EVALUATION OF A DIVERSE PANEL OF BIOCONTROL AGENTS AGAINST INFECTION OF BLUEBERRY FLOWERS BY *MONILINIA VACCINII-CORYMBOSI*

by

HOLLY ANN THORNTON

(Under the Direction of Harald Scherm)

ABSTRACT

Seven microorganisms were evaluated for their biocontrol potential against *Monilinia vaccinii-corymbosi* which causes mummy berry disease through stigmatic infection of blueberry flowers: the bacteria *Bacillus subtilis*, *B. mojavensis*, *B. mycoides*, and *Pantoea agglomerans*; the yeast *Wickerhamiella australiensis*; and the filamentous fungi *Trichoderma harzianum* and *Gliocladium roseum*. All organisms were tested for antibiosis using dual cultures and for nutrient competition (niche overlap) using Biolog microplates. Their population dynamics on detached blueberry flowers were investigated to estimate epiphytic fitness, and such flowers, co-inoculated with *M. vaccinii-corymbosi*, were used to determine efficacy in reducing pathogen ingress into the styles. The most promising candidates were *B. subtilis*, which showed strong antibiotic activity and considerably reduced pathogen ingress, despite having limited epiphytic fitness; *G. roseum*, which exhibited complete niche overlap with the pathogen and consistently suppressed stylar penetration; and *P. agglomerans*, which showed favorable population dynamics but produced more variable results in reducing stylar infection.

INDEX WORDS: Antibiosis, Biocontrol, Blueberry, Competition, Epiphytic fitness, Mummy berry, *Monilinia vaccinii-corymbosi*, *Bacillus subtilis*, *Bacillus*

mycoides, *Bacillus mojavensis*, *Pantoea agglomerans*, *Wickerhamiella australiensis*, *Trichoderma harzianum*, *Gliocladium roseum*

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HOLLY ANN THORNTON

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HOLLY ANN THORNTON

Major Professor: Harald Scherm

Committee: Charles W. Mims
Phillip M. Brannen

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

I dedicate this thesis to my mom, who has been a role model and inspiration to me through her constant hard work and ability to remain strong during both good times and bad.

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CHAPTER 1

INTRODUCTION

The discomycete *Monilinia vaccinii-corymbosi* causes mummy berry disease of blueberries (*Vaccinium* Section *Cyanococcus*), a significant problem in most blueberry-producing regions in North America (Batra 1983; Hildebrand et al. 1985). Symptoms of the primary, ascosporic infection stage of the disease include blighting of leaves and shoots in early spring. It is on these infected vegetative tissues that conidia are produced and carried to open flowers by wind, rain, or pollinating insects (Batra and Batra 1985). The secondary infection stage is characterized by the deposition of conidia on the stigmatic surfaces of open flowers and their subsequent germination and ingress of the ensuing hyphae into the flower styles, following the same pathway as pollen tubes (Ngugi and Scherm 2004; Shinners and Olson 1996). The pathogen subsequently infects the ovary of the flower, and – as the fruit matures – an infected, sclerotinized berry results, eventually falling to the ground and overwintering as a mummy (Batra 1983; Milholland 1977).

Although currently controlled with repeated fungicide applications during bloom (Scherm and Krewer 2003; Scherm and Stanaland 2001; Stanaland et al. 2004, 2005), alternatives to synthetic fungicides are desirable to reduce costs, avoid fungicide resistance that could develop following repeated application of the same active ingredient(s), and for organic and pick-your-own blueberry farmers who need non-chemical alternatives for control of the disease. Such alternatives include cultural controls, which are most effective against primary infection (Ngugi et al. 2002b), as well as biological control, which has been targeted chiefly

against secondary infection (Dedej et al. 2004; Scherm et al. 2004). The secondary infection pathway of *M. vaccinii-corymbosi* is a particularly promising target for biocontrol for several reasons (Scherm and Stanaland 2001). First, season-long protection is unnecessary because fruit infection can take place only during bloom, which represents a relatively short period of host development (3 to 4 weeks). Second, the copious exudate on the stigmatic surface of the blueberry flower may provide the antagonist with the moisture and nutrients needed for successful colonization and growth. Lastly, *M. vaccinii-corymbosi* is a relatively slow-growing pathogen, which should allow the antagonist sufficient time to act against the pathogen prior to its ingress into the ovary.

In previous work in this laboratory, Scherm et al. (2004) examined the potential of two bacteria, *Pseudomonas fluorescens* A506 and *Bacillus subtilis* QRD137, to control mummy berry disease. These antagonists were applied to detached blueberry flowers in the laboratory, either alone to assess how well each could colonize and grow on the stigmas, or together with *M. vaccinii-corymbosi* to determine whether the antagonist was capable of preventing or reducing pathogen ingress into the style. Of the two antagonists, *B. subtilis* was considerably more effective, reducing growth rates of *M. vaccinii-corymbosi* in blueberry styles to less than one-fifth of those of the untreated control. Unfortunately, *B. subtilis* had limited epiphytic fitness on the flower stigma, and its population density dropped to undetectable levels within 3 days after application.

The overall goal of this study was to investigate a diverse panel of microorganisms in order to identify an antagonist(s) that combines high efficacy against secondary infection by the pathogen with favorable epiphytic fitness on the stigmatic surface. The organisms included four bacterial species, one yeast, and two filamentous fungi, all selected based on their performance

or specific inherent properties as determined in previous biocontrol studies with other pathosystems. Specific objectives were to: (1) monitor the population dynamics of the microorganisms following their application to stigmas of blueberry flowers as an indicator of epiphytic fitness; (2) determine the efficacy of the microorganisms in terms of suppressing hyphal ingress of *M. vaccinii-corymbosi* into such flowers; and (3) explore the mode(s) of action utilized by the antagonists, with an emphasis on antibiosis and nutrient competition.

CHAPTER 2

LITERATURE REVIEW

2.1 Disease cycle of mummy berry

Mummy berry disease, caused by the discomycete *Monilinia vaccinii-corymbosi*, is among the most important diseases of blueberries (*Vaccinium* Section *Cyanococcus*) in North America (Batra 1983; Hildebrand et al. 1985). The pathogen and the host have synchronized developmental stages that increase the likelihood that the fungus can infect and reproduce (Lehman and Oudemans 2000). In fact, in this disease system, phenological development of pathogen and host must be coupled closely for disease development to occur (Lehman and Oudemans 2000), as will become clear from the description of the pathogen's life cycle below.

Overwintering pseudosclerotia (mummies) on the ground are the only means by which the fungus survives the fall and winter and continues to infect blueberry in subsequent seasons (Batra 1983). Milholland (1977) erroneously described the overwintering structure as a sclerotium composed entirely of fungal tissue, rather than a pseudosclerotium that also contains host tissue within it. After an adequate chilling period during the winter, the pseudosclerotia germinate carpogenically to produce apothecia, thereby beginning the primary, sexual infection phase of *M. vaccinii-corymbosi*. The development of apothecia is inhibited if the overwintering structure is buried deeper than 2.5 cm below the soil surface (Milholland 1974; Ngugi et al. 2002b). The chill-hour requirement for apothecium production in Georgia is low compared with that required for populations of the fungus in North Carolina (900 to 1200 hours minimum) and Washington state (700 hours) (Scherm et al. 2000), indicating pathogen adaptation to low-chill

conditions. In fact, pseudosclerotia collected from Georgia blueberry plantings can germinate even after low chilling during unusually warm winters, in that high heat unit (degree-day) accumulation during late winter can compensate for low chill-hour accumulation earlier in the winter (Scherm et al. 2000). The apothecia then produce ascospores, which are forcibly discharged and wind-dispersed to nearby blueberry bushes (Cox and Scherm 2001). Ascospores infect primarily young, expanding, vegetative tissues, leading to blighting of leaves and shoots (Batra 1983). Here, the synchronous timing of the life cycles of the pathogen and the host is critical, because the young blueberry tissue is susceptible only for a short period of time (Lehman and Oudemans 1997).

The secondary, asexual infection phase of the disease cycle is characterized by the production of conidia on the blighted leaves and shoots and the transfer of conidia (typically by pollinating insects) to the stigmatic surfaces of the flowers where they germinate (Batra 1983). Leaves blighted by *M. vaccinii-corymbosi* produce ultraviolet-reflective patterns that resemble flowers containing nectar, thus attracting the insect vectors necessary to disseminate conidia (Batra and Batra 1985). Heavily blighted blueberry bushes are said to have a characteristic odor similar to fermented dark tea leaves (Batra 1983), further serving to attract insects. Following its transfer onto the stigma, the fungus grows into the style and, following the transmitting tract (a hollow stylar canal), reaches the ovary within 4 to 7 days (Ngugi et al. 2002a). As the blueberry flower ages, its susceptibility to the pathogen is reduced rapidly, and the fastest hyphal growth of *M. vaccinii-corymbosi* occurs in flowers inoculated on the day of anthesis (Ngugi et al. 2002a). Ngugi and Scherm (2004) showed that *M. vaccinii-corymbosi* mimics pollen tube growth through the stylar canal into the ovary of blueberry flowers both anatomically and physiologically. This mimicry ability is important because it is thought to allow the pathogen to

evade the plant host defenses. Once it has traversed the entire length of the style, *M. vaccinii-corymbosi* colonizes the ovary of the flower and, as the fruit mature, the exocarp becomes stromatized and a pseudosclerotium results. In summary, the mummy berry disease cycle consists of a single primary infection phase in the spring resulting in leaf and shoot blight, a single secondary infection phase in the summer resulting in fruit mummification, and a dormant period during the fall and winter.

2.2 Mummy berry control

Mummy berry caused $211,600 worth of direct damage in Georgia in 2001, and the (indirect) cost to control the disease averaged around $500,000 that year (Williams-Woodward 2002). The disease is controlled by sanitation efforts that either remove blueberry mummies or bury them at least 2.5 cm below the soil surface (Milholland 1974) and by repeated fungicide applications from vegetative bud break through the end of bloom (Scherm and Stanaland 2001; Stanaland et al. 2004, 2005). Ngugi et al. (2002b) assessed the vertical distribution profiles of pseudosclerotia in the soil following different soil cultivation practices in the row middles between blueberry bushes and found that, for most cultivation methods employed by commercial blueberry producers, a large proportion of pseudosclerotia was not buried deep enough to prevent apothecium emergence. Therefore, mechanical cultivation alone is not an effective means of mummy berry management, and fungicides typically must be used in addition to or instead of cultural control. Of the fungicides currently available, Indar (fenbuconazole), which is available via a Section 18 label, and Pristine (pyraclostrobin + boscalid) are most effective in controlling the disease (Stanaland et al. 2004, 2005). Abound (azoxystrobin) also is labeled but has lower efficacy (Scherm and Stanaland 2001; Stanaland et al. 2004). All three fungicides are also used

after harvest to control leaf diseases such as Septoria, anthracnose, and/or rust. The resulting high number of applications with the same active ingredients highlights the need for active fungicide resistance management. This has been recognized in the most recent Section 18 label for Indar in Georgia (for the 2005 season), which mandates that two sequential applications of Pristine be utilized before Indar can be applied (P.M. Brannen, *personal communication*).

2.3 Biocontrol of plant diseases

Biocontrol of plant disease uses microorganisms to reduce inoculum or disease progress rates of pathogens (Roberts and Lohrke 2003). The first recorded use of a biocontrol agent was in 1921 by Hartley, who demonstrated that microbes could control damping-off of tree seedlings caused by *Pythium aphanidermatum* (Deacon 1991). From the early 1920s to present, hundreds of biocontrol agents have been tested for their effectiveness in controlling certain diseases, but only a few (around 5%) have achieved that goal consistently (Desai et al. 2002). This low level of success can be attributed to several factors, including a lack of knowledge about the best screening process for the biocontrol agents; certain aspects of the ecology of the biocontrol agents (e.g., their inability to colonize certain niches); problems arising from the three-way interactions between the biocontrol agent, the pathogen, and the host; the inconsistencies between *in vitro* and *in vivo* results; formulation difficulties; and lengthy governmental registration processes (Roberts and Lohrke 2003). In addition, safety issues involving the introduction of microorganisms into the environment are a common concern in the public sector (Cook 1996). Although many factors seem to be working against the successful use of biocontrol agents in plant disease control, biocontrol may offer a viable long-term alternative to plant

disease management as pathogens develop fungicide resistance and other, more labor-intensive methods (e.g., sanitation) become more expensive.

There are several important factors to consider when choosing an appropriate antagonist, including its mode of action, the population dynamics of the biocontrol agent on the plant, the formulation of the biocontrol agent, its cost and method of application, and its compatibility with fungicides and other disease control methods. The mode of action of the biocontrol agent should be determined so that one can make generalizations as to how it will perform in a range of environments. Indeed, the most successful mode of action is dependent on the interactions between the pathogen and the biocontrol agent in the natural environment (Marois and Coleman 1995). Guetsky et al. (2002) demonstrated that using multiple antagonists with different modes of action (*Pichia guilermondii* and *Bacillus mycoides*, in their example) resulted in additive effects relative to suppressing *Botrytis cinerea* on strawberry.

Modes of action by which a biocontrol agent can suppress a plant pathogen include, but are not limited to antibiosis, competition, and mycoparasitism. Antibiosis, which involves the production of metabolites by the antagonist that are detrimental to the pathogen, is a common mode of action for bacteria and fungi. Low-molecular-weight diffusible molecules, antibiotics, small toxic molecules, volatiles, and lytic enzymes are all compounds by which one microorganism can inhibit another through antibiosis (Chet et al. 1997). For example, *Gliocladium virens* produces the antibiotic gliovirin that kills the damping-off pathogen *Pythium ultimum* through coagulation of its protoplasm (Chet et al. 1997). *Trichoderma harzianum* reduces damping-off in lettuce caused by *Rhizoctonia solani* through the production of antifungal, volatile alkyl pyrones (Chet et al. 1997). This method is relatively easy to test for *in vitro* by applying both the antagonist and the pathogen to an appropriate medium in Petri dishes

and determining if zones of inhibition are formed around the pathogen. *Pseudomonas fluorescens* strain 2-79 produces three antifungal compounds that help suppress take-all of wheat caused by *Gaeumannomyces graminis* (Milner et al. 1997).

Competition for nutrients and space is the mode of action utilized by many yeast, fungal, and bacterial biocontrol agents. Competition can be defined as "the negative effects which one organism has upon another by consuming, or controlling access to, a resource that is limited in availability" (Keddy 1989). In the blueberry-*M. vaccinii-corymbosi* pathosystem, the limiting resource may be the nutrients (i.e., carbohydrates or lipids) that comprise the exudate produced on the stigma of the blueberry flower, the infection court for *M. vaccinii-corymbosi* and the potential site of biocontrol application. Different fungi, for example, have varying abilities to utilize certain carbohydrates. 'Sugar fungi,' such as *Mucor* species, have high competitive saprophytic abilities and poor enzymatic abilities to degrade the more complex carbohydrates (Widden 1997). Other fungi, such as *Penicillium* and *Trichoderma,* have broad enzymatic abilities and are capable of degrading and utilizing the more complex carbohydrates (Widden 1997). Therefore, when choosing candidates as potential antagonists, some fungi will compete with the pathogen as sugar fungi, which typically germinate and grow faster; while others will compete through their enzymatic abilities. One way to determine competition as a mode of action is to monitor the population density of both the antagonist and the pathogen after application (Marois and Coleman 1995). If the antagonist and pathogen populations both decline, then it is likely that they are competing for the same space and nutrients. For example, *Alternaria alternata* and *Cladosporium cladosporioides* control infection of *Sclerotinia sclerotiorum* by competition (Marois and Coleman 1995). Niche overlap between two particular organisms may be an indicator for nutrient competition and may be tested for by determining which nutrients are

utilized by both the pathogen and the antagonist. If similar nutrients are being utilized, then niche overlap and the potential for nutrient competition are greater. In addition to competition for nutrients, there may be competition for physical space on the stigma between antagonists and *M. vaccinii-corymbosi*.

Mycoparasitism, a common mode of action for fungal biocontrol agents, is the direct attack on the thallus of the pathogen followed by nutrient utilization by the mycoparasite (Chet et al. 1997). Barnett and Binder (1973) divided mycoparasitism into two classes. Necrotrophic mycoparasitism results in death and destruction of the host thallus, and the fungi in this class are usually more aggressive and have a broad host range; whereas biotrophic mycoparasitism is a more balanced parasitic relationship, and the representatives of this class produce specialized structures used to absorb the nutrients from their host (Chet et al. 1997). *Ampelomyces quisqualis*, an example of a biotrophic hyperparasite, parasitizes powdery mildews, as do *Eudarluca* spp. on rusts (Chet et al. 1997; Marois and Coleman 1995). *Trichoderma harzianum* and *G. roseum* are described as necrotrophic mycoparasites that directly attack the mycelium or produce toxic substances, respectively. A problem that arises with these systems is obtaining high levels of parasitism to overcome the fast reproductive cycles of the pathogen.

Mycoparasitism can be tested *in vitro* by dual culture on specific media. Transmission electron microscopy (TEM) is used with ultra-thin sections of mycelial samples taken from the interaction zones of such cultures to view the specific interactions that occur between the pathogen and the antagonist (Elad et al. 1983). Another method used to visualize the interactions between certain fungi involves growing the two fungi on a membrane and taking sections of the membrane to view using scanning electron microscopy (SEM) (Elad et al. 1983). The mycelia of each of the fungi are distinguishable because the mycoparasite is typically smaller and tends to

coil around the pathogen using small outgrowths from the hyphae to penetrate and parasitize the host fungus (Gao et al. 2005; Inbar et al. 1996; Roy and Sayre 1984).

The population dynamics of the antagonist on the plant are also important when choosing an appropriate antagonist. The antagonist generally must reach and maintain sufficiently high population densities on the plant to suppress the pathogen as poor population dynamics can allow the pathogen to overcome the biocontrol agent. If antibiosis is the mode of action and the antagonist cannot reach and maintain sufficiently high numbers on the infection court, then the limited production of antibiotics would prevent control of the disease. Population dynamics of bacterial and yeast biocontrol agents are assessed readily as population counts following dilution-plating of the colonized host tissue, but this method is not generally suitable for fungi. For the latter group of organisms, direct microscopic observation of the plant host tissue to quantify the extent of colonization may be needed.

Apart from its mode of actions, the formulation of the antagonist is also an important consideration. The agent can be applied as a liquid spray, wettable powder, dust, or in granular form. However, to maximize its efficacy, one needs to determine which formulation offers optimum coverage of the plant (Desai et al. 2002). Moreover, the shelf life of the formulation is important for biocontrol activity. Antagonists having a long shelf life, possibly through the production of survival structures (in the case of fungi or spore-forming bacteria) have a better chance of becoming commercialized (Desai et al. 2002). Different formulations contain different relative amounts of the antagonist. Therefore, the type of formulation used will affect costs and application rates. For example, there are several formulations of *Bacillus subtilis* in the marketplace [e.g., Companion, HiStick N/T, Kodiak, Rhizo-Plus, Serenade (WP, AS, and MAX formulation), Subtilex], but improvements to current formulations may be needed to enhance

disease control (Schisler et al. 2004). Such improvements include producing formulations that have moisture-retaining polymers and/or nutrient amendments that enhance antagonist survival once applied (Schisler et al. 2004). Also, mixtures of antagonists may enhance biocontrol activity by broadening the spectrum of activity and enhancing both the efficacy and reliability of the biocontrol as long as the antagonists do not interfere with each other (Guetsky et al. 2002; Janisiewicz 1996).

Compatibility of the prospective antagonist also must be assessed during the screening process. If certain antagonists are compatible with other management practices, implementation of biocontrol in production systems will increase (Desai et al. 2002). As management approaches continue to move toward the ideal of Integrated Pest Management (IPM), this factor will become increasingly important because the antagonist will need to be compatible with current practices that often involve fungicides (Elad et al. 1993). For example, combining *Trichoderma* spp. with fungicides to manage *Sclerotium rolfsii* in peanut is effective (Desai et al. 2002).

2.4 Biocontrol of mummy berry

Limited research has been published on the biocontrol of mummy berry disease in blueberry. As stated above, the blueberry-mummy berry system is an excellent candidate for biocontrol research for several reasons. These include a small infection court that needs to be protected (the flower stigma), the short window of opportunity for the fungus to infect the flower (less than 5 days after anthesis for individual flowers) (Ngugi et al. 2002a), and the large amounts of exudate on the stigma, which could provide moisture and nutrients for the biocontrol agent. Further, *M. vaccinii-corymbosi* is a slow-growing fungus, so a fast-multiplying biocontrol agent would be well-positioned to outcompete the pathogen prior to its ingress into the flower

ovary. Scherm et al. (2004) examined two bacterial biocontrol agents, *Pseudomonas fluorescens* A506 (BlightBan) and *Bacillus subtilis* QRD137 (Serenade WP), and found *B. subtilis* to be a promising candidate for control of secondary infection by *M. vaccinii-corymbosi* based on inoculation experiments on detached flowers. Complementary *in vitro* experiments suggested antibiosis as a major mode of action of *B. subtilis* against the pathogen. In subsequent field experiments with caged blueberry bushes, Dedej et al. (2004) documented partial suppression of mummy berry disease using bee-vectored *B. subtilis* (formulated as Serenade WP, AgraQuest, Davis, CA). However, one of the potentially problematic issues was the poor growth of *B. subtilis* on the flower stigma (Scherm et al. 2004). Indeed, *B. subtilis* population numbers were variable 1 and 2 days after application and then dropped consistently by days 3 and 4. Therefore, efforts should continue to identify a biocontrol agent that is not only effective in model experiments (*in vitro* and on detached flowers), but also has a favorable epiphytic ability, allowing it to colonize the flower stigma rapidly. In the present study, we particularly were interested in assessing the biocontrol potential against *M. vaccinii-corymbosi* of the bacteria *Bacillus mycoides*, *B. mojavensis*, and *Pantoea agglomerans*; the yeast *Wickerhamiella australiensis*; and the filamentous fungi *T. harzianum* and *G. roseum*. The selection of these organisms was based on features and properties described below.

Bacillus mojavensis is an endophytic bacterium that provides protection against *Fusarium verticilloides* (and associated mycotoxin formation) in maize seedlings (Bacon and Hinton 1999; Bacon et al. 2001). The organism's endophytic nature in plants may be useful for a pathosystem such as mummy berry disease in which the pathogen invades through a natural opening, i.e., the stylar canal. Bacon et al. (2001) found that their patented *B. mojavensis* strain RRC101 is antagonistic to many species of fungi. The bacterium causes zones of inhibition when grown in

dual culture with fungi, and hyphae that come in contact with the bacterium appear lysed and necrotic (Bacon and Hinton 2002). Certain isolates of *B. mycoides* are systemic resistance-inducing biocontrol organisms capable of controlling leaf spot on sugar beet (Bargabus et al. 2002). This organism has not been evaluated previously for disease suppression in flower pathosystems. *Pantoea agglomerans*, formerly known as *Erwinia herbicola*, is noted for its ability to exclude (a method of competition) certain pathogens from the stigmas of crop plants such as *Erwinia amylovora* on pome fruits (Johnson and Stockwell 1998; Pusey 1999). Ishimaru et al. (1988) demonstrated the ability of *P. agglomerans* to produce multiple antibiotics that are capable of controlling several plant pathogens. Yeasts are known for their ability to act as biocontrol agents through competition for nutrients and have been shown to reduce conidial germination in certain plant-pathogenic fungi (Elad et al. 1994). Most of the yeast species evaluated previously for biocontrol are phylloplane yeasts such as *Rhodotorula glutinis* and *Cryptococcus albidus.* However, some yeast species, such as *Wickerhamiella australiensis*, can be recovered from flowers. Such yeasts readily hydrolyze lipids, which may be advantageous for biocontrol in the mummy berry system since the stigmatic exudate of blueberry flowers contains large amounts of lipids (Heslop-Harrison and Shivannah 1977). Thus, the stigmatic surface may present a particularly favorable niche for *W. australiensis*. The biocontrol potential of *Trichoderma* spp. was documented as early as the 1930s (Weindling 1932). Presently, the list of plant diseases controlled by various species of *Trichoderma* is substantial. *Trichoderma* spp. are excellent candidates for biocontrol because of the many strategies they use to combat plant pathogens. Mycoparasitism (Chet 1987; Weindling 1932), antibiosis (Howell et al. 1993), competition (Harman 2000), the production of enzymes causing cell lysis (Mecalf and Wilson 2001), and induced host resistance (Yedidia et al. 1999) are all mechanisms employed by

Trichoderma spp. Both mycoparasitism and competition for nutrients and/or space are modes of action used by *G. roseum* to exclude plant pathogens. The organism has been used previously to control *B. cinerea*, a flower-infecting fungus, on red raspberry and other crops (Yu and Sutton 1999).

CHAPTER 3

MATERIALS AND METHODS

3.1 Test organisms

Seven microorganisms were evaluated for their potential to suppress infection of blueberry flowers by *Monilinia vaccinii-corymbosi*: the bacteria *Bacillus subtilis* QRD137, *B. mycoides* 7IIC4, *B. mojavensis* RRC101, and *Pantoea agglomerans* C9-1S; the yeast *Wickerhamiella australiensis* Y-27360; and the filamentous fungi *Trichoderma harzianum* KRL-AG2 and *Gliocladium roseum*. Sources and properties of these strains are given in Table 3.1. *Bacillus subtilis* QRD137 was included as a standard because of its use in previous biocontrol research with *M. vaccinii-corymbosi* (Scherm et al. 2004). The bacteria and yeast species were stored in 40% glycerol at −80ºC, while the filamentous fungi were maintained on potato dextrose agar (PDA) slants at 4ºC. The pathogen isolate was *M. vaccinii-corymbosi* HT2-R.

3.2 Antibiosis *in vitro*

Antibiotic activity of the seven test organisms against *M. vaccinii-corymbosi* was evaluated by measuring zones of inhibition in dual cultures (Baker and Cook 1974). PDA-containing Petri dishes (10 cm diameter) were inoculated with 3-mm-diameter mycelial plugs of the pathogen placed 2 cm from the edge of the dish (one plug per dish). The cultures were allowed to grow for 1 week at 23ºC in the dark. Radial growth of *M. vaccinii-corymbosi* was then measured with a ruler (in mm) and recorded.

After the initial 1-week growth period of the pathogen, fresh colonies of the bacteria or yeast growing on nutrient yeast dextrose (NYD) agar were applied to the culture dishes as a streak in a single line across the surface of the agar at a distance of about 3 cm from the *M. vaccinii-corymbosi* colony. At the same time, the fungal species were applied as culture filtrates to elongated wells (approximately the same length as the bacterial and yeast streaks) that had been cut out of the agar 3 cm from the *M. vaccinii-corymbosi* colony. The culture filtrate was prepared by growing *T. harzianum* and *G. roseum* in potato dextrose broth for 2 and 4 days, respectively. Conidia and mycelia were filtered out of the liquid culture using vacuum and syringe filters (0.2 μm pore size). The culture filtrate in the wells was replenished twice during the subsequent 10-day incubation period of the dual culture dishes. Six dishes were prepared for each test organism, eight additional dishes served as controls, and the entire experiment was repeated three times.

Following co-inoculation with the test organisms, dual-culture dishes were incubated at 23^{o}C in the dark. Radial growth of *M. vaccinii-corymbosi* was measured 7 and 10 days after co-inoculation and converted to a growth rate (mm/day) after subtraction of the initial growth that occurred during the previous 1-week incubation of *M. vaccinii-corymbosi*. Data then were analyzed by one-way ANOVA for a randomized complete block design using the three experimental runs as replicates. Treatment means were compared with Tukey's studentized range test ($\alpha = 0.05$).

3.3 Niche overlap *in vitro*

To determine the potential for nutrient competition between the seven test organisms and *M. vaccinii-corymbosi*, nutritional niche overlap was assessed using 96-well Biolog microplates

(Biolog, Hayward, CA). Each well in these plates contains a different carbon source/ amino acid that would either be utilized by the organism or not. A large overlap in the carbon source/ amino acid utilization profiles between a test organism and the pathogen indicates greater potential for nutrient competition (Wilson and Lindow 1994a, b).

All filamentous fungi, which included *M. vaccinii-corymbosi* as well as *T. harzianum* and *G. roseum*, were grown at 23ºC on oat bran agar (20 g of oat bran and 10 g agar in 750 ml of deionized water) until a sporulating culture resulted (4 days for the two test fungi and up to 3 weeks for *M. vaccinii-corymbosi*). Conidia were harvested by rubbing a moistened cotton swab across the surface of the fungal growth. A conidial suspension having 60% transmittance in a spectrophotometer (λ = 590 nm) was prepared in 13.5 ml of sterile gel-forming colloid, 0.2% Carageenan Type II (Sigma Chemical Co., St. Louis, MO) and then diluted ten-fold. Biolog SF-P2 microplates (not containing tetrazolium dye that can be toxic to fungi) were inoculated with 100 µl per well of the diluted conidial suspension. Microplates were incubated at 23ºC in a moist chamber, monitored every 24 hours for growth based on turbidity (or presence of mycelium and/or sporulation), and given a positive or negative score for each well based on a comparison with the water control well. Assessments were carried out for 1 to 5 days with the two test fungi or until a stable pattern had formed (up to 4½ weeks) for the slow-growing *M. vaccinii-corymbosi*. There was one microplate per organism, and the experiment was repeated four times. Nutrient wells showing growth in at least three of the four experimental runs were considered positive.

Cultures of the test bacteria and yeast were grown on BUG agar (Biolog) amended with 0.25% maltose at 23ºC. Sodium thioglycolate (7.66%) was added to the culture dishes prior to inoculation to prevent the *Bacillus* spp. from forming pellicles, skin-like clumps that make it

difficult to prepare a uniform cell suspension. After 24 to 48 hours of growth, the test organisms were swabbed from the agar surface using a sterile, moistened cotton swab and were suspended in a 0.85% NaCl inoculating fluid containing 7.66% sodium thioglycolate at manufacturer-specified transmittance values in a spectrophotometer (λ = 590 nm): 52% for *P. agglomerans*, 28% for all *Bacillus* spp., and 47% for *W. australiensis*. Then, 150 µl of the suspension was inoculated into GP2 microplates for all bacteria, GN2 microplates for the Gram-negative *P. agglomerans*, and SF-P2 microplates for *W. australiensis*. The plates were incubated at 23ºC for 4 to 48 hours, after which growth was scored as positive or negative based on color change in the wells. There was one microplate per organism, and the experiment was repeated four times. Nutrient wells showing a color reaction in at least three of the four experimental runs were considered positive.

Niche overlap indices (NOIs) were calculated for each test organism based on the number of carbon/ nitrogen sources utilized by the organism that were also utilized by *M. vaccinii-corymbosi*, divided by the total number of nutrient sources utilized by *M. vaccinii-corymbosi* (Wilson and Lindow 1994a, b).

3.4 Stigmatic colonization of blueberry flowers

Four- to five-year-old rabbiteye blueberry (*Vaccinium ashei* 'Tifblue') plants were maintained in 11.4-liter pots in a greenhouse (24 to 28°C) following vernalization in a cold room (5 to 6°C) during the winter. Individual flower buds were monitored daily, and flowers were marked the day they opened. These flowers were detached 1 day later and placed (stigma facing upward) in 96-well microtiter plates containing 150 µl of sterile deionized water per well.

For the test bacteria and yeast, culture tubes containing 12 ml of NYD broth were inoculated and, after 18 to 24 hours, the broth cultures for each organism were harvested by centrifugation (7 min at 4500 rpm). This time point was well before cells of *B. subtilis* reach the stationary phase prior to spore formation (Clifton 1957, Branda et al. 2001). The resulting pellet was washed in potassium phosphate buffer (0.01M) containing Tween 20, centrifuged, the supernatant discarded, and the pellet washed again in buffer. Cell suspensions were adjusted to 0.3 absorbance using a spectrophotometer (λ = 590 nm) and diluted ten-fold for *P. agglomerans* or five-fold for *B. mojavensis*; suspensions of *B. subtilis, B. mycoides,* and *W. australiensis* remained undiluted. The suspensions were applied under a dissecting microscope to individual flower stigmas as 0.5-µl droplets using a Hamilton microsyringe (Hamilton, Reno, NV). Each test organism was applied to 16 flowers. Controls were made by adding a 0.5-µl droplet of sterile deionized water to the stigma. The microtiter plates containing the flowers were incubated in a moist chamber at 23ºC under 12 h light for 0, 1, 2, or 4 days. On each of these days, four flowers were removed from the moist chamber and assayed for population densities of the test organisms. The corollas of the flowers were removed and the styles excised and placed, in groups of two, in sterile microcentrifuge tubes with 1 ml potassium phosphate buffer (0.01M) containing Tween 20. The tubes were placed in a sonicating bath for 1 min, followed by vortexing for 30 sec to resuspend the organisms. Aliquots (0.1 ml) of the suspensions were dilution-plated in triplicate on NYD agar. Dishes were incubated at 23ºC and colonies counted after 18 to 36 hours. Colony counts were converted to population densities (colony-forming units, CFU) per stigma based on the most appropriate dilution for each organism. The experiment was repeated four times.

The theoretical detection limit of the dilution-plating technique used in this study was between 1 and 2 CFU per stigma. The practical detection limit, which will be higher due to imperfect removal of cells from the stigma and cell viability <100%, was not determined.

The procedure to determine the suitability of the stigmatic surface as a niche for growth of *T. harzianum* and *G. roseum* was different from that used for the bacteria and yeast. Twelve flowers per experimental run were inoculated with each of the two fungi using a microsyringe to apply 0.5-µl droplets of conidial suspension (approx. 1×10^6 per ml), similar to the procedure described previously for the bacteria and yeast. On day 0 (i.e., immediately following application of the inoculum droplet), four styles per organism were washed, vortexed, sonicated, and dilution-plated as described above, except that PDA was used for plating instead of NYD agar. Single fungal colonies were counted 3 to 4 days after plating, and the data were used as a baseline to determine how many viable conidia were applied per stigma. On days 2 and 4, four flowers each per organism were sampled for microscopic examination of the colonization of stigma and style. Externally, the length of the stylar necrosis was measured under a dissecting microscope relative to a water control. To determine internal colonization of the style, pistils were fixed, cleared, and stained for fluorescence microscopy as described by Ngugi et al. (2002), and the distance from the stigmatic surface to the hyphal front within the stylar canal was measured using an ocular micrometer. Four separate experimental runs were conducted for the day-0 experiment, while three were carried out for each of the assessments on days 2 and 4. Means and standard errors were calculated across runs.

A separate set of experiments was carried out to determine the effect of the stigmatic exudate on conidial germination of *T. harzianum* and *G. roseum*. First, flowers were marked and collected from greenhouse-grown plants as described above. On the day of collection, flowers

were stripped of their corollas and the nectar was absorbed using a Kimwipe tissue. Pistils were placed with their stigmas facing downward in microtiter wells containing 200 µl of sterile deionized water. Four pistils were placed into each well and shaken to remove the exudate off of the stigma. With their tips still submerged in the microtiter wells, pistils were placed in a vacuum for 10 min to extract additional exudate. This was repeated until a total of 12 pistils had been placed in each of the wells. A total of 3 ml of this aqueous suspension of exudate was collected and stored at −80°C in sterile microcentrifuge tubes until use. Conidia were harvested from sporulating cultures of *T. harzianum* and *G. roseum* in sterile deionized water, and conidial suspensions were adjusted to a density of 5×10^4 per ml. Fifty microliters of these suspensions were added to 200 µl of exudate, and 25-µl drops of the mixture were placed into the wells of depression slides and covered with a cover slip. For each of the two test fungi, four slides were prepared with conidia in either exudate or sterile deionized water (control). After 15 and 12 hours for *T. harzianum* and *G. roseum*, respectively, germination was assessed microscopically, with a conidium considered germinated when the germ tube length had reached approximately twice the width of the conidium. At least 120 conidia per drop were assessed to determine percent germination. The experiment was repeated four times, and percentage germination between exudate and water were compared with *t*-tests for each of the two fungi using the four experimental runs as replicates.

3.5 Effect against floral infection by *M. vaccinii-corymbosi*

Plants were maintained in a greenhouse as described above for the stigmatic colonization study. Individual flowers were monitored and detached 1 day after anthesis. They were placed with the stigma facing upward in 96-well microtiter plates containing 150 µl of sterile deionized

water. On the same day, flowers were inoculated with conidia of *M. vaccinii-corymbosi* taken from the edge of a sporulating culture grown on half-strength oat bran agar for 2 to 3 weeks using a dry-inoculation procedure (Ngugi et al. 2002). Immediately thereafter, the test organisms were applied in a 0.5-µl droplet of either potassium phosphate buffer (0.01M) containing Tween 20 for the bacteria and yeast or sterile deionized water for the filamentous fungi as described for the stigmatic colonization study above. This was done on nine flowers for each test organism (and a water control), which were used subsequently for microscopic determination of hyphal ingress of *M. vaccinii-corymbosi* into the stylar canal. After 4 days, the corollas were removed and the styles fixed, cleared, and stained with decolorized aniline blue as described previously (Ngugi et al. 2002). Using a fluorescence microscope, the number of hyphae penetrating at least 1/5 down the style was recorded. In addition, the lengths of the eight longest hyphae were recorded for each style and converted to hyphal growth rates (mm/day). Data were obtained from two separate experiments, in each of which hyphal numbers and growth rates were expressed as percent reduction compared with the water control. Separately for the two experimental runs, data were subjected to one-way ANOVA for a completely randomized design using individual flowers as replications. Means among the treatments were compared with Tukey's studentized range test ($\alpha = 0.05$).

Table 3.1. List of test organisms evaluated as potential biocontrol agents against infection of blueberry flowers by *Monilinia vaccinii-corymbosi*.

Organism	Strain	Source	Comments
Bacillus subtilis	QRD137	Serenade WP, AgraQuest, Davis, CA	Highly effective against secondary infection by *M. vaccinii-corymbosi*, but limited epiphytic fitness on flower stigmas (Scherm et al. 2004); used as a standard in this study
Bacillus mycoides	7IIC4	Isolated from blueberry flowers in Alma, GA (2003)	Strains of this species induce systemic acquired resistance in sugar beet leaves (Bargabus et al. 2002)
Bacillus mojavensis	RRC101	Dr. C.W. Bacon, USDA-ARS, Athens, GA	Endophyte in maize; antibiotic activity against most fungi (Bacon et al. 2001)
Pantoea agglomerans	C9-1S	Dr. K.B. Johnson, Oregon State University, Corvallis, OR	Epiphytic fitness on flower stigmas of pome fruits; antibiosis (Ishimaru et al. 1987; Stockwell et al. 2002) and competition (Pusey 1999)
Wickerhamiella australiensis	Y-27360	USDA-ARS MWA NCAUR, Peoria, IL	Saprophytic, lipophylic yeast (Lachance 1998)
Trichoderma harzianum	KRL-AG2	PlantShield HC, BioWorks, Geneva, NY	Mycoparasitism (Weindling 1932; Chet 1987), antibiosis (Howell et al. 1993), competition (Harman 2000), production of enzymes causing cell lysis (Mecalf and Wilson 2001), and induced host resistance (Yedidia et al. 1999)
Gliocladium roseum = *Clonostachys rosea* (Schroers et al. 1999)		Dr. R.T. Hanlin, UGA	Mycoparasitism and competition (Yu and Sutton 1999)

CHAPTER 4

RESULTS

4.1 Antibiosis *in vitro*

ANOVA revealed a highly significant reduction in radial growth of *M. vaccinii-corymbosi* due to the antagonist treatments for both the 7-day ($P < 0.0001$) and 10-day ($P = 0.0002$) assessments, whereas there were no significant differences among experimental runs ($P > 0.05$) (Table 4.1). At both assessment dates, *B. subtilis*, *B. mojavensis*, and *W. australiensis* resulted in growth rates of *M. vaccinii-corymbosi* that were significantly lower than the water control, with *B. subtilis* consistently showing the strongest pathogen suppression (Fig. 4.1). Antibiotic activities of *B. mycoides* and *P. agglomeran*s were intermediate, whereas the culture filtrates of *T. harzianum* and *G. roseum* resulted in no pathogen growth reduction compared with the water control.

In addition to reducing the growth rate of the pathogen, *B. subtilis* and *B. mojavensis* caused necrosis of the mycelial front of *M. vaccinii-corymbosi* (Fig. 4.2). *Pantoea agglomerans* also caused a slight necrosis of the mycelia but failed to stop colony growth.

4.2 Niche overlap *in vitro*

Monilinia vaccinii-corymbosi utilized 38 of the carbon and nitrogen sources in the 96-well Biolog microplates (see Appendix). Wells were rated positive when they contained mycelial strands of the pathogen. *Trichoderma harzianum* and *G. roseum* were less fastidious, utilizing 95 and 96 of the 96 nutrient sources, respectively, with L-rhamnose being the only carbon source

not utilized by *T. harzianum*. In contrast to *M. vaccinii-corymbosi*, both test fungi grew extensively in the wells, producing abundant mycelia and eventually conidiophores and conidia within 4 to 5 days after inoculation. *Bacillus subtilis*, *B. mycoides*, *B. mojavensis*, *P. agglomerans*, and *W. australiensis* utilized 40, 38, 50, 40, and 96 of the 96 nutrient sources, respectively.

Wickerhamiella australiensis, *T. harzianum*, and *G. roseum* had NOI values of 1.0 relative to *M. vaccinii-corymbosi*, meaning that they shared all 38 nutrient sources utilized by the pathogen. Nutritional overlap and, by extension, potential for nutrient competition was lower for *B. subtilis* (NOI = 0.605), *B. mycoides* (NOI = 0.395), *B. mojavensis* (NOI = 0.684), and *P. agglomerans* (NOI = 0.474).

4.3 Stigmatic colonization of blueberry flowers

Population densities of *B. subtilis* and *B. mycoides* consistently dropped to undetectable levels (or very close to it) within 4 days after application (Fig. 4.3A), indicating that the blueberry stigma is unsuitable for population growth by these organisms. Results for *B. mojavensis* were somewhat inconsistent in that there was an initial population increase (to an average population density of 2.1×10^4 CFU/stigma) in two experimental runs which was not observed in the other two runs (Fig. 4.3B). Nonetheless, populations of this *Bacillus* species also declined to undetectable levels within 4 days after application.

Both *P. agglomerans* and *W. australiensis* had high epiphytic fitness on the flower stigma, reaching peak population densities of 3.4×10^5 and 2.2×10^4 CFU/stigma within 2 days after application, respectively (Fig. 4.3C). Both organisms still had population densities above 10^4 CFU/stigma 4 days after application.

Based on the results of dilution-plating immediately following application, 190.4 ± 35.8 and 330.4 ± 40.0 conidia were applied per stigma for *T. harzianum* and *G. roseum*, respectively. Conidia of both fungi readily germinated in the stigmatic exudate, as documented in the depression-slide experiments (Fig. 4.4). In fact, for *T. harzianum,* there was evidence for significantly ($P = 0.0101$) enhanced germination in exudate compared with deionized water, whereas there was no such difference for *G. roseum* ($P = 0.2453$).

When blueberry stigmas were treated with the two test fungi and assessed microscopically after 2 and 4 days, enhanced stylar necrosis was noted compared with the water control (Fig. 4.5, 4.6). This effect was statistically significant for the comparison between *T. harzianum* and water at the 4-day assessment ($P = 0.0254$). Internally, there was no fungal colonization of the styles by the antagonists 2 days after inoculation. At the 4-day assessment, however, 5 out of 21 styles treated with *T. harzianum* showed hyphal growth of that species (Fig. 4.7A). The corresponding value for *G. roseum* was 11 out of 29 styles (Fig. 4.7B). Growth appeared as a front of mycelium within the stylar canal.

4.4 Effect against floral infection by *M. vaccinii-corymbosi*

In both trials, ANOVA revealed significant effects of the test organisms in reducing the numbers of hyphae of *M. vaccinii-corymbosi* penetrating into the stylar canal as well as the growth rates of these hyphae (Table 4.2). In terms of hyphal numbers, the strongest pathogen suppression was observed for *B. subtilis* (reduction to about one-fifth of the water control) and *G. roseum* (reduction to less than one-half of the control) (Fig. 4.8A). *Pantoea agglomerans* and *T. harzianum* reduced pathogen penetration in the second but not the first trial, whereas the remaining three organisms (*B. mycoides*, *B. mojavensis*, and *W. australiensis*) showed

consistently weak effects. Data on hyphal growth rates of *M. vaccinii-corymbosi* in the stylar canal mirrored those of the hyphal numbers (Fig. 4.8B).

Table 4.1. Results of analyses of variance to determine the effects of seven test organisms[a] in reducing the radial growth rate of *Monilinia vaccinii-corymbosi* on potato-dextrose agar in the laboratory[b].

		Assessment after 7 days			Assessment after 10 days		
Source	df	Mean Square	*F*	*P>F*	Mean Square	*F*	*P>F*
Organism	7	1.090	24.37	<0.0001	1.055	8.69	0.0002
Replication	3	0.125	2.80	0.0734	0.283	2.33	0.1125
Error	16	0.045	---	---	0.121	---	---

[a]*Bacillus subtilis*, *B. mycoides*, *B. mojavensis*, *Pantoea agglomerans*, *Wickerhamiella australiensis*, *Trichoderma harzianum*, and *Gliocladium roseum* were tested along with an untreated control.

[b]*Monilinia vaccinii-corymbosi* was grown for 1 week before the test organisms were either streaked (bacteria and yeast) or applied as a culture filtrate to elongated wells (fungi) onto the medium distally from the pathogen colony.

Table 4.2. Results of analyses of variance to determine the effects of seven test organisms[a] in reducing hyphal penetration of *Monilinia vaccinii-corymbosi* into styles of detached blueberry flowers[b].

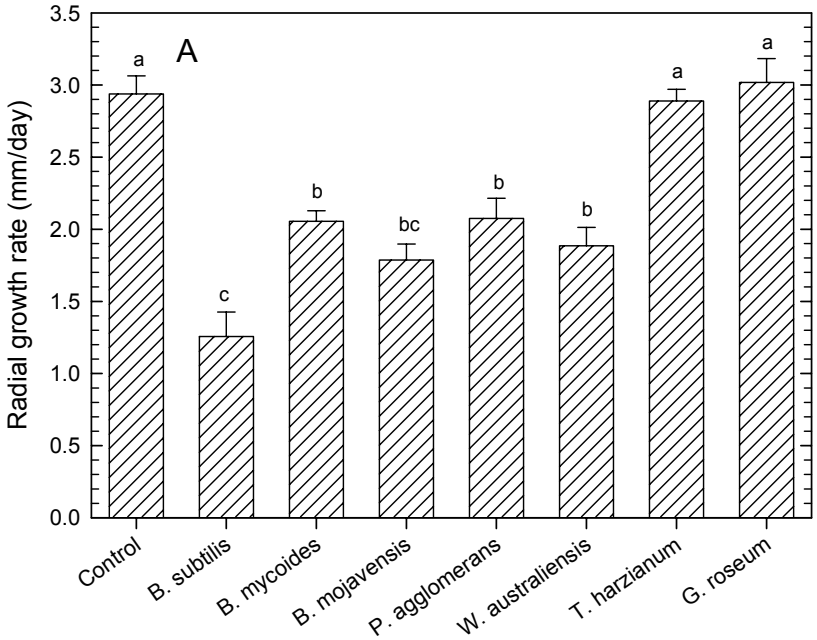
		Number of hyphae per style[c]			Hyphal growth rate[c]		
Source	df	Mean Square	*F*	*P>F*	Mean Square	*F*	*P>F*
Run 1:							
Organism	6	4488.7	3.71	0.0050	4500.6	7.55	<0.0001
Error	40	1208.6	---	---	596.4	---	---
Run 2:							
Organism	6	5635.7	5.83	0.0001	5216.3	4.08	0.0021
Error	49	966.4	---	---	1278.3	---	---

[a]*Bacillus subtilis*, *B. mycoides*, *B. mojavensis*, *Pantoea agglomerans*, *Wickerhamiella australiensis*, *Trichoderma harzianum*, and *Gliocladium roseum*.

[b]Flower stigmas were inoculated with *M. vaccinii-corymbosi* 1 day after they opened, followed immediately by application of the test organisms. Styles were fixed for microscopic examination of pathogen ingress 4 days after inoculation.

[c]For both variables, values were expressed as percentage of the water control.

Fig. 4.1. Radial growth rate of *Monilinia vaccinii-corymbosi* on potato-dextrose agar in the presence of seven test organisms evaluated as potential biocontrol agents. *Monilinia vaccinii-corymbosi* was grown for 1 week before the test organisms were either streaked (bacteria and yeast) or applied as a culture filtrate to elongated wells (fungi) onto the medium distally from the fungal colony. Pathogen growth was measured 1 week (**A**) and 10 days later (**B**). Values are means and standard errors of eight culture dishes per treatment. Means followed by the same letters are not significantly different from each other based on Tukey's studentized range test ($\alpha = 0.05$).


A
Radial growth rate (mm/day)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
c
b
bc
b
b
a
a
Control
B. subtilis
B. mycoides
B. mojavensis
P. agglomerans
W. australiensis
T. harzianum
G. roseum

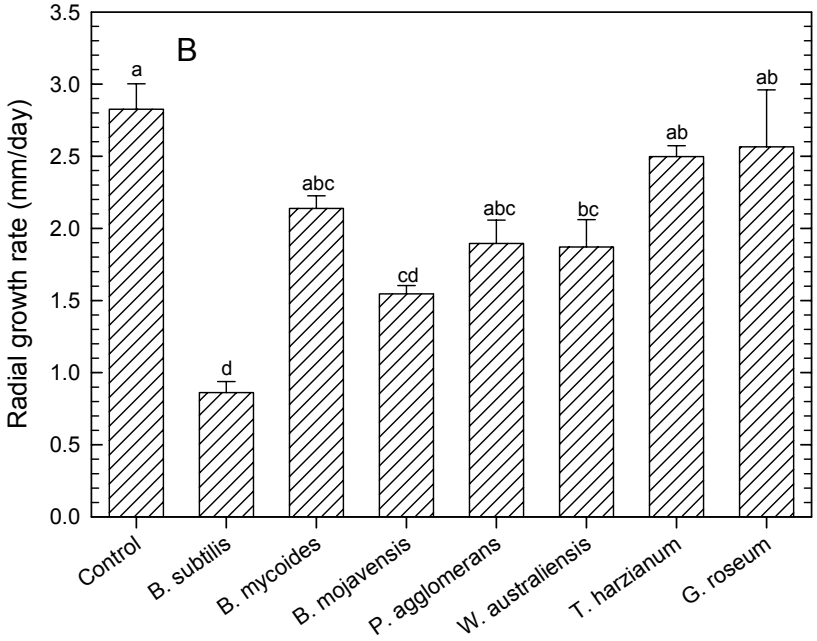

B
Radial growth rate (mm/day)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
d
abc
cd
abc
bc
ab
ab
Control
B. subtilis
B. mycoides
B. mojavensis
P. agglomerans
W. australiensis
T. harzianum
G. roseum

Fig. 4.2. Images showing interactions between *Monilinia vaccinii-corymbosi* (left) and each of the seven test organisms evaluated as potential biocontrol agents (right) 10 days after co-inoculation on dual-culture plates on which *M. vaccinii-corymbosi* had been growing for 7 days previously. (**A**) Control, (**B**) *Bacillus subtilis*, (**C**) *B. mycoides*, (**D**) *B. mojavensis*, (**E**) *Pantoea agglomerans*, (**F**) *Wickerhamiella australiensis*, (**G**) *Trichoderma harzianum*, and (**H**) *Gliocladium roseum*. Test organisms were either streaked (bacteria and yeast) or applied as a culture filtrate to elongated wells (fungi) onto the medium distally from the pathogen colony.

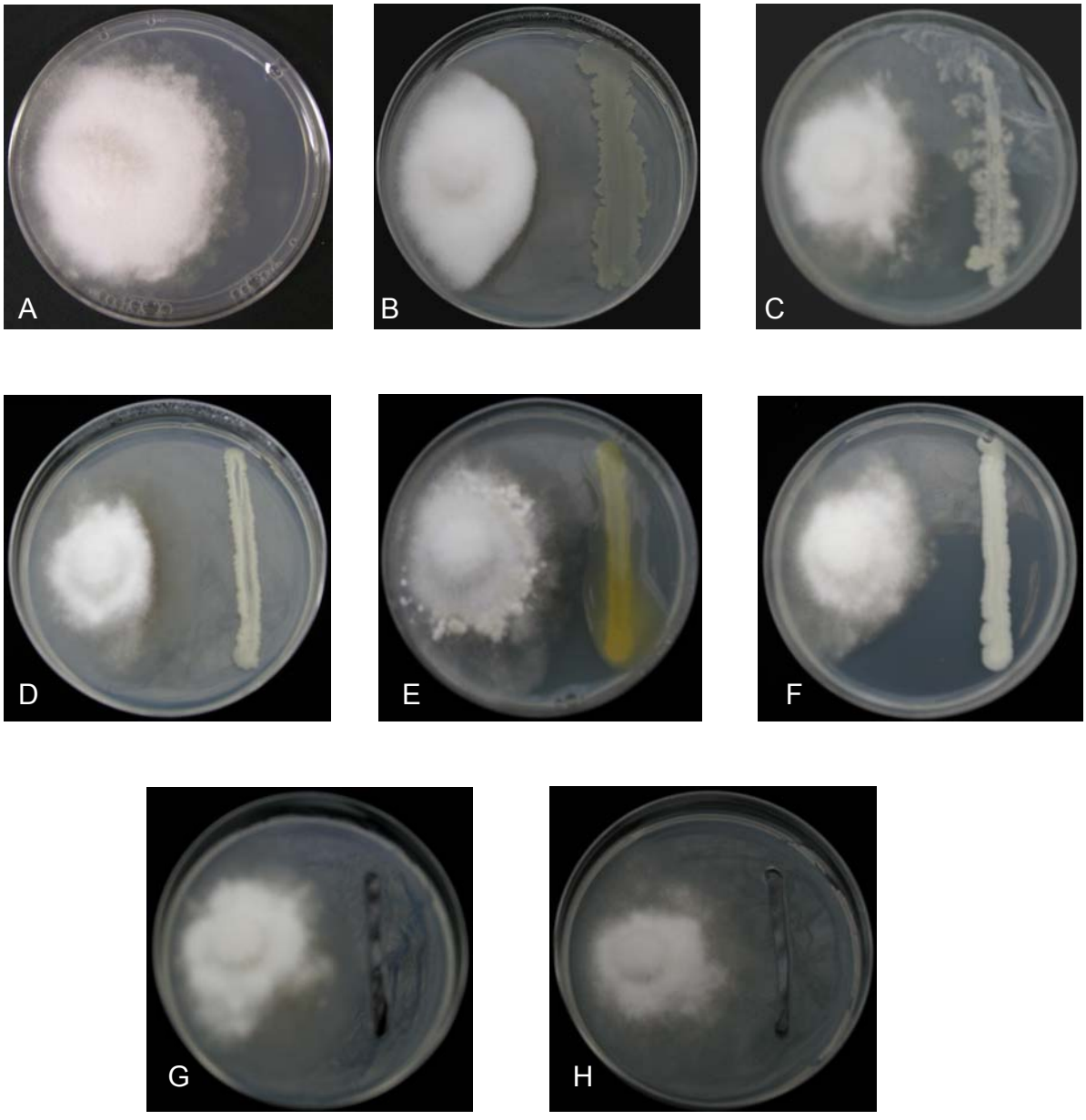

A
B
C
D
E
F
G
H

Fig. 4.3. Population dynamics of five test organisms following their application to the stigmatic surface of detached blueberry flowers 1 day after the flowers opened. Values are means and standard errors of four flowers per sampling date, processed in groups of two. Note logarithmic scale in **C**. CFU = colony-forming units.

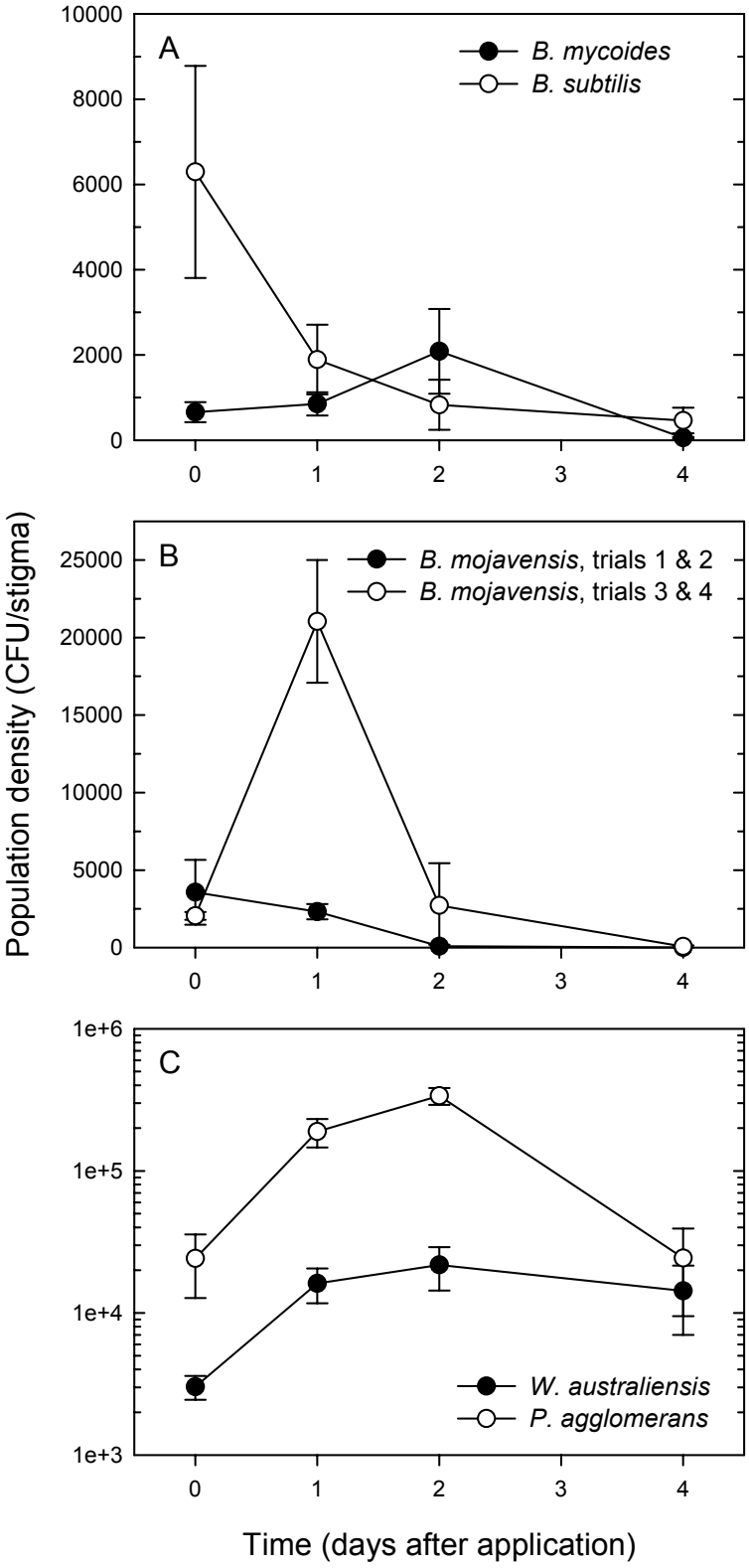

A
B. mycoides
B. subtilis
B
B. mojavensis, trials 1 & 2
B. mojavensis, trials 3 & 4
C
W. australiensis
P. agglomerans
Population density (CFU/stigma)
Time (days after application)

Fig. 4.4. Conidial germination of *Trichoderma harzianum* and *Gliocladium roseum* after 15 and 12 hours, respectively, of incubation in water versus blueberry stigmatic exudate. Values are means and standard errors of four replications. Means followed by the same upper- or lowercase letters are not significantly different from each other based on *t*-tests ($\alpha = 0.05$).

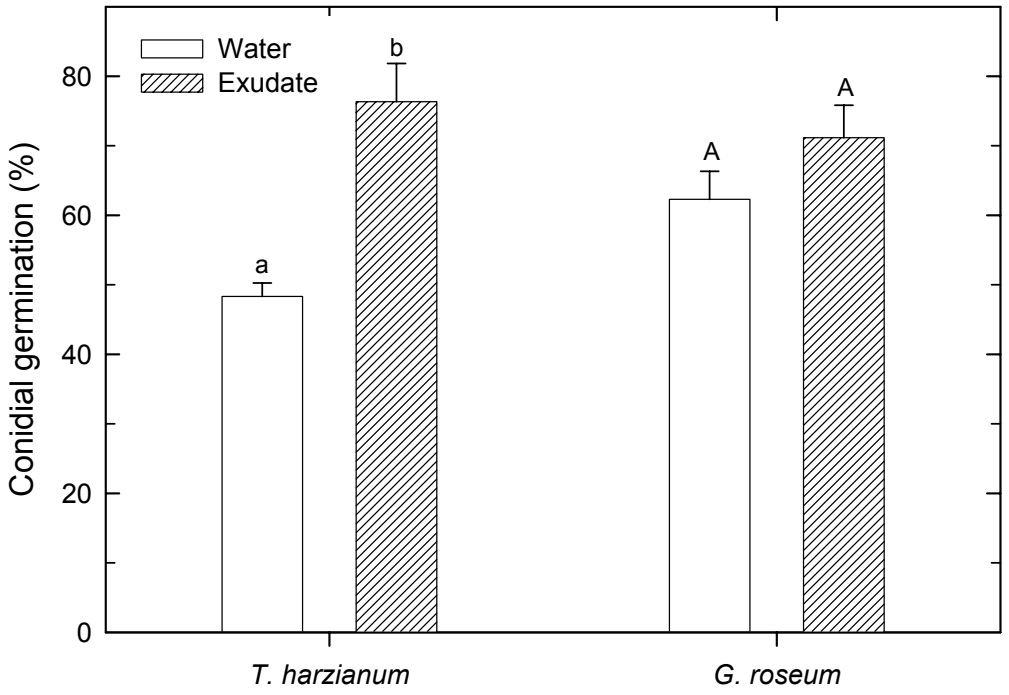

Water
Exudate
Conidial germination (%)
0
20
40
60
80
a
b
A
A
T. harzianum
G. roseum

Fig. 4.5. External necrosis on detached blueberry styles following application of *Trichoderma harzianum* or *Gliocladium roseum* to the stigmatic surface. Values are means and standard errors of ten styles per treatment. Means followed by the same letters are not significantly different from each other based on Tukey's studentized range test ($\alpha = 0.05$).

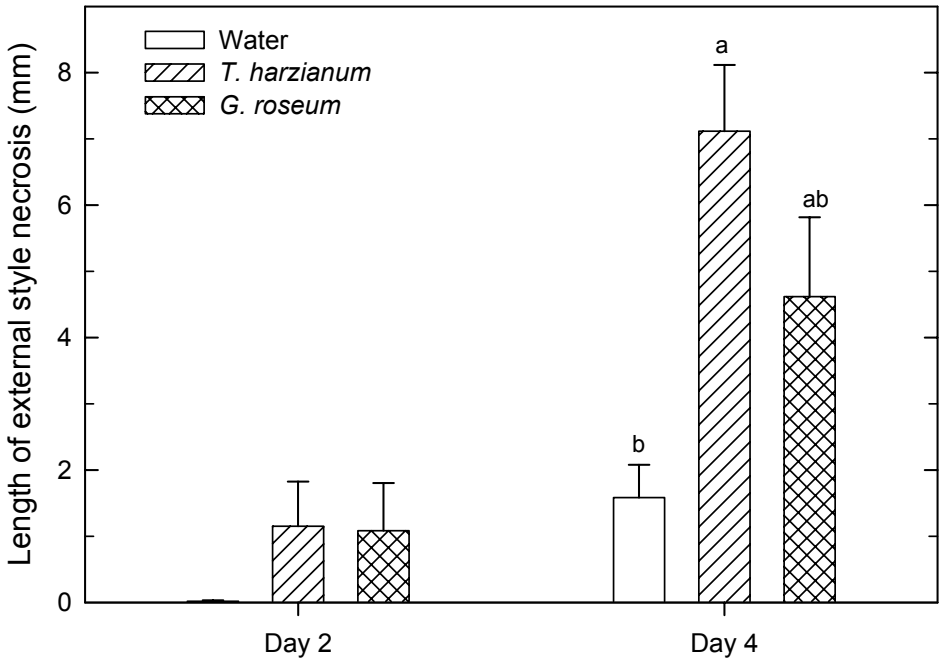

Water
T. harzianum
G. roseum
Length of external style necrosis (mm)
0
2
4
6
8
b
a
ab
Day 2
Day 4

Fig. 4.6. Examples of external style necrosis and stigmatic colonization 4 days after application of *Trichoderma harzianum* (**A**, **B**) and *Gliocladium roseum* (**C, D**) to the stigmas of detached blueberry flowers. Scale bar = 1 mm.

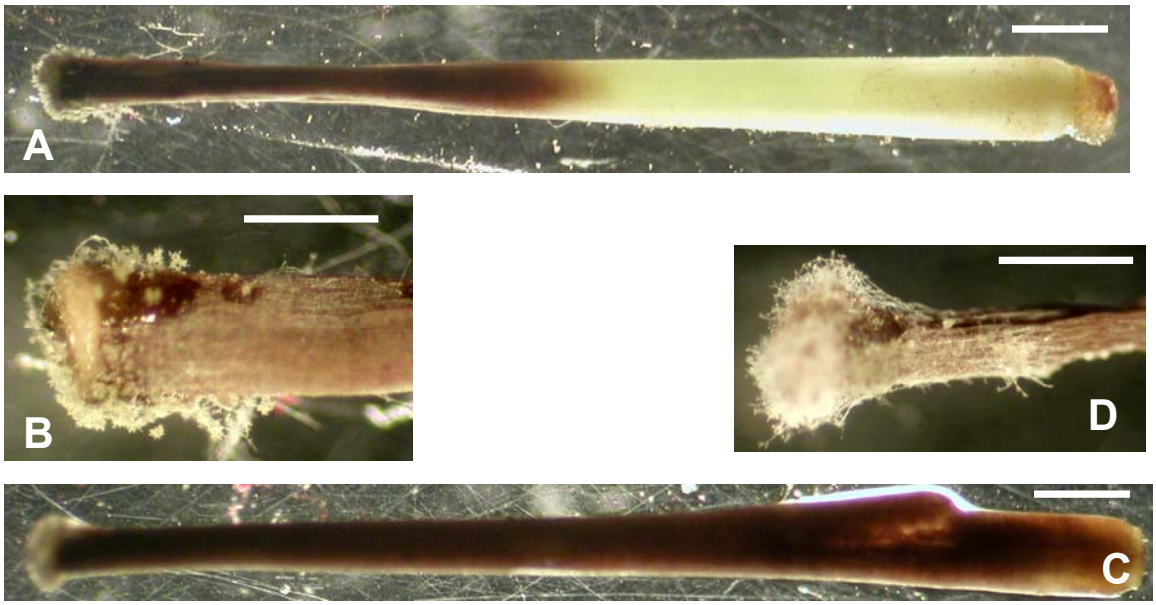
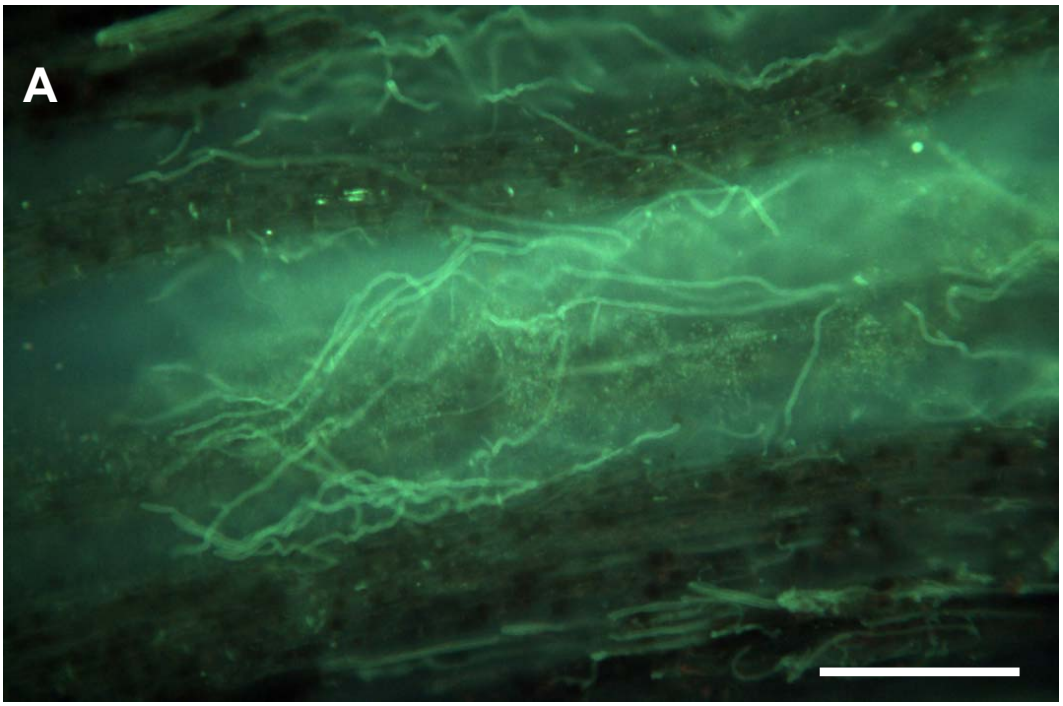

A
B
D
C

Fig. 4.7. Hyphal growth of *Trichoderma harzianum* (**A**) and *Gliocladium roseum* (**B**) in the stylar canal of detached blueberry flowers 4 days after application to the stigmatic surface. Scale bar = 100 μm.


A

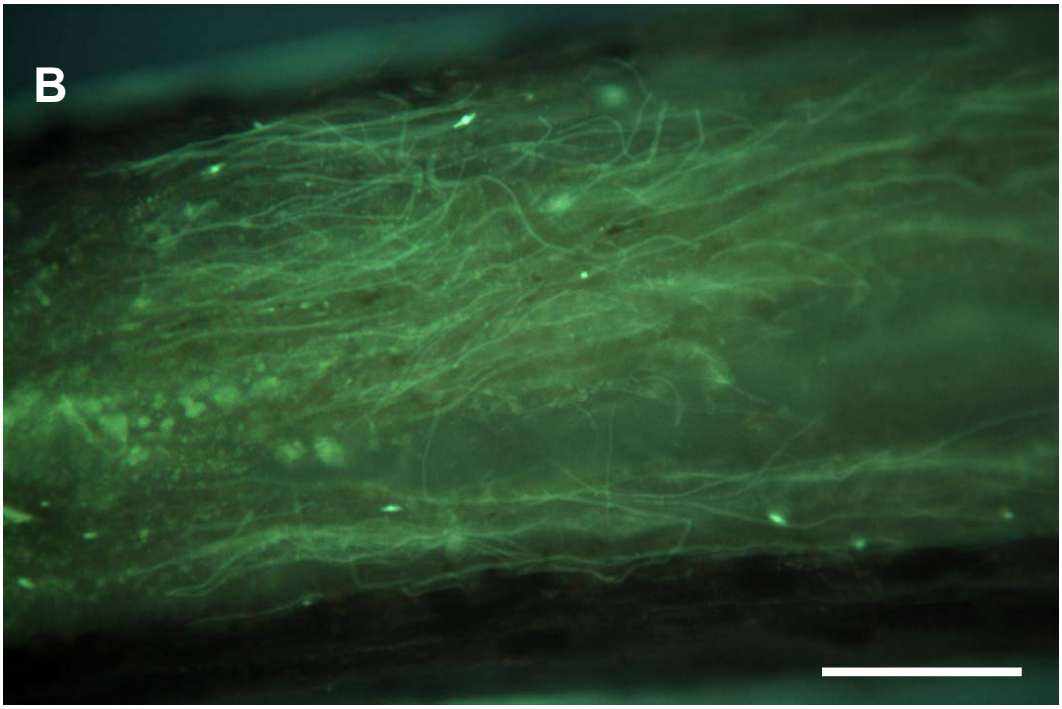
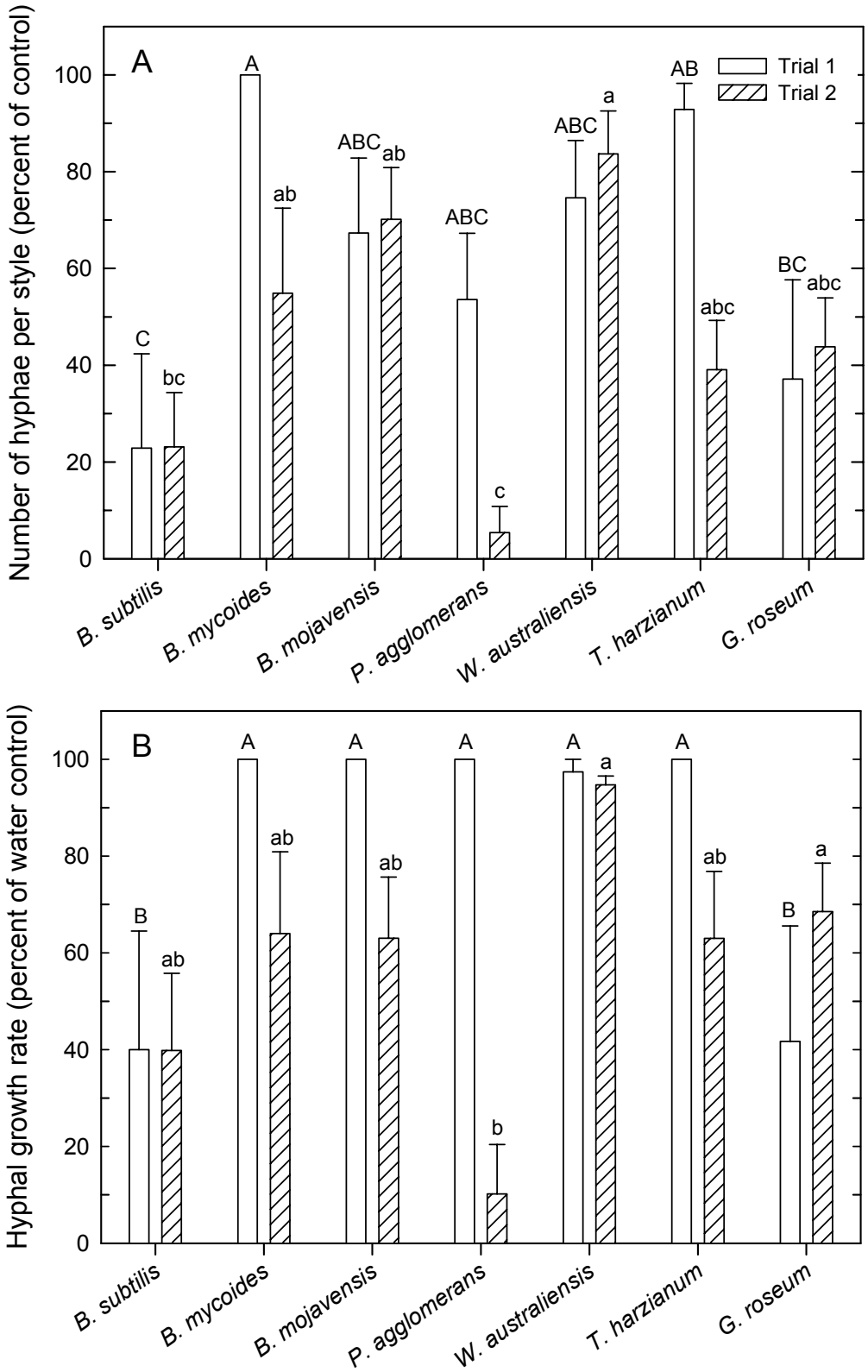

B

Fig. 4.8. Effect of seven test organisms on hyphal ingress of *Monilinia vaccinii-corymbosi* into styles of detached blueberry flowers, expressed as percentage of the water control. Flower stigmas were inoculated with *M. vaccinii-corymbosi* 1 day after they opened, followed immediately by application of the test organisms. Fungal ingress was assessed as number of hyphae per style (**A**) or hyphal growth rate through the style (**B**). Values are means and standard errors of nine styles per treatment. Means followed by the same letters are not significantly different from each other based on Tukey's studentized range test ($\alpha = 0.05$). For the water control, average hyphal numbers were 7.0 (Trial 1) and 10.6 (Trial 2) per style, and average hyphal growth rates were 1.1 (Trial 1) and 1.8 (Trial 2) mm/day.


A
Number of hyphae per style (percent of control)
Trial 1
Trial 2
0
20
40
60
80
100
C
bc
A
ab
ABC
ab
ABC
c
ABC
a
AB
abc
BC
abc
B. subtilis
B. mycoides
B. mojavensis
P. agglomerans
W. australiensis
T. harzianum
G. roseum
B
Hyphal growth rate (percent of water control)
0
20
40
60
80
100
B
ab
A
ab
A
ab
A
b
A
a
A
ab
B
a
B. subtilis
B. mycoides
B. mojavensis
P. agglomerans
W. australiensis
T. harzianum
G. roseum

CHAPTER 5

DISCUSSION

This study showed that *Bacillus subtilis* QRD137 continues to be the most promising candidate for control of blueberry flower infection by *Monilinia vaccinii-corymbosi*. Among a diverse panel of potential biocontrol organisms evaluated in this study, this bacterium showed the strongest antibiotic activity against the pathogen *in vitro* and, most importantly, suppressed pathogen ingress into the pistil significantly and consistently. The relatively high NOI for *B. subtilis* further suggests that the antagonist may compete with *M. vaccinii-corymbosi* for nutrients and/ or space on the stigmatic surface, in addition to having antibiotic activity. Unfortunately, similar to all other *Bacillus* species evaluated in this study, *B. subtilis* had limited epiphytic fitness on the stigmatic surface, with populations dropping to undetectable levels within 2 to 4 days after application.

The observation of unfavorable population dynamics of *B. subtilis* on flower stigmas is consistent with previous reports from blueberry (Scherm et al. 2004) and pome fruits (Laux and Zeller 2002; Zeller and Laux 2001). The fact that individual blueberry flowers are susceptible to infection by *M. vaccinii-corymbosi* for less than 5 days after they open (Ngugi et al. 2002a) would suggest that a prolonged epiphytic phase on the stigma is not needed for a biocontrol agent to be effective. However, without population increase on the stigma, there will be very limited opportunity for secondary spread of the biocontrol organism from treated flowers to newly opened flowers by pollinators. Such flower-to-flower spread following application has been important for improving bacterial biocontrol efficacy against fire blight of pear (Nuclo et al.

1998), another pathosystem in which the stigmatic surface is the target of biocontrol. Without secondary spread of *B. subtilis*, frequent applications during the 3 to 4-week bloom period of blueberry would be needed to ensure that newly opened flowers are protected continuously. As an alternative to frequent spray applications, Dedej et al. (2004) investigated the use of honey bees to vector commercially formulated *B. subtilis* from bee hives to blueberry flowers, with the goal of achieving continuous delivery of the antagonist to flowers as soon as they open. However, this approach was only partly successful due to certain aspects of honey bee behavior in blueberry, especially their tendency to engage in nectar-robbing through cuts in the flower corolla made previously by carpenter bees (Dedej and Delaplane 2004); this results in flower visits without stigmatic contact, presumably without transmission of the biocontrol agent to the infection court of *M. vaccinii-corymbosi*.

Bacillus subtilis is primarily a soil bacterium; as such, it is inherently incapable of the rapid epiphytic population growth exhibited by typically plant-associated bacteria such as *Pseudomonas fluorescens* or *Pantoea agglomerans*. Nonetheless, it may be possible to improve persistence of *B. subtilis* via the addition of limiting nutrient(s) during application. In the fire blight system, Temple et al. (2004) found that the floral stigma offers an abundant supply of nutrients, but these tended to be short-lived. One of the nutrients limiting growth of bacterial biocontrol agents on floral stigmas was iron, and the addition of iron upon application of the antagonist (either through multiple sprays or through bee vectors) can increase the epiphytic fitness of such organisms (Temple et al. 2004). It currently is unknown whether iron is limiting in the stigmatic exudate of blueberry or to what extent iron supplements can improve the epiphytic fitness of *B. subtilis*.

Of the biocontrol candidates included in this study, *P. agglomerans* showed the most favorable population dynamics on blueberry stigmas, which is consistent with the fact that this bacterium is a common epiphyte of plant surfaces (Thompson et al. 1993). Although the organism showed only limited antibiosis toward and moderate niche overlap with *M. vaccinii-corymbosi*, its application to the flower stigma did result in considerable suppression of pathogen ingress into the pistil in one trial. Future research should seek to determine the cause(s) for the variability in efficacy observed for *P. agglomerans* in this study. On pome fruit blossoms, combination of *P. agglomerans* with other biocontrol agents can reduce variability of biocontrol (Stockwell et al. 2001).

Wickerhamiella australiensis, *Trichoderma harzianum*, and *Gliocladium roseum* all showed complete nutritional overlap with *M. vaccinii-corymbosi* on the Biolog microplates, suggesting the potential for biocontrol via nutrient competition. Of these three test organisms, only *G. roseum* appeared promising in reducing penetration and growth of the pathogen within the stylar canal. Conidia of this species also germinated as well in the exudate as in water, and as evidenced by extensive hyphal growth, *G. roseum* was able to grow on and into the style. Although stylar necrotization following application of *G. roseum* (in the absence of *M. vaccinii-corymbosi*) was not significantly different from the water control, future work with this antagonist should determine the risk, if any, posed by its growth and colonization on and within the style relative to pollination. A similar study has been undertaken recently with *B. subtilis* (Ngugi et al. 2004), for which no negative effect on pollination was found, except in conditions where pollination was already marginal in the field.

Similar to *G. roseum*, *T. harzianum* was able to penetrate into and grow within the blueberry styles. Interestingly, conidia of this fungus germinated better in stigmatic exudate than

in water. The ability of both test fungi to germinate readily in exudate and to penetrate the stylar canal is unusual in light of the results of Jung (1956) who concluded – based on an extensive survey with pathogenic and saprophytic fungi inoculated onto stigmas of a wide range of plants – that the stigmatic exudate has antimicrobial properties based on the failure of a large proportion of the fungi to germinate and/or grow. Apparently, the blueberry exudate lacks such antimicrobial properties. Ngugi and Scherm (2004) showed that *Monilinia fructicola*, a non-pathogen of blueberry, was able to penetrate into the stylar canal of blueberry flowers but failed to reach the ovary where fruit mummification by its relative *M. vaccinii-corymbosi* occurs. Thus, specificity in the mummy berry pathosystem seems to be determined not by the ability to germinate on the stigma and penetrate into the style, but rather by the capacity for directed growth through the stylar canal into the ovary.

Despite having excellent population dynamics on the floral stigma, a moderately antibiotic effect, and a high NOI with *M. vaccinii-corymbosi*, *W. australiensis* did not reduce pathogen penetration into blueberry styles. This yeast was included in our study because it is known to be a flower inhabitant (Lachance et al. 1998). Further, *W. australiensis* is lipophylic, which we considered important in light of the fact that stigmatic exudates generally are lipid-rich (Heslop-Harrison and Shivannah 1977). Larger numbers of yeast isolates and species should be screened to determine whether there are genotypes that combine these favorable attributes with higher efficacy against the target pathogen *M. vaccinii-corymbosi*.

In conclusion, this study highlighted the opportunity and need for additional research on antagonists for the biological control of mummy berry disease. Future work should focus on improving the population dynamics of *B. subtilis*, which for now continues to be the most efficacious antagonist against *M. vaccinii-corymbosi*. On the other hand, the factor(s) leading to

variability in the efficacy of *P. agglomerans* need to be addressed as well. In addition, combinations of potential antagonists on the stigma should be evaluated to determine whether there are additive effects or whether variability in biocontrol can be reduced. Finally, risk assessment studies need to be performed to ensure that there are no negative effects of the antagonists on pollination or fruit set.

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APPENDIX

Nutrient utilization profiles of *Monilinia vaccinii-corymbosi* and seven test organisms evaluated as potential biocontrol agents in Biolog microplates. Assays conducted in GP2 plates for all bacteria, GN2 plates for *Pantoea agglomerans*, and SF-P2 plates for the yeast and filamentous fungi. The experiment was repeated four times, and nutrient wells showing a positive reaction in at least three of the four runs were considered positive.

	Organism[a]							
Nutrient	***M. v.-c.***	***B. subtilis***	***B. myc.***	***B. mojav.***	***P. aggl.***	***W. austr.***	***T. harz.***	***G. ros.***
Water	−	−	−	−	−	+	+	+
α-cyclodextrin	−	−	+	−	−	+	+	+
β-cyclodextrin	−	−	+	−	−	+	+	+
Dextrin	−	+	+	+	+	+	+	+
Glycogen	−	+	+	+	−	+	+	+
Tween 40	+	−	+	−	+	+	+	+
Tween 80	−	−	−	−	+	+	+	+
Inulin	−	−	−	−	−	+	+	+
Mannan	−	−	−	−	−	+	+	+
N-acetyl-D-glucosamine	−	−	+	+	+	+	+	+
L-arabinose	+	−	−	−	+	+	+	+
D-arabitol	+	−	−	−	−	+	+	+
Cellobiose	+	+	−	+	−	+	+	+
N-acetyl-D-mannosamine	−	−	−	−	−	+	+	+
Amygdalin	−	+	−	+	−	+	+	+
D-fructose	+	+	+	+	+	+	+	+
Arbutin	+	+	+	+	+	+	+	+
L-fucose	+	−	−	−	−	+	+	+
D-galactose	+	−	−	+	+	+	+	+
Gentiobiose	+	+	−	+	−	+	+	+
α-D-glucose	+	+	+	+	+	+	+	+
D-galacturonic acid	−	−	−	−	+	+	+	+
D-gluconic acid	−	+	−	+	+	+	+	+
m-inositol	+	+	−	+	+	+	+	+
α-D-lactose	+	−	−	−	−	+	+	+
lactulose	+	−	−	−	−	+	+	+
Maltose	+	+	+	+	+	+	+	+
D-mannitol	+	+	−	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+
Maltotriose	+	+	+	+	+	+	+	+

D-melibiose	+	−	−	−	−	+	+	+
D-melezitose	+	−	−	+	−	+	+	+
β-methyl D-glucoside	+	+	−	+	+	+	+	+
D-psicose	+	+	+	+	−	+	+	+
D-raffinose	+	+	−	+	−	+	+	+
L-rhamnose	−	−	−	−	+	+	−	+
D-sorbitol	+	+	−	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
D-trehalose	+	+	+	+	+	+	+	+
Turanose	+	+	+	+	−	+	+	+
Xylitol	+	−	−	−	−	+	+	+
Methyl pyruvate	+	+	+	+	+	+	+	+
Mono-methyl succinate	−	−	−	−	−	+	+	+
Acetic acid	+	−	+	+	−	+	+	+
α-methyl D-galactoside	−	−	−	−	−	+	+	+
β-methyl D-galactoside	−	−	−	−	−	+	+	+
3-methyl glucose	−	+	−	+	−	+	+	+
α-methyl D-glucoside	+	+	−	+	−	+	+	+
α-methyl D-mannoside	+	−	−	+	−	+	+	+
Palatinose	+	+	+	+	−	+	+	+
α-hydroxybutryic acid	−	−	−	−	−	+	+	+
β-hydroxybutryic acid	−	−	−	−	−	+	+	+
γ-hydroxybutryic acid	−	−	−	−	−	+	+	+
p-hydroxy phenylacetic acid	−	−	−	−	−	+	+	+
α-keto glutaric acid	−	−	−	−	−	+	+	+
D-ribose	−	−	−	−	−	+	+	+
Salicin	−	−	+	+	+	+	+	+
Sedoheptulosan	+	−	−	+	−	+	+	+
Stachyose	+	+	−	−	−	+	+	+
D-tagatose	+	−	−	−	−	+	+	+
D-xylose	+	−	−	−	−	+	+	+
α-keto valeric acid	−	−	+	+	−	+	+	+
Propionic acid	−	−	+	−	−	+	+	+
Succinic acid	−	−	−	−	+	+	+	+
Succinamic acid	−	−	−	−	−	+	+	+
Alaninamide	−	−	−	−	−	+	+	+
D-alanine	−	−	−	−	−	+	+	+
L-alanine	−	+	+	+	+	+	+	+
L-alanyl glycine	−	−	+	−	+	+	+	+

L-asparagine	+	+	−	+	+	+	+	+
L-glutamic acid	−	+	−	+	+	+	+	+
Glycyl-L-glutamic acid	−	−	+	−	+	+	+	+
L-pyroglutamic acid	−	+	−	+	−	+	+	+
L-serine	−	+	+	+	+	+	+	+
Inosine	−	+	+	+	+	+	+	+
Uridine	−	+	+	+	−	+	+	+
Thymidine	−	+	+	+	+	+	+	+
Putrescine	−	−	−	−	−	+	+	+
2,3-butanediol	−	−	−	+	−	+	+	+
Glycerol	+	+	+	+	+	+	+	+
D,L-α-glycerol phosphate	−	+	+	+	+	+	+	+
Glucose-1-phosphate	−	−	−	−	+	+	+	+
Glucose-6-phosphate	−	−	−	−	+	+	+	+
Lactamide	−	−	−	−	−	+	+	+
D-lactic acid methyl ester	−	−	−	−	−	+	+	+
L-lactic acid	−	−	−	+	+	+	+	+
D-malic acid	−	−	−	−	−	+	+	+
L-malic acid	−	+	+	+	+	+	+	+
Pyruvic acid	−	+	+	+	−	+	+	+
N-acetyl L-glutamic acid	−	−	−	+	−	+	+	+
Adenosine	−	+	+	+	−	+	+	+
2'-deoxy adenosine	−	+	+	+	−	+	+	+
Adenosine-5'-monophosphate	−	−	+	−	+	+	+	+
Thymidine-5'-monophosphate	−	−	+	−	−	+	+	+
Uridine-5'-monophosphate	−	−	+	−	−	+	+	+
Fructose-6-phosphate	−	−	−	−	+	+	+	+

[a]*M. v.-c. = Monilinia vaccinii-corymbosi*; *B. subtilis = Bacillus subtilis*; *B. myc. = Bacillus mycoides*; *B. mojav. = Bacillus mojavensis*; *P. aggl. = Pantoea agglomerans*; *W. austr. = Wickerhamiella australiensis*; *T. harz. = Trichoderma harzianum*; *G. ros. = Gliocladium roseum*.