1976; Cox and Scherm, 2001a). The desiccant ammonium thiosulfate also inhibits apothecial development in the laboratory and provides control over a wider range of apothecial development compared with other compounds (Cox and Scherm, 2001a). It has further appeal in mummy berry control because it is also a fertilizer and broadleaf herbicide (Agamalian, 1984). A potentially more benign compound, soybean oil, also decreases ascospore production but is more inconsistent than other compounds affecting apothecial development (Cox and Scherm, 2001a).

Although limited research has been done on biological control of mummy berry disease, Scherm and Stanaland (2001) make the case that the secondary infection stage of mummy berry is an ideal target for biological control. The infection court for secondary infection by *M. vaccinii-corymbosi* is small, consisting only of the stigmatic surface of the blueberry flower. This surface, with its copious exudate, would provide a suitable niche for a biological control agent to survive and multiply. There is also a short time period when infection may take place (during bloom), so the antagonist would have to be active only for a period of a few weeks (or a few

days on individual flowers). Lastly, flower infection by *M. vaccinii-corymbosi* is relatively slow, so a biocontrol agent may be able to exclude the pathogen effectively before infection takes place.

The biocontrol product Serenade WP (formulated *Bacillus subtilis*) shows strong antibiosis against *M. vaccinii-corymbosi in vitro* (Scherm et al., 2004). When applied to blueberry stigmas 24 hours before or after inoculation with the pathogen, the product significantly and substantially decreased the number of *M. vaccinii-corymbosi* hyphae advancing through the stylar canal. Pollinator bees can vector Serenade to blueberry flowers, decreasing the incidence of mummified berries in the field (Dedej et al., 2004).

Fungicides manage both primary and secondary infection. Triforine (Funginex), a demethylation inhibitor (DMI), was the long-time standard for mummy berry control, and it effectively limited infection by both ascospores and conidia (Ramsdell et al., 1975a, b). Benomyl (Benlate), which – like triforine – is no longer labeled, reduces the amount of secondary infection but has limited action against ascospore infection (Ramsdell et al., 1975a, b). A standard practice has been to apply captan and benomyl together, thus achieving satisfactory control of both disease stages (Cline and Milholland, 1993; Cline et al., 1999a). In a grower survey carried out in Georgia in 1999 (Scherm et al., 2001a), over 80% of growers applied benomyl, captan, and triforine on over 80% of their hectarage twice or three times per season. Since then, other fungicidal compounds, primarily DMI and Quinone outside inhibitor (Q_oI) fungicides, have replaced triforine and benomyl in blueberry. Two DMI compounds, propiconazole (Orbit) and fenbuconazole (Indar), decreased the incidence of shoot blight and fruit mummification development in numerous studies (Lambert, 1996a, b; Bristow and Windham, 1996, 1997; Cline et al., 1999b, c; Stanaland et al., 2000, 2004; Scherm and

Stanaland, 2001), with fenbuconazole showing somewhat better control (Bristow and Windham, 1996; 1997). However, the U.S. Environmental Protection Agency (EPA) is reluctant to grant full registration of fenbuconazole for use on blueberries because the triazoles are classified as potential carcinogens (Anonymous, 2001). A Section 18 emergency permit has been issued for several states allowing limited use of fenbuconazole on blueberries. This permit must be renewed annually, and further research supporting the use of fenbuconazole as the most effective fungicide option for mummy berry is needed to support renewal of the permit and the eventual full registration of the product.

Fenbuconazole, a DMI fungicide in the triazole chemical group, stops ergosterol synthesis by inhibiting 14 α-methylation by binding to the cytochrome P450 component of the C-14 demethylase (Worthington, 1989). The systemic activity of triazoles in leaves has been well-documented (Garland et al., 1999; Tsuda et al., 2004), as well as their post-infection activity (O'Leary and Sutton, 1986; Bushong and Timmer, 2000; Hoffman and Wilcox, 2003). Fenbuconazole was found to have translaminar systemic activity in leaves, preventing disease development in a distal direction, but lacks vapor-phase activity (Tsuda et al., 2004). However, the activity of DMI fungicides in flowers, the target tissue for use against mummy berry disease, has not been characterized.

Two recently labeled Q_oI fungicides, azoxystrobin (Abound) and pyraclostrobin + boscalid (Pristine) provide an alternative to the DMIs for managing mummy berry disease. These fungicides block electron transfer in cytochrome b and c_1, thus inhibiting mitochondrial respiration in fungal pathogens (Ypema and Gold, 1999; Bartlett et al., 2002). Azoxystrobin's systemic activity is limited (Wong and Wilcox; 2001); it has been characterized as moving translaminarly and through the xylem, but having low movement into leaves (Bartlett et al.,

2002). Azoxystrobin has limited curative ability: while decreasing symptoms and sporulation in developing lesions when applied after infection, it cannot stop symptom development (Wong and Wilcox, 2001; Hoffman and Wilcox, 2003). The compound is considered a reduced-risk fungicide by the EPA, i.e., is credited with having low toxicity to mammals and other non-target organisms as well as a low risk of groundwater contamination (Anonymous, 1997). However, azoxystrobin does not control mummy berry disease as well as fenbuconazole (Scherm and Stanaland, 2001; Stanaland et al., 2000, 2004). It may be that translocation of this active ingredient in blueberry flowers is more limited than that of fenbuconazole, or that it has less of a curative ability in these tissues. However, as stated above, fungicide activity and translocation in flowers have not been studied previously.

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CHAPTER 3

ACTIVITY OF FUNGICIDES AGAINST THE MUMMY BERRY PATHOGEN *MONILINIA VACCINII-CORYMBOSI* IN DIFFERENT DEVELOPMENTAL STAGES OF BLUEBERRY FLOWERS[1]

[1] Tarnowski, T.L.B, and Scherm, H. To be submitted to *Plant Disease*.

ABSTRACT

The activity of fenbuconazole and azoxystrobin applied at different development stages of blueberry flowers against gynoecial infection by the mummy berry fungus *Monilinia vaccinii-corymbosi* was assessed in greenhouse and field experiments. In the greenhouse, flower clusters at five distinct developmental stages (from 15 days before to the day of anthesis) were treated with the two fungicides and then inoculated with *M. vaccinii-corymbosi* 1 day after the flowers had opened. Four days after inoculation, hyphal ingress into the style was determined microscopically as a measure of fungicide efficacy. Results revealed a significant flower stage effect ($P < 0.0001$) but no difference between the two fungicides ($P \geq 0.5040$). Pre-anthesis treatments were ineffective for both fungicides, and only flowers sprayed at anthesis were protected from infection. In the field, flower clusters at anthesis and at three pre-anthesis stages were treated with the two fungicides and exposed to natural inoculum. Mummy berry incidence in fruit developing from the treated clusters was used to determine fungicide efficacy. Azoxystrobin proved ineffective regardless of flower stage at the time of application, whereas fenbuconazole lowered disease incidence significantly for all developmental stages. This study suggests that azoxystrobin and fenbuconazole applied prior to anthesis are not translocated into flower styles to protect against gynoecial infection by *M. vaccinii-corymbosi* once the flowers have opened. The significant activity of pre-anthesis applications of fenbuconazole in the field may have been due to an indirect effect via a reduction of inoculum available for flower infection or a direct effect via residual activity in the ovary which was exposed to the sprays at the time of treatment. To prescribe the most effective management program for flower-infecting fungi, the systemic activity of fungicides in flowers must be better understood.

Additional keywords: Indar, Abound, demethylation inhibitor, DMI, Quinone outside inhibitor, Q_oI, rabbiteye blueberry, *Vaccinium ashei*.

INTRODUCTION

During the past 25 years, blueberry (*Vaccinium* section *Cyanococcus*) has emerged as an important crop in Georgia, becoming the second most valuable fruit crop in the state. In part due to the recent recognition of the health benefits of blueberry fruit (McCord, 1999; Zheng and Wang, 2003), the production area of blueberries statewide has risen from less than 500 hectares in the 1980s to over 3,200 in 2003 (Scherm and Krewer, 2003; Boatright and McKissick, 2004)

Mummy berry disease, caused by the fungus *Monilinia vaccinii-corymbosi*, is a major disease confronting blueberry growers in North America (Honey, 1936; Batra, 1983; Eck, 1988). In a survey published in 2001, over 75% of Georgia blueberry producers reported mummy berry as a moderate to major disease problem (Scherm et al., 2001). As a result of a near-zero tolerance for mummified fruit in commercial blueberry shipments, most losses associated with the disease are due to the rejection or downgrading of affected fruit loads in the packinghouse after harvest (Scherm and Copes, 1999).

Fruit mummification by *M. vaccinii-corymbosi* is the result of conidial infection of open flowers through the stigma and style, whereby hyphae grow through the gynoecial pathway into the ovary to colonize the developing fruit internally (Shinners and Olson, 1996). As the fruit develop, the ovaries are filled with dense mycelial growth. The hyphae migrate into the pericarp and sclerotinize during fruit maturation, forming a pseudosclerotium (mummy). Mummified fruit fall to the ground where they oversummer and overwinter, serving as the source of primary

inoculum in the following spring (Milholland, 1977; Batra, 1983). Ascospores, produced in apothecia formed on overwintered mummies, incite primary infection in late winter/ early spring, leading to a leaf and shoot blight. Conidia produced on blighted tissue subsequently result in secondary infection, i.e., fruit mummification via infection of open flowers during bloom.

Mummy berry disease is managed through repeated applications of fungicide from vegetative bud break through bloom. To minimize the number of sprays needed for control of the disease in Georgia, spray schedules linked more closely to host phenology have been developed (Copes and Stanaland, 1999; Stanaland et al., 2000, 2004, 2005; Scherm and Stanaland, 2001). In Georgia, two to three spray applications during bloom proved effective when utilizing a benomyl/captan mix (traditionally used for mummy berry control) or fenbuconazole (Indar) (Scherm and Stanaland, 2001). The latter active ingredient (a demethylation inhibitor, DMI) is currently registered under a Section 18 emergency use label in Georgia and other blueberry-producing states. Since the late 1990s, this compound has become the standard for managing both primary and secondary infection by *M. vaccinii-corymbosi* (Stanaland et al., 2000, 2004, 2005; Scherm and Stanaland, 2001). Other active ingredients currently labeled against mummy berry disease include the Quinone outside inhibitors (Q_oI) azoxystrobin (Abound) and pyraclostrobin + boscalid (Pristine).

A problem with timing bloom sprays against mummy berry disease is that a large proportion of flowers can open within just a few days if the weather is suitable for bloom progression. Thus, numerous new infection courts (stigmatic surfaces) become exposed to inoculum within a relatively short time. It is unknown to what degree stigmas and styles in flowers treated before anthesis are protected from infection, even if the fungicide application was made just a few days before the flowers opened. This raises the question as to the pre-anthesis

activity of fungicides in flowers, which should be related, at least in part, to the ability of the applied active ingredient to be translocated from the ovary (the only exposed part of the pistil during spray application to unopened flowers) to the style and stigma. In general, DMIs have more pronounced systemic properties (Garland et al., 1999; Tsuda et al., 2004) and post-infection activity (Bushong and Timmer, 2000; O'Leary and Sutton, 1986; Hoffman and Wilcox, 2003) than Q_oI fungicides, whose translocation is often limited to translaminar movement (Bartlett et al., 2002). However, there is no information on mobility and activity of different fungicidal compounds in flowers. In the field, fenbuconazole is consistently more effective against mummy berry disease than azoxystrobin (Scherm and Stanaland, 2001; Stanaland et al., 2000, 2004), and this difference may be due in part to different degrees of mobility of the two compounds in the floral tissues through which *M. vaccinii-corymbosi* infects.

This study sought to determine the efficacy against *M. vaccinii-corymbosi* of the fungicides fenbuconazole and azoxystrobin in blueberry flowers treated at different developmental stages. Experiments were carried out in the laboratory by assessing hyphal ingress of *M. vaccinii-corymbosi* into artificially inoculated, detached flower styles previously treated with the two fungicides, as well as in the field under natural inoculum by comparing the incidence of fruit mummification in flowers treated with the fungicides at distinct developmental stages. Information from this study could help optimize the timing of fungicide applications against mummy berry disease during the bloom period.

MATERIALS AND METHODS

Detached flower experiments. Four to five-year-old rabbiteye blueberry plants (*Vaccinium ashei* 'Climax') were maintained in 11.4-liter pots in a cold room (5 to 6^oC) during

the winter to allow accumulation of adequate chill-hours for subsequent flower production. At intervals from early January to early May, groups of plants were moved to a greenhouse maintained at 24 to 28°C. Each individual run of the experiment was carried out when a large enough number of plants with the desired flower stages (see below) was available.

The experimental design was a split-plot with fungicide (three levels) as the main-plot and flower development stage (five levels) as the sub-plot. Each main-plot consisted of a single plant having all five flower development stages present simultaneously. Fungicide treatments consisted of fenbuconazole (Indar 75WSP, applied at 300 mg formulated product per liter), azoxystrobin (Abound SC, 1.94 ml formulated product per liter), and a water control. Numerical flower development stages (Spiers, 1978) at the time of inoculation were: 3 (bud scales separated, apices of flowers visible); 4 (individual flowers distinguishable); early 5 (individual flowers distinctly separated; corollas small, unexpanded, and closed); late 5 (individual flowers distinctly separated; corollas larger and expanded, but still closed); and early 6 (corollas completely expanded, flowers open). On the day that stage-6 flowers reached anthesis, each plant was treated with approximately 300 ml of fungicide suspension or water using a 7.6-liter handheld sprayer. After treatment, six flower clusters per stage were tagged on each plant using colored embroidery thread. The experiment was carried out five times over time, and the individual experimental runs were considered replicates (blocks) in the analysis.

Flowers at stage 6 (at anthesis at the time of fungicide application) were detached 1 day after treatment and inoculated with conidia of *M. vaccinii-corymbosi* produced on oat bran agar (20 g of oat bran and 10 g agar in 750 ml of deionized water) as described by Ngugi et al. (2002). Flowers treated with fungicide at phenologically earlier development stages were also detached 1 day after anthesis and inoculated similarly. Four days after inoculation, corollas and ovaries of

inoculated flowers were removed and styles fixed and stained with decolorized aniline blue for fluorescence microscopy (Ngugi et al., 2002). They were cut longitudinally with a scalpel, and hyphal ingress by *M. vaccinii-corymbosi* was measured microscopically using an ocular micrometer. As in Ngugi et al. (2002), the number of hyphae penetrating at least one-fifth the length of the style was counted. In addition, the lengths of the eight longest hyphae per style were measured to determine hyphal growth rates. Averages of the two variables were calculated across all flowers belonging to the same flower stage on each plant.

Data on numbers of hyphae per style and hyphal growth rates were subjected to analysis of variance for a split-plot design (Littell et al., 1991). All data were expressed as a percentage of the water control prior to the analysis. Tukey's test ($\alpha = 0.05$) was applied to compare means within main-plots and sub-plots. All data analyses were performed using SAS (v. 8.02; SAS Institute, Cary, N.C.).

Field experiments. A field study to determine the effect of fenbuconazole and azoxystrobin applied at different flower development stages was carried out at the University of Georgia Horticulture Farm near Watkinsville in 2004 and 2005 and in a commercial blueberry planting near Social Circle in 2005. Both plantings contained mature rabbiteye blueberry plants, and experimental treatments were applied to rows of cultivars 'Climax' and 'Tifblue' at Watkinsville and Social Circle, respectively. In each case, the experimental design was a split-plot with fungicide (three levels: fenbuconazole, azoxystrobin, and untreated) in the main-plot and flower development stage (four levels: stages 4, early 5, late 5, and 6) in the sub-plot. Each main-plot consisted of a single blueberry plant surrounded by untreated buffer plants and replicated four times.

On each bush, 12 flower clusters at the designated stages of development were tagged with colored embroidery thread just prior to fungicide application. Fungicide treatments (at the rates and volume described for the detached flower study above) were applied with a handheld sprayer when stage-6 flowers had opened; all other tagged clusters were still closed at treatment time. No artificial inoculations were carried out at either site. All treated clusters were harvested when green berries reached a diameter of 7 to 10 mm Berries were bisected to determine incidence of infection by *M. vaccinii-corymbos*i as evident by colonized placentas, visible mycelial strands or mycelial matrix in the locules, or presence of a mature pseudosclerotium.

The number of blighted shoots infected by *M. vaccinii-corymbos*i in each bush was counted when symptoms first appeared.

Data on incidence of fruit infection by *M. vaccinii-corymbosi* was subjected to analysis of variance for a split-plot design (Littell et al., 1991). Tukey's test ($\alpha = 0.05$) was applied to compare means within main-plots and sub-plots.

RESULTS

Detached flower experiments. For both response variables (number of hyphae per style and hyphal growth rates), analysis of variance revealed highly significant effects of bloom stage at the time of application, whereas fungicide (fenbuconazole vs. azoxystrobin) and fungicide × bloom stage interaction were not significant (Table 3.1). For both fungicides, pathogen ingress into the style for flowers treated at stages 3 through late 5 was not significantly different from the water control, as shown by the overlapping standard errors in Fig. 3.1. Only flowers treated at anthesis (stage 6) showed significantly lower pathogen ingress based on Tukey's test across bloom stages. In those flowers, an average of 2.7 and 1.6 hyphae grew into styles treated with

fenbuconazole and azoxystrobin, respectively, compared with 10.9 hyphae in water-treated styles (Fig. 3.1A). Similarly, growth rates in styles treated with fenbuconazole or azoxystrobin were 0.24 and 0.13 mm/day respectively, compared with 1.40 mm/day for hyphae in the water control styles (Figure 3.1B). Conidia inoculated on flowers treated with fenbuconazole at anthesis often showed some germination, but no penetration, while those inoculated on flowers treated with azoxystrobin seldom showed any germination (*data not shown*).

Field experiments. Although mummy berry disease is endemic at the Horticulture Farm and disease levels have been high in previous years, only very low levels of fruit mummification developed in both 2004 and 2005 (Appendix Table 1). Thus, only the field trial carried out in the commercial planting in 2005 yielded data that could be analyzed. Inoculum pressure varied during the bloom period, as evidenced by the varying disease levels obtained for the water control at the different bloom stages (the stigmas of which were exposed to natural inoculum at different times) (Fig. 3.2). Inoculum levels among the bushes (as determined by the number of blighted shoots per bush) did not differ for the different fungicide treatments (means ± standard errors of 12.75 ± 2.53, 9.00 ± 2.35, and 9.75 ± 1.11 for azoxystrobin, fenbuconazole, and the control, respectively). The highest fruit mummification incidence in the water control of 9.6% was observed for flowers at early stage 5 at the time of treatment. When data for the two fungicides were expressed as a percentage of the water control to adjust for the varying inoculum levels, analysis of variance revealed a highly significant fungicide effect (Table 3.2), with fenbuconazole showing better disease control than azoxystrobin (Fig. 3.2). In fact, disease incidence in the azoxystrobin treatment was not significantly different from the water control for all flower stages, as shown by the overlapping standard errors in Fig. 3.2. There was no

significant flower stage main effect, nor was there a fungicide × flower stage interaction (Table 3.2).

DISCUSSION

Repeated fungicide sprays during bloom can be an effective management strategy for mummy berry disease of blueberry (Stanaland et al., 2000, 2004, 2005; Scherm and Stanaland, 2001). However, in the field, many flowers open between fungicide applications, thus their stigmatic surfaces are not directly covered by fungicide sprays. Therefore, the pre-anthesis activity provided by fungicides before the infection court of *M. vaccinii-corymbosi* becomes exposed is important in determining the application timing and frequency necessary to protect against infection by this pathogen. In recent field studies, fungicides have been applied according to bloom phenology rather than based on calendar days, resulting in considerably shorter application intervals than specified by the product labels in years when bloom progressed very rapidly (Stanaland et al., 2004, 2005). Timing and frequency of applications could be improved further if the pre-infection activity of fungicides applied prior to anthesis could be quantified. This was intended in the present study.

The greenhouse study showed that for both fenbuconazole and azoxystrobin, only flowers sprayed at anthesis (stigmas exposed to direct fungicide coverage) were protected from infection by *M. vaccinii-corymbosi* following artificial inoculation. Any flowers sprayed prior to anthesis showed no decrease in hyphal penetration when they were inoculated after the flowers had opened 1 to 15 days after application. This suggests that all flowers that open after a fungicide application will be accessible to infection by *M. vaccinii-corymbosi*. Although DMIs are generally more systemic than Q_oI fungicides, fenbuconazole appears to have translaminar rather

than fully systemic activity (Tsuda et al., 2004), not unlike that reported for azoxystrobin (Bartlett et al., 2002). As such, the lack of protection from stylar ingress in flowers treated prior to anthesis may represent the failure of both active ingredients to translocate from the ovary, the only exposed part of the pistil in pre-anthesis flowers, to the style and stigma where fungal penetration occurs. In light of this, it would be useful to screen a wider range of active ingredients for better systemic movement in flowers. The lack of conidial germination in stage-6 flowers treated with azoxystrobin is consistent with the compound's activity as a spore germination inhibitor, while fenbuconazole, as a triazole fungicide, does not inhibit spore germination and early germ tube growth (Bartlett et al., 2002).

Because only one of the field experiments resulted in disease levels high enough to warrant analysis, conclusions are somewhat tentative. In that trial, azoxystrobin failed to decrease mummy berry incidence, even in clusters sprayed at anthesis. Fenbuconazole, on the other hand, was very effective, having lower incidence than the water control for all developmental stages. This is consistent with previous reports (Scherm and Stanaland, 2001, Stanaland et al., 2004), according to which azoxystrobin is relatively ineffective in managing mummy berry disease. The compound acts mostly by inhibiting spore germination (Bartlett et al., 2002), requiring application before germination of conidia to be most effective. The low efficacy of azoxystrobin in the field, even in flowers sprayed at anthesis, could have been due to the fact that flower phenology could not be controlled as precisely in the field as in the greenhouse study. In the greenhouse, stage-6 flowers were sprayed the day they opened and inoculated 1 day later. Such precise timing was not possible in the field where it was not possible to determine how long stage-6 flowers had been open before fungicide application or how soon before or after application they were exposed to conidia of *M. vaccinii-corymbosi*. Thus, the

flowers may have already been infected at the time of treatment, or infected after azoxystrobin was most effective.

The high efficacy of fenbuconazole in the field at all developmental stages is at odds with the finding of the greenhouse study, where the compound was effective only when applied to open flowers. The higher efficacy of pre-anthesis applications in the field could have been due to the fact that such applications may have reduced conidial inoculum levels available for flower infection during bloom. The application of fenbuconazole did not reduce the incidence of primary infection (number of blighted shoots), and so any reduction in conidial inoculum would likely be due to a suppression of sporulation on infected shoots rather than a reduction of primary infection by the pathogen.

Another explanation for the higher efficacy of pre-bloom applications of fenbuconazole in the field is that the material may have had a more pronounced residual activity in the ovary. The ovary is the only part of the pistil directly covered by fungicide in flowers sprayed before anthesis. Although fenbuconazole does not appear to move systemically from the ovary into the style (as shown by the greenhouse study), it may translocate in the ovary and inhibit colonization of the ovary by *M. vaccinii-corymbosi* after the hyphae have penetrated the style.

In general, DMI fungicides show some curative ability when applied to leaf tissue shortly after infection (O'Leary and Sutton, 1986; Bushong and Timmer, 2001; Hoffman and Wilcox, 2003; Tsuda et al., 2004). Such curative ability could also be active in flowers, and along with any existent pre-anthesis activity due to indirect effects via production of secondary inoculum, could explain why some control has been noted in the field even when sprays are applied at early flower development stages or at longer intervals (Copes and Stanaland, 1999; Stanaland et al., 2004).

In a broader context, this study highlights the need to determine mobility and activity of fungicidal active ingredients in flower tissues, as flowers are physiologically different from leaves. Knowledge of a compound's activity in leaves cannot be extrapolated to flowers, and so for the numerous pathosystems involving flowers (Ngugi and Scherm, 2005) it is more difficult to prescribe chemical control measures. Further research investigating the pre-anthesis and curative activity of different active ingredients against *M. vaccinii-corymbosi* would be useful, including documentation of the inhibition of mycelial colonization via residual activity in the ovary after successful penetration of the style has occurred. By understanding the activity of fungicides in flowers, better management decisions can be made, and the timing of fungicide applications before and during bloom can become more precise.

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Table 3.1. Results of a split-plot analysis of variance to determine the effects of fungicide, flower stage at the time of fungicide application, and their interaction on penetration of *Monilinia vaccinii-corymbosi* into flower styles of 'Climax' rabbiteye blueberry [a].

		Number of hyphae per style [b]			Average growth rate of hyphae [b]		
Source	df	Mean Square	*F*	*P* > *F*	Mean Square	*F*	*P* > *F*
Replication [c]	4	1298.2	2.12	0.2424	828.6	2.27	0.2232
Fungicide [d]	1	128.7	0.21	0.6704	132.3	0.36	0.5795
Main-plot error	4	612.5	---	---	364.8	---	---
Flower stage [e]	4	7288.7	10.25	<0.0001	9112.3	25.47	<0.0001
Fungicide × Flower stage interaction	4	389.3	0.55	0.7021	434.4	1.21	0.3247
Sub-plot error	31	711.0	---	---	357.7	---	---

[a] Flowers were treated with fungicide at the stages indicated below; detached and inoculated with conidia of *M. vaccinii-corymbosi* 1 day after anthesis; and fixed for microscopic assessment 4 days later.

[b] Analysis based on values expressed as a percentage of the water control.

[c] Five separate experiments, each with eight styles per treatment combination.

[d] Indar 75WSP (fenbuconazole) or Abound (azoxystrobin) applied as formulated product at 0.3 g/l and 1.9 ml/l.

[e] Bloom stages at fungicide application were 3 (bud scales separated, apices of flowers visible), 4 (individual flowers distinguishable), early 5 (individual flowers distinctly separated; corollas small, unexpanded and closed), late 5 (individual flowers distinctly separated; corollas larger, unexpanded and closed), or 6 (corollas completely expanded and open) (Spiers, 1978).

Table 3.2. Results of a split-plot analysis of variance to determine the effects of fungicide, flower stage at the time of fungicide application, and their interaction on the incidence of fruit mummification by *Monilinia vaccinii-corymbosi* on 'Tifblue' rabbiteye blueberry in the field [a].

Source	df	Mean Square [b]	*F*	*P* > *F*
Replication	3	948.7	4.08	0.1393
Fungicide [c]	1	12783.2	54.94	0.0051
Main plot error	3	232.7	---	---
Flower stage [d]	3	1396.3	1.37	0.2851
Fungicide × Flower stage interaction	3	340.8	0.33	0.8014
Subplot error	18	1022.4	---	---

[a] Flower clusters were treated with fungicide at the stages indicated below; exposed to natural inoculum of *M. vaccinii-corymbosi*; and fruit developing from these clusters were harvested and assessed for disease incidence.

[b] Analysis based on values expressed as a percentage of the water control.

[c] Indar 75WSP (fenbuconazole) or Abound (azoxystrobin) applied as formulated products at 0.3 g/l and 1.9 ml/l, respectively.

[d] Bloom stages at fungicide application were 4 (individual flowers distinguishable), early 5 (individual flowers distinctly separated; corollas small, unexpanded and closed), late 5 (individual flowers distinctly separated; corollas larger, unexpanded and closed), or 6 (corollas completely expanded and open) (Spiers, 1978).

Figure 3.1. Hyphal ingress of *Monilinia vaccinii-corymbosi* into flower styles of ‘Climax’ rabbiteye blueberry in relation to fungicide active ingredient and flower stage at the time of fungicide application. **A**) Number of hyphae that penetrated at least 20% of the length of the style. **B**) Average hyphal growth rate per style. Flowers were treated with fungicide at the stages indicated; detached and inoculated with conidia of *M. vaccinii-corymbosi* 1 day after anthesis; and fixed for microscopic assessment 4 days later. Values are means and standard errors of five replicate experiments, each with eight styles per treatment combination.

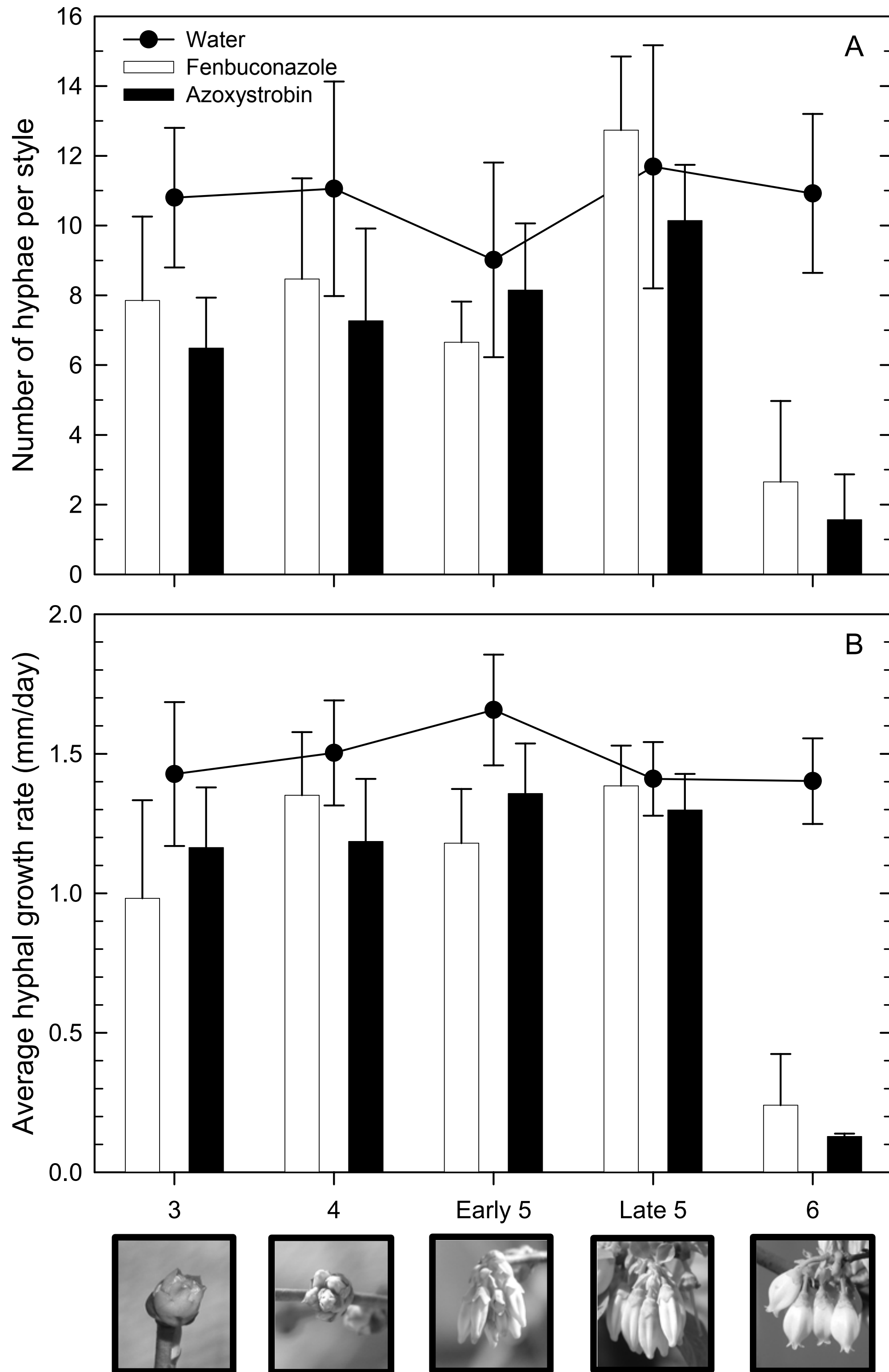
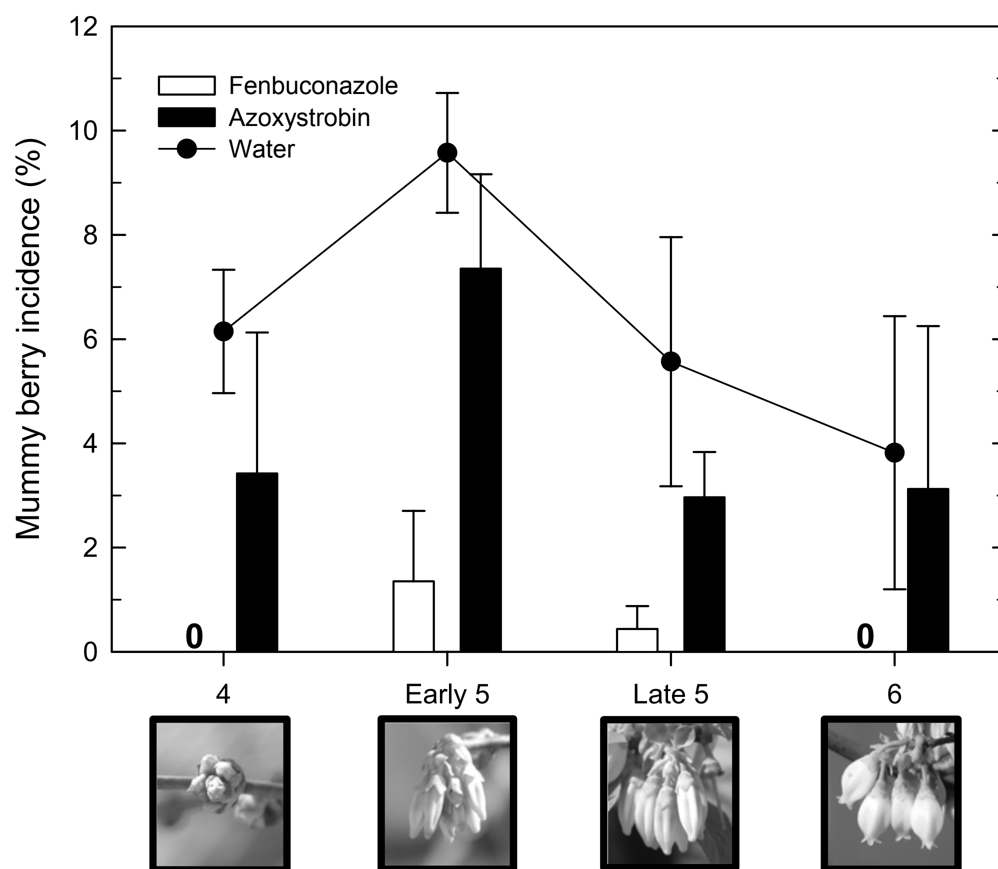

Water
Fenbuconazole
Azoxystrobin
A
B
Number of hyphae per style
Average hyphal growth rate (mm/day)
0
2
4
6
8
10
12
14
16
0.0
0.5
1.0
1.5
2.0
3
4
Early 5
Late 5
6

Figure 3.2. Incidence of fruit mummification by *Monilinia vaccinii-corymbosi* on 'Tifblue' rabbiteye blueberry in the field in relation to fungicide active ingredient and flower stage at the time of fungicide application. Flower clusters were treated with fungicide at the stages indicated; exposed to natural inoculum of *M. vaccinii-corymbosi*; and fruit developing from these clusters were harvested and assessed for disease incidence. Values are means and standard errors of four replicate plots, each with 12 flower clusters per treatment combination.



Mummy berry incidence (%)
12
10
8
6
4
2
0
Fenbuconazole
Azoxystrobin
Water
0
0
4
Early 5
Late 5
6

CHAPTER 4

cDNA SYNTHESIS FROM BLUEBERRY PISTILS INFECTED BY *MONILINIA VACCINII-CORYMBOSI*

INTRODUCTION

Mummy berry, caused by *Monilinia vaccinii-corymbosi*, is an important disease of blueberry (*Vaccinium* section *Cyanococcus*) in North America (Honey, 1936; Batra, 1983; Eck, 1988). In a survey published in 2001, over 75% of Georgia blueberry producers reported mummy berry to be a moderate to major disease problem (Scherm et al., 2001). As a result of a near-zero tolerance for mummified fruit in commercial blueberry shipments, most losses associated with the disease are due to the rejection or downgrading of affected fruit loads in the packinghouse after harvest (Scherm and Copes, 1999).

The mummy berry pathosystem consists of three distinct stages: a primary infection cycle, where ascospores infect young, expanding leaves and shoots causing a blight; a secondary infection cycle, where conidia produced on the blighted tissues infect the open flower through the gynoecial pathway, colonizing the developing fruit internally via the ovary and leading to the formation of a fruit mummy (pseudosclerotium); and the oversummering and overwintering of the mummy, on which apothecia are produced in early spring (Honey, 1936; Batra, 1983).

Because conidia of *M. vaccinii-corymbosi* use the same pathway for infection as the host's pollen tubes use for fertilization, it has been proposed that the fungus practices a form of pollen mimicry (Ngugi et al., 2004). Indeed, conidia of the pathogen are dispersed from the

blighted shoots and deposited on flower styles primarily by insect pollinators (Batra and Batra, 1985). They proceed to germinate in the exudate on the stigmatic surface and grow through the stylar canal to the ovaries (Shinners and Olsen, 1996). In addition, similar to pollen tubes, conidia of *M. vaccinii-corymbosi* adhered to imprints of stylar transmitting tract tissue *in vitro*, and when tested *in vivo*, grew unidirectionally through the style toward the ovary (Ngugi and Scherm, 2004). By contrast, germ tubes of *Monilinia fructicola,* a relative of *M. vaccinii-corymbosi* that is non-pathogenic on blueberry, was able to advance into blueberry styles but showed no directional growth in the stylar canal, indicating that they lack the ability to recognize specific host signals in the pistil that allow *M. vaccinii-corymbosi* to advance through the transmitting tract in a highly directional manner. Based on these observations, the pollen mimicry hypothesis states that the host is not able to distinguish conidia and hyphae of the highly specialized *M. vaccinii-corymbosi* from pollen grains and pollen tubes of its own congruent pollen. Molecular data (e.g., a comparison of host gene expression during pollination vs. infection) would help confirm or refute this hypothesis about pathogenesis in the blueberry-mummy berry system.

Microarrays and cDNA-AFLP (amplified fragment length polymorphisms) have been used in gene expression studies (Reijans et al., 2003). As a hybridization-based analysis, microarrays require knowledge of the genome of the organism being studied. This poses a challenge to scientists working with non-model organisms (such as blueberry or *M. vaccinii-corymbosi*) whose genomes have not been widely characterized. An alternative is cDNA-AFLP, a PCR-based transcript profiling approach that possesses specificity, sensitivity, and reproducibility similar to microarrays (Reijans et al., 2003). This method requires no prior

knowledge of an organism's genome, and so is appropriate for gene expression studies in blueberry, where little of the genome has been characterized.

cDNA-AFLP has been used to characterize gene expression during pollination in some plant species, and in the plant host and pathogen during infection in several pathosystems. A study comparing gene expression in unpollinated and pollinated rice pistils showed a large degree of down-regulation of genes during pollination (Chen et al., 2001). Gene expression profiles in plant hosts when challenged with a pathogen have been characterized using cDNA-AFLP in sugar cane (Carmona et al., 2004), barley (Eckey et al., 2004), and *Arabidopsis* (Tao et al., 2003). Expression in cell types of *Phytophthora infestans* shortly before and during the early stages of potato infection was profiled using cDNA-AFLP (Avrova et al., 2003), as was gene expression in germinating conidia of the barley net blotch pathogen, *Pyrenophora teres* (Dilger et al., 2003). Some previous studies using cDNA-AFLP went on to sequence and identify the genes and functions of the cDNA transcripts that were identified in the AFLP analysis as being up- or down-regulated. This is accomplished by sequencing transcripts of interest and finding homologous genes in a sequence database such as GenBank.

The objective of this study was to optimize mRNA isolation and cDNA synthesis from blueberry styles to lay the groundwork for cDNA-AFLP expression profiling to compare host gene expression in infected and pollinated styles to determine how similar these two processes are from the host's perspective.

MATERIALS AND METHODS

Tissue collection. *Blueberry pistils.* Four to five-year-old rabbiteye blueberry plants (*Vaccinium ashei* 'Tifblue' and 'Powderblue') were maintained in 11.4-liter pots in a cold room

(5 to 6°C) during the winter to allow accumulation of adequate chill-hours for subsequent flower production. At intervals from early January to early May, groups of plants were moved to a greenhouse maintained at 24 to 28°C, resulting in plants with staggered bloom periods from early winter through late spring. Plants were monitored for bloom progression, and individual 'Tifblue' flowers were marked the day they opened. One day later, flowers were subjected to one of three treatments: 1) pollinated with fresh 'Powderblue' pollen, 2) inoculated with conidia of *M. vaccinii-corymbosi*, or 3) untreated control. Pollination was carried out using a dental pick, whereby approximately 50 'Powderblue' pollen grains were applied per 'Tifblue' flower. For inoculation, *M. vaccinii-corymbosi* isolate HT2 was grown on oat bran agar (20 g of oat bran and 10 g agar in 750 ml of deionized water), and 'Tifblue' flowers were inoculated by touching the stigmatic surface with a sporulating mycelial plug of *M. vaccinii-corymbosi*. Two days after treatment, styles were detached from the plants, frozen immediately in liquid nitrogen, and stored in sterile 2.5-ml microcentrifuge tubes at –80°C. Thirty styles per treatment were pooled and stored in a single tube, resulting in tissue samples weighing approximately 0.1 g. Between 15 and 18 such pooled stylar samples were obtained during the flowering period for each treatment.

Blueberry pollen tubes. Fresh 'Powderblue' pollen (approximately 0.25 ml volume) was collected and placed in liquid pollen germination medium (PGM; 50 g sucrose, 0.05 g H_3BO_3, 0.15 g $Ca(NO_3)_2{\cdot}H_2O$, 0.10 g $MgSO_4{\cdot}7\ H_2O$, and 0.05 g KNO_3 in 50 ml deionized water) at room temperature (23 to 25°C) under light. After 24 hours, the suspension containing the germinated pollen was centrifuged (4500 rpm for 5 min), then rinsed with sterile deionized water and split into 0.1-g aliquots in sterile 2.5-ml microcentrifuge tubes. The aliquots were rinsed in sterile water and centrifuged at 12000 rpm for 2 min three times. Nine such samples were stored at –80°C.

Conidial germ tubes. Conidia of *M. vaccinii-corymbosi* were collected from 15-day-old sporulating cultures grown on oat bran agar as described above. They were placed on Petri dishes containing 1% water agar covered with a thin layer of PGM (approximately 10 ml) and incubated at room temperature under light for 12 hours to allow germination. The PGM containing the germinated conidia was poured off into 15 ml of fresh PGM in a 50-ml flask and incubated on a shaker for 4 hours. After incubation, the suspension was centrifuged (4500 rpm for 5 min) and the pellet rinsed with sterile deionized water. Aliquots (0.1 g) of the pellet were deposited into sterile 2.5-ml microcentrifuge tubes. The aliquots were rinsed, centrifuged, and stored as described above for the samples containing pollen tubes. A total of three 0.1-g samples were prepared.

mRNA extraction and quantitation. mRNA was extracted from treated styles, pollen tubes, and conidial germ tubes using the Dynabead mRNA DIRECT Kit (Dynal Biotech, Oslo, Norway). Briefly, one aliquot of tissue (0.1 g) was ground in its microcentrifuge tube over liquid nitrogen to a fine powder and treated in lysis buffer for 2 min before adding 250 µl oligo$(dT)_{25}$ microbeads. mRNA was eluted from the microbeads into 10 µl Tris-HCl (pH 7.5) in sterile 250-µl microcentrifuge tubes by incubation at 72°C for 2 min, followed by storage at −80°C. mRNA was quantified using the Quant-iT RNA Assay kit (Molecular Probes, Eugene, OR), a fluorescence-based quantitation system. One microliter of mRNA from each sample was diluted in 4 µl Tris-HCl. Along with a concentration series of RNA standards (0 to 100 ng per well), 2 µl of diluted samples were combined with 200 µl of Quant-iT reagent in wells of a 96-well microplate. Fluorescence was determined using a FL500 fluorescence microplate reader (Bio-Tek, Winooski, VT) at excitation and emission wave lengths of 590 and 645 nm, respectively.

Fluorescence values were converted to mRNA quantities (µg/g tissue or µg/style) using a calibration line derived from linear regression analysis (SAS, v. 8.02; SAS Institute, Cary, N.C.).

cDNA synthesis and quantitation. cDNA was synthesized from extracted mRNA using the Just cDNA Double-Stranded cDNA Synthesis Kit (Stratagene, La Jolla, CA). This kit uses Stratascript reverse transcriptase for first-strand cDNA synthesis, DNA polymerase I and RNase H for second-strand synthesis, and *Pfu* DNA polymerase to blunt cDNA transcript termini. Three mRNA samples were pooled for each reaction. After synthesis, cDNA was eluted in 9 µl TE Buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) and stored at −80°C.

cDNA was quantified using a similar procedure as for the quantitation of the mRNA samples. The fluorescent dye Hoechst 33258 (bisbenzamide) was prepared in a stock solution of 1 mg/ml in distilled water, and then sterilized by filtration through a 0.22-µm filter. It was stored at 4°C in the dark. Assay buffer (2M NaCl, 50 mM NaH_2PO_4, pH 7.4) was prepared, sterilized and stored at 4°C. Fresh working solution was prepared prior to running the assay by adding 0.1 µl of dye stock to each ml of assay buffer. Six standard DNA quantities (0, 20, 50, 100, 200, and 500 ng DNA) were prepared in TE buffer and used to construct a calibration curve. Fluorescence of DNA samples was determined by adding 1-2 µl of each sample to 200 µl of working solution in a microplate well, and reading fluorescence in a FL500 fluorescence microplate reader (excitation and emission wave lengths of 360 and 460 nm, respectively).

The quality of cDNA was assessed using gel electrophoresis. Five microliters of each sample were combined with 2 µl of 6× blue/orange loading dye (Promega, Madison, WI) and 10 µl of sterile water and loaded into a 1% agarose gel. Samples were run alongside a 100 bp ladder (Promega) in 1× TBE (Tris-borate-EDTA, pH 8.2) buffer at 60 V for approximately 120 min. Gels were visualized and imaged using the Eagle Eye II transilluminator system (Stratagene).

RESULTS AND DISCUSSION

mRNA was extracted successfully from all test tissues, although some procedural hurdles had to be overcome. Tissue grinding was difficult due to the sticky nature of the exudate in the style tissue, and so the frozen, powdered tissue would stick to the glass mortar and pestle first used. This was overcome by using a small polypropylene pestle to grind tissue in plastic microcentrifuge tubes. RNA extraction also proved difficult. Originally, total RNA was extracted from tissues using the Trizol reagent. Again, the exudate in the style tissue presented a problem by forming a sticky lipid layer in the lysate of ground tissue. This layer would not pellet during centrifugation, and so it was difficult to retrieve lysate free of plant material. The problem was solved by using the magnetic Dynabeads, which isolated mRNA directly without the lipid layer in the lysate interfering with recovery of the mRNA.

The calibration line for mRNA quantitation with the Quant-iT kit was $y = 0.224\ x$ ($r^2 = 0.98$, $n = 8$), where y is mRNA quantity (ng/well) and x is the fluorescence emission at 645 nm (Fig. 4.1). Amounts of mRNA extracted from the various tissue types were relatively consistent, resulting in low standard errors except for mRNA from hyphae (Table 4.1). This was probably due to the difficulty in achieving consistent conidial germination. Indeed, when conidia were not of the correct age, germination decreased drastically (*data not shown*). Numerically, inoculated and pollinated styles resulted in the most mRNA extracted, while pollen tubes yielded the least (Table 4.1). Because of the relatively low amounts of mRNA in the tissue samples, purity values could not be estimated reliably as the small amount of mRNA could not be visualized in a gel and spectrophotometer readings were variable. Sufficient purity of the mRNA was assumed upon successful cDNA synthesis.

The calibration curve for cDNA quantitation using Hoechst 33258 fluorescent dye was y=0.2633x (r^2 = 0.82, n = 6). Synthesis reactions produced calculated cDNA quantities of 0.08 to 4.90 µg/g tissue (Table 4.2). The highest amounts of nucleic acid resulted from inoculated and pollinated styles, as well as from pollen tubes. Standard errors could not be calculated for cDNA quantities because only one sample was synthesized for each tissue. On agarose gel, cDNA from the style samples appeared as faint smears from 100 to 1500 kb (Fig. 4.2). This is characteristic of cDNA, which contains fragments of many different lengths. The intensities of the smears correspond to the relative concentrations measured by spectrophotometry (Table 4.2). cDNA from inoculated styles and pollinated style produced more intense bands (as they had higher concentrations), and cDNA from control styles the faintest band. The results obtained with the gel indicate that the synthesized cDNA was of sufficient quality to be used in the cDNA-AFLP step.

For cDNA-AFLP in future work, the cDNA synthesized from the five tissues would be digested by two different restriction enzymes, then ligated to specific adapters (Bachem et al., 1998). Primers corresponding to these adapters would then be used in PCR to amplify the different fragments of cDNA. To generate a transcript profile, the PCR products are loaded into a gene sequencer, which produces electropherograms showing peaks where bands occur. The expression profiles from the different tissue types would then be compared for degree of similarity. A similar profile between pollinated and inoculated styles would support the pollen-mimicry hypothesis

In summary, this study optimized the steps involved in tissue preparation and processing, mRNA extraction, and cDNA synthesis for the five tissue types necessary to study gene expression in blueberry pistils pollinated or inoculated with *M. vaccinii-corymbosi*. The

groundwork has been laid for cDNA-AFLP to be used to investigate the role of pollen mimicry in the mummy berry pathosystem on a molecular level.

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Table 4.1. mRNA quantities extracted from blueberry styles pollinated or inoculated with *Monilinia vaccinii-corymbosi* [a].

	mRNA quantity [b]	
Tissue type	(μg/g tissue)	(μg/style)
Untreated styles (control)	4.98 ± 0.56	0.017 ± 0.002
Inoculated styles	9.78 ± 1.53	0.033 ± 0.005
Pollinated styles	6.59 ± 1.21	0.022 ± 0.004
Germinated conidia of *M. vaccinii-corymbosi*	10.56 ± 6.28	---
Blueberry pollen tubes	3.57 ± 0.82	---

[a] mRNA was extracted using magnetic Oligo(dT)$_{25}$ microbeads, and quantities were determined fluorometrically.

[b] Values are means and standard errors of three extractions, each from approximately 0.1 g of tissue.

Table 4.2. cDNA quantities synthesized from blueberry styles pollinated or inoculated with *Monilinia vaccinii-corymbosi* [a].

	cDNA quantity [b]	
Tissue type	(μg/g tissue)	(ng/style)
Untreated styles (control)	0.33	1.10
Inoculated styles	2.63	8.77
Pollinated styles	4.90	16.34
Germinated conidia of *M. vaccinii-corymbosi*	0.38	---
Blueberry pollen tubes	6.93	---

[a] cDNA was synthesized from mRNA using Stratascript reverse transcriptase for first-strand synthesis and DNA polymerase I and RNase H for second-strand synthesis.

[b] cDNA quantities were determined fluorometrically.

Fig 4.1. Calibration line relating RNA quantities to fluorescence readings in a FL500 microplate reader using the Quant-iT RNA Assay kit. The equation corresponding to the line is $y = 0.224\ x$ ($r^2 = 0.98$, $n = 8$), where y is RNA quantity (ng/well) and x is the fluorescence emission at 645 nm.

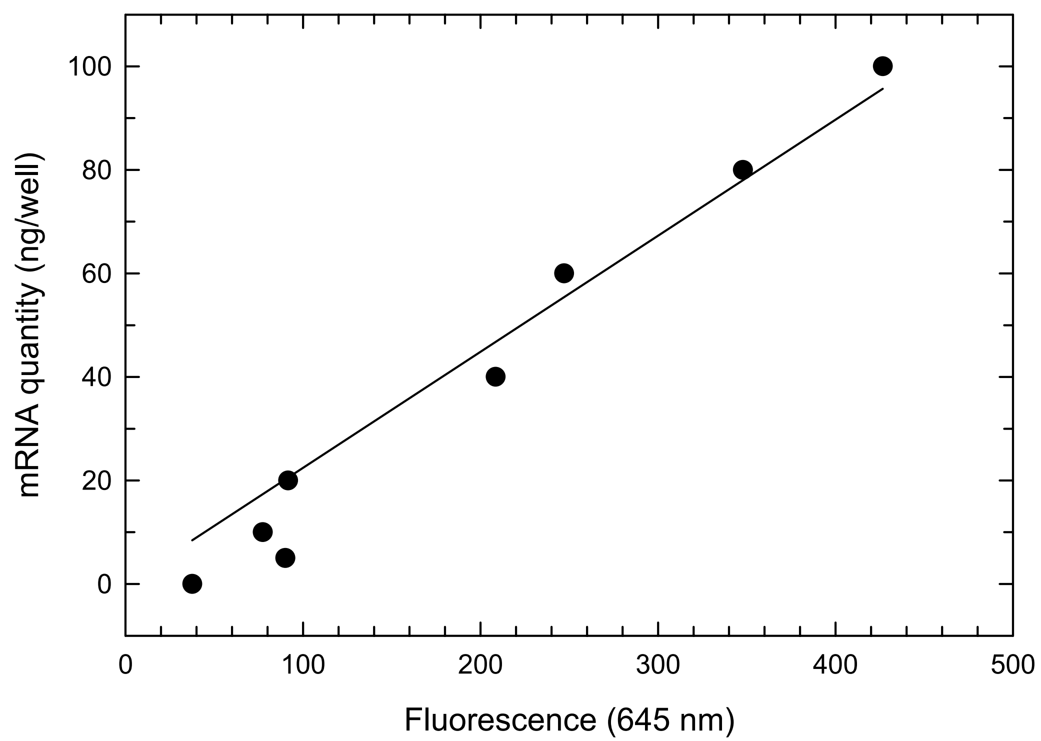
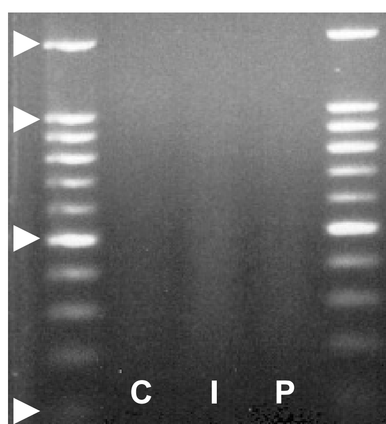

mRNA quantity (ng/well)
Fluorescence (645 nm)
0
20
40
60
80
100
0
100
200
300
400
500

Fig. 4.2. Agarose gel (1%) showing smears of cDNA synthesized from blueberry styles that were either pollinated (P), inoculated with *Monilinia vaccinii-corymbosi* (I), or left untreated (C). mRNA was extracted from styles using magnetic Oligo(dT)$_{25}$ microbeads, and cDNA was synthesized using Stratascript reverse transcriptase for first-strand synthesis and DNA polymerase I and RNase H for second-strand synthesis. Arrows indicate 100, 500, 1000, and 1500 kb markers.


C
I
P

CHAPTER 5

CONCLUSIONS

A lack of understanding of the efficacy of fungicide applications before versus after anthesis in managing floral infection by the mummy berry fungus *Monilinia vaccinii-corymbosi* prompted me to conduct a study investigating the in-flower activity of fenbuconazole and azoxystrobin, two fungicidal active ingredients currently labeled for use against the disease. Specifically, the two compounds were assessed for their efficacy in flowers sprayed at several stages before and at anthesis. In the greenhouse, only flowers sprayed at anthesis showed reduced pathogen growth into styles for both fungicides compared with the water control. In contrast, styles of flowers sprayed at pre-anthesis developmental stages were not protected from hyphal penetration, suggesting the two fungicides are not translocated from the ovary (the only part of the pistil exposed to fungicide in pre-anthesis flowers) into the style. Any flowers closed at the time of a fungicide application would thus be accessible to infection by *M. vaccinii-corymbosi*, regardless of how close to anthesis the spray was applied.

In the field, azoxystrobin did not decrease mummy incidence, even when sprayed at anthesis. In the field experiment, flower phenology could not be controlled as precisely as in the greenhouse (i.e., it was not known how many days before treatment flowers had opened), and flowers sprayed at anthesis in the field could already have been infected before treatment, or after azoxystrobin was most effective. Unlike azoxystrobin, fenbuconazole treatments reduced disease when sprayed at all flower stages, even in flowers sprayed before anthesis. This is at odds with the findings of the greenhouse study and suggests that factors other than inhibition of

hyphal penetration through the style are responsible for the efficacy of pre-anthesis fenbuconazole treatments. Pre-bloom sprays may decrease the levels of inoculum available for flower infection, or the residual activity of fenbuconazole in the ovaries may inhibit colonization of the developing fruit by *M. vaccinii-corymbosi* following its successful growth through the style.

A more thorough investigation of the systemic activity of fungicides in flowers is necessary to make effective chemical management choices for pathosystems involving flowers. We cannot assume that the systemicity of a compound in leaves will extend to floral organs. For the mummy berry system, a more systemic compound may provide better protection from floral infection. A range of active ingredients should be tested, and their systemic and curative action characterized in treated flowers in order to design the most effective spray regime.

Another interesting and important aspect of the mummy berry pathosystem is the possible role of pollen mimicry in the gynoecial infection of blueberry flowers by *M. vaccinii-corymbosi*. There are numerous ecological, anatomical, and physiological similarities between the dissemination of pollen grains and that of conidia (Batra and Batra, 1985) and between the stylar ingress of pollen tubes and that of hyphae (Shinners and Olsen, 1996; Ngugi and Scherm, 2004) which suggest that the flower recognizes the pathogen in the same way as congruent pollen tubes. To corroborate or refute this theory, a molecular study characterizing host gene expression in infected and pollinated styles is needed. cDNA-AFLP (amplified fragment length polymorphism) is an appropriate approach to such a study (Chen et al., 2001; Reijans et al., 2003). but requires that the steps involved in tissue preparation and processing, mRNA extraction, and cDNA synthesis be optimized. These preliminary steps have been completed in the current study, and the groundwork has been laid to apply cDNA-AFLP to determine the

similarity in gene expression profiles between flowers that are pollinated vs. those infected by *M. vaccinii-corymbosi*.

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APPENDIX A

DISEASE INCIDENCE DATA FROM HORTICULTURE FARM FIELD TRIALS, 2004-2005

Table A.1. Incidence of fruit mummification by *Monilinia vaccinii-corymbosi* on 'Climax' rabbiteye blueberry at the University of Georgia Horticulture Farm in relation to fungicide active ingredient and flower stage at the time of fungicide application [a].

	Incidence of mummified fruit (%) [b]							
	2004				2005			
	Replication				Replication			
Treatment [c] and stage [d]	1	2	3	4	1	2	3	4
Untreated								
Stage 4	2.63	0	0	1.69	1.89	4.55	4.17	3.23
Stage early 5	0	0	1.45	12.00	12.77	2.04	12.50	0
Stage late 5	0	0	4.00	1.61	0	0	0	0
Stage 6	2.27	0	7.32	2.13	0	0	0	0
Fenbuconazole								
Stage 4	4.00	0	2.78	8.00	0	3.85	2.27	0
Stage early 5	1.61	0	5.36	0	0	0	0	0
Stage late 5	3.45	2.38	1.75	1.67	0	0	0	0
Stage 6	2.17	0	6.06	0	0	0	0	0
Azoxystrobin								
Stage 4	0	0	0	2.50	2.13	12.50	0	0
Stage early 5	0	0	8.82	0	2.17	0	0	0
Stage late 5	0	0	13.79	0	0	0	0	4.26
Stage 6	2.22	1.92	19.05	2.08	0	0	0	0

[a] Flower clusters were treated with fungicide at the stages indicated below; exposed to natural inoculum of *M. vaccinii-corymbosi*; and fruit developing from these clusters were harvested and assessed for disease incidence.

[b] Values based on 12 flower clusters per treatment combination.

[c] Indar 75WSP (fenbuconazole) or Abound (azoxystrobin) applied as formulated products at 0.3 g/l and 1.9 ml/l, respectively.

[d] Bloom stages at fungicide application were 4 (individual flowers distinguishable), early 5 (individual flowers distinctly separated; corollas small, unexpanded and closed), late 5 (individual flowers distinctly separated; corollas larger, unexpanded and closed), or 6 (corollas completely expanded and open) (Spiers, 1978).