

Effectiveness of honey bees in delivering the biocontrol agent *Bacillus subtilis* to blueberry flowers to suppress mummy berry disease

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Abstract

Honey bees are important pollinators of commercial blueberries in the southeastern United States, and blueberry producers often use supplemental bees to achieve adequate fruit set. However, honey bees also vector the plant pathogenic fungus *Monilinia vaccinii-corymbosi* which infects open blueberry flowers through the gynoeceal pathway causing mummy berry disease. Here, we report the results of a 3-year field study to test the hypothesis that using bee hives equipped with dispensers containing the biocontrol product Serenade, a commercial formulation of the bacterium *Bacillus subtilis* which has shown activity against flower infection by *M. vaccinii-corymbosi* in laboratory experiments, can reduce mummy berry disease incidence when honey bees are used as pollinators in blueberries. Individual honey bees carried $5.1\text{--}6.4 \times 10^5$ colony-forming units (CFU) of *B. subtilis* when exiting hive-mounted dispensers with Serenade. On caged rabbiteye blueberry bushes in the field, population densities of *B. subtilis* vectored by honey bees reached a carrying capacity of $<10^3$ CFU per flower stigma within 2 days of exposure, and there was a highly significant non-linear relationship between *B. subtilis* populations per stigma and bee activity, expressed as number of legitimate flower visits per time interval per cage ($R = 0.6928$, $P < 0.0001$, $n = 32$). Honey bee density (1600 or 6400 individuals per 5.8-m³ cage) and Serenade treatment (presence or absence of the product in hive-mounted dispensers) significantly ($P < 0.05$) affected the incidence of fruit mummification on caged bushes, whereby increasing bee density increased disease incidence and application of Serenade reduced disease levels. Taken together, results of this study suggest that use of a hive-dispersed biocontrol product such as Serenade as a supplement during pollination can reduce the risk of mummy berry disease. This may be a prudent practice that optimizes the benefits to pollination of high bee densities while reducing the associated disease-vectoring risk.

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1. Introduction

The blueberry industry in Georgia and other southeastern states has expanded considerably during the past decade (Krewer and NeSmith, 2002; Scherm and Krewer, 2003). Despite the steady increase in acreage, however, total blueberry production has remained variable among years. Failure to produce good blueberry

yields is often the result of freeze injury during bloom (NeSmith, 1999), poor pollination (Filmer and Marucci, 1963; Lyrene, 2004), and/or presence of disease (Scherm et al., 2001). Among the diseases affecting blueberry in Georgia, mummy berry disease, caused by the fungus *Monilinia vaccinii-corymbosi* Reade (Honey), has the greatest economical impact on the industry (Scherm et al., 2001). Symptoms are manifested in the blighting of emerging leaves and shoots during early spring (primary infection) and in the mummification of maturing fruit in early summer

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(secondary infection). Fruit mummification results from the infection of open flowers by conidia of the pathogen via the gynoeical pathway (Batra, 1983; Millholland, 1977; Ngugi et al., 2002; Shinnors and Olson, 1996). These conidia are transported from blighted leaves, where they are produced, to the floral stigmas by wind, rain, and especially pollinating insects (Batra and Batra, 1985; Cox and Scherm, 2001; Woronin, 1888). Among these pollinators, honey bees (*Apis mellifera* L.) have been reported to be the most numerous visitors on blooming blueberries in Georgia (Delaplane, 1995). The involvement of pollinating insects in vectoring of *M. vaccinii-corymbosi* poses a dilemma for commercial blueberry producers: on the one hand, enhancing pollinator activity, most commonly achieved by supplementing honey bees in the field (Scherm et al., 2001), is essential for ensuring effective pollination and adequate fruit set (Dedej and Delaplane, 2003; Sampson and Cane, 2000). On the other hand, increased bee density is likely to lead to increased vectoring of the mummy berry pathogen. Thus, strategies are needed to reduce the potential for disease transmission when honey bees are used as blueberry pollinators.

We recently documented that the biofungicide Serenade, a commercial formulation of the bacterium *Bacillus subtilis* (Ehrenberg) Cohn, effectively controlled flower infection by *M. vaccinii-corymbosi* when applied directly to the stigmas of open flowers in the laboratory (Scherm et al., 2004). Part of the biocontrol activity of Serenade appeared to be due to preformed antibiotics contained within the commercially formulated product, in addition to the production of antibiotics by *B. subtilis* in situ. In these experiments, the biocontrol product was delivered manually in small quantities (~10 µg per stigma) as a wettable powder formulation using a transfer needle. This type of dry-application could be mimicked in the field by using honey bees as vectors, given their documented ability to adsorb (Prier et al., 2001) and deliver formulated biocontrol agents in other pathosystems involving flower infection (Johnson and Stockwell, 1998; Kovach et al., 2000; Maccagnani et al., 1999; Peng et al., 1992; Thomson et al., 1992; Yu and Sutton, 1997). Bee delivery of Serenade to open blueberry flowers in the field could reduce flower infection by *M. vaccinii-corymbosi*, regardless of whether conidia of the pathogen are vectored by the bees themselves or by other means.

Based on these considerations, this study was undertaken to (i) determine the effectiveness of honey bees in transmitting *B. subtilis* from bee hive-mounted dispensers containing Serenade to open blueberry flowers in the field and (ii) evaluate the efficacy of this biocontrol product vectored by honey bees in reducing the incidence of fruit mummification caused by *M. vaccinii-corymbosi*.

2. Materials and methods

2.1. Field site and experimental design

The study was carried out in a research blueberry planting at the Horticulture Farm of the University of Georgia (Oconee County) from 2001 to 2003. The planting was established in 1988 and consisted of alternating rows of 'Climax' and 'Premier' rabbiteye blueberry (*Vaccinium ashei* Reade). Maintenance of the planting, including fertilization, pruning, and weed control, followed commercially recommended practice (Austin, 1994). Plants remained untreated with fungicide and insecticide throughout the 3-year period. Supplemental overhead irrigation was applied as needed.

Experimental plots were delineated by caging blueberry bushes with $1.8 \times 1.8 \times 1.8\text{-m}^3$ frames covered with insect-proof Lumite screen (Bioquip, Gardena, CA). Each cage contained two adjacent 'Climax' plants along with two potted 2- or 3-year-old 'Tifblue' plants that served as pollenizers. At the onset of bloom in early spring, bee hives containing target populations of 0, 1600, or 6400 honey bees were placed into the cages. Bee populations were established by the gravimetric method of Delaplane and Hood (1997). Average population densities during the 3 years were 1178 ± 61.5 (mean \pm SE, $n = 10$) and 4267 ± 85.4 for the 1600 and 6400-bee treatments, respectively, but for convenience we will refer to the treatments in terms of their initial target densities. Bee colonies were fed regularly with sugar syrup and socially stabilized with synthetic queen mandibular pheromone (Bee-Boost; Phero Tech, Delta, BC, Canada) (Currie et al., 1994). The pheromone was used in lieu of a queen to eliminate confounding effects of differential brood production resulting from variable bee populations. Honey bee colonies were removed from the cages at the end of bloom.

Each bee hive was equipped with a hive-mounted dispenser (Gross et al., 1994) permitting bees to acquire the biocontrol product and to disseminate it within the cage. Dispensers were filled with Serenade (QRD 132 WP; Agrquest, Davis, CA) to a depth of about 0.5 cm, and the biocontrol product was replenished as needed. In 2001, the experiment consisted of two replicate cages for each bee density, all of which contained hive-mounted dispensers supplied with Serenade. In 2002 and 2003, six additional cages in which hives were not supplied with Serenade were included in the study. Thus, in the latter 2 years, the experiment included a second treatment factor, presence or absence of Serenade, in addition to the three bee density treatments described above.

2.2. Acquisition of *B. subtilis* by honey bees

In 2001, 10 bees each from the cages with 1600 and 6400 honey bees were captured emerging from the hive

dispensers. At the same time, an additional 20 bees were sampled at the apiary of the University of Georgia, located within 200 m of the experimental site, as they emerged from hives not supplemented with Serenade; these bees were used as a background control. Each bee was placed into a glass vial and killed by freezing. Bees were washed individually in 5 ml of sterile potassium phosphate buffer (0.01 M, pH 6.7) for 15 s, the wash water was placed in a sonicating bath (Bransonic Model 2200; Branson, Shelton, CT) for 60 s to resuspend the bacteria, and population densities of *B. subtilis* were determined by dilution-plating in triplicate onto nutrient–yeast extract–dextrose agar (Lelliott and Stead, 1987). Culture dishes were incubated at 23 °C, and the number of colony-forming units (CFU) of *B. subtilis* per bee was determined between 1.5 and 2 days later. Colonies of *B. subtilis* were identified based on their rapid growth rate and characteristic colony morphology (Buchanan and Gibbons, 1987) in comparison with reference cultures isolated directly from Serenade.

2.3. Vectoring of *B. subtilis* to open flowers

In 2001 and 2002, individual flowers were sampled from within the cages and analyzed for population densities of *B. subtilis* on the stigmatic surface. In each cage, 40–50 unopened flowers were labeled on their corollas with a permanent marker and monitored for time of anthesis; ten of these flowers were detached and assayed for population densities of *B. subtilis* 2 days after opening and exposure to the bees within the cage. Styles were removed from the flowers and placed individually in 1 ml of sterile potassium phosphate buffer in microcentrifuge tubes, followed by vortexing for 15 s and incubation in an ultrasonic bath for 60 s. Serial dilutions were made and plated as described above. Population densities of *B. subtilis* were expressed as CFU per stigma. As bloom progressed, five and three independent experimental runs (consisting of non-overlapping 2-day exposure periods) were carried out in 2001 and 2002, respectively.

During each experimental run, honey bee activity (primarily a function of bee density and weather) was estimated by counting the number of legitimate flower visits on all bushes within each cage for one 2-min period per day during normal flight hours (11:00–16:00 h). Visits were considered legitimate if the bee probed the terminal aperture of the flower (Dedej and Delaplane, 2003).

2.4. Incidence of fruit mummification

At the end of bloom in 2002 and 2003, the screens surrounding the cages were replaced with poultry netting to protect fruit from animals and unauthorized harvesting. When fruit were fully developed but still green,

30 fruit clusters were selected arbitrarily from each of the two ‘Climax’ bushes within each cage, and all fruit on these clusters were collected to determine the incidence of fruit mummification per bush. Each fruit was bisected individually, and the presence or absence of mycelia or pseudosclerotia of *M. vaccinii-corymbosi* was determined (Scherm and Copes, 1999).

To obtain an estimate of disease pressure for the 2 years, incidence of fruit mummification was determined as described above for two ‘open plots’ each year. Similar to the caged plots, the open plots consisted of two adjacent ‘Climax’ bushes. However, these bushes were not surrounded by screen cages, and no supplemental bees, Serenade, or potted pollenizer plants were added.

2.5. Statistical analyses

To quantify the Serenade-vectoring ability of honey bees, the relationship between population density of *B. subtilis* per stigma and bee activity was analyzed using non-linear regression analysis (SigmaPlot v. 8.02; SPSS, Chicago, IL) utilizing combined data from all eight experimental runs but omitting the data from control cages without bees. The regression model was of the form $y = a(1 - b^x)$, where y is the population density of *B. subtilis* (CFU per stigma), x is bee activity (number of legitimate visits per 2 min per cage), and a and b are parameters to be estimated.

The effect of honey bee density, presence or absence of Serenade, and their interaction on the incidence of fruit mummification per bush was determined using two-way analysis of variance (ANOVA) for a completely randomized design (SAS v. 8.02; SAS Institute, Cary, NC). Data from cages without bees were omitted from the analysis because of low fruit set and because no Serenade was vectored in these cages.

3. Results

3.1. Acquisition of *B. subtilis* by honey bees

Individual bees carried $5.1 \times 10^5 \pm 1.2 \times 10^5$ and $6.4 \times 10^5 \pm 5.3 \times 10^4$ CFU of *B. subtilis* (mean \pm SE) when exiting hive-mounted dispensers containing Serenade in cages with 1600 and 6400 bees, respectively. No colonies of *B. subtilis* were obtained from control bees.

3.2. Vectoring of *B. subtilis* to open flowers

Bee activity varied between 0 and 48 legitimate visits per cage per 2-min period, while population densities of *B. subtilis* reached within 2 days of exposure ranged from 0 to 5.1×10^3 CFU per stigma (Fig. 1). The regres-

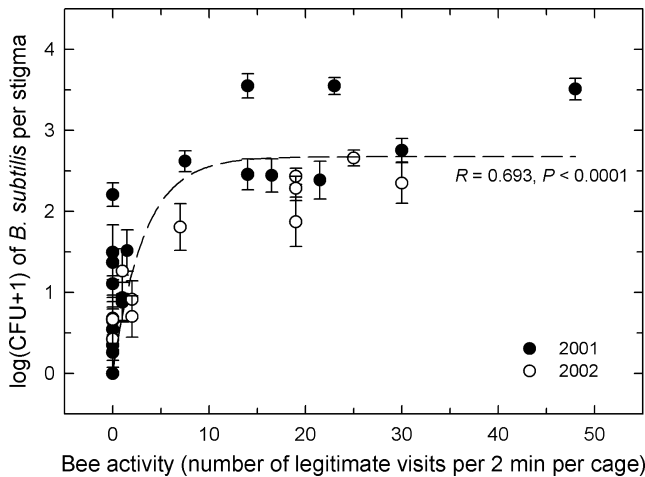


Fig. 1. Vectoring by honey bees of Serenade biofungicide (*Bacillus subtilis* strain QRD132) from hive-mounted dispensers to the stigmas of open flowers on caged 'Climax' rabbiteye blueberry in the field. Population densities of *B. subtilis* were determined by dilution-plating from individual stigmas 2 days after the flowers had opened; values are means and standard errors of ten flowers per cage. The regression equation is of the form $y = a(1 - b^x)$. There were five and three independent experiments in 2001 and 2002, respectively. CFU = colony-forming units.

sion between bee activity and bacterial population density was highly significant ($R = 0.6928$, $P < 0.0001$, $n = 32$) and indicated an apparent carrying capacity of $<10^3$ CFU of *B. subtilis* per stigma attained for bee activities ≥ 10 visits per 2-min period per cage (Fig. 1).

3.3. Incidence of fruit mummification

The incidence of fruit infected by *M. vaccinii-corymbosi* was greater in 2003 than in 2002, presumably because of more frequent rainfall and warmer temperatures during spring of 2003. For example, average disease incidence in the most severely affected treatment (having 6400 bees per cage without Serenade application) was 21.1% in 2002 vs. 66.5% in 2003 (Fig. 2). Similarly, disease incidence in bushes in open plots (exposed to ambient bee activity without use of Serenade) was greater in 2003 (30.5%) than in 2002 (14.2%).

Both bee density and presence of Serenade significantly ($P < 0.05$) affected disease incidence in the 2 years (Table 1). In general, increasing bee density increased disease incidence, while application of Serenade reduced disease levels (Fig. 2). However, a significant bee density \times Serenade interaction was observed in 2002 (Table 1); this was due to absence of disease in the cages containing 1600 bees with no Serenade, while a low incidence of disease (3.4%) was observed in cages with the same bee density in the presence of Serenade (Fig. 2A). In the cages having 6400 bees, application of Serenade reduced disease incidence from 21.1 to 6.6% in 2002 and from 66.5 to 43.5% in 2003.

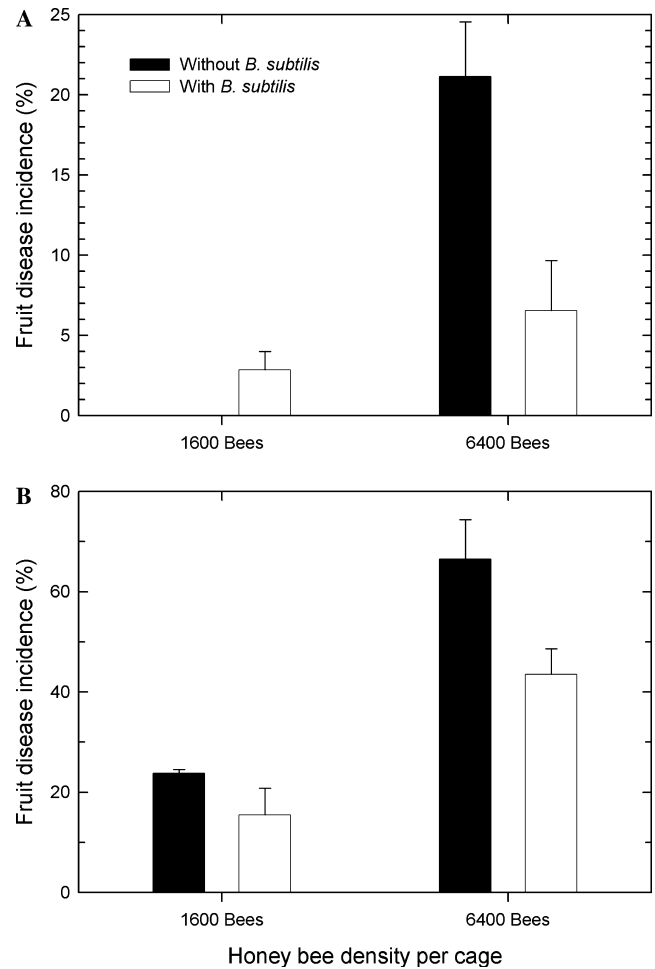


Fig. 2. Incidence of fruit mummification caused by *Monilinia vaccinii-corymbosi* on caged 'Climax' rabbiteye blueberry in the field in relation to honey bee density and presence or absence of Serenade Biofungicide (*Bacillus subtilis* strain QRD132) in hive-mounted dispensers within each cage. Values are means and standard errors of four bushes per treatment and are shown for experiments carried out in 2002 (A) and 2003 (B). Disease incidence in bushes without cages, supplemental bees, and no Serenade application was $14.2 \pm 3.4\%$ and $30.5 \pm 2.8\%$ in 2002 and 2003, respectively.

4. Discussion

This study shed light on several important aspects of the three-way interaction between a pollinator (honey bee), a flower-infecting fungus (*M. vaccinii-corymbosi*), and a bacterial biocontrol agent (*B. subtilis*) on blueberry in the field. First, we confirmed the importance of bees as vectoring agents for the pathogen, as evidenced by a higher incidence of fruit mummification in cages with higher bee densities. Second, we documented the ability of bees to acquire a commercial formulation of the biocontrol agent and vector it to open blueberry flowers where *M. vaccinii-corymbosi* infects. There was a positive, non-linear relationship between bee activity and the resulting population density of *B. subtilis* on the flower stigma. Third, our results showed that

Table 1

Analysis of variance to determine the effects of honey bee density, presence or absence of Serenade Biofungicide (*Bacillus subtilis* strain QRD132), and their interaction on the incidence of fruit mummification caused by *Monilinia vaccinii-corymbosi* on caged 'Climax' rabbiteye blueberry in the field

Source	df	2002			2003		
		Mean square	F	P > F	Mean square	F	P > F
Bee density ^a	1	617.7	27.2	0.0002	5004.3	42.7	<0.0001
Serenade ^b	1	137.9	6.07	0.0299	976.9	8.33	0.0137
Bee density × Serenade interaction	1	303.9	13.4	0.0033	216.8	1.85	0.1990
Error	12	22.7	—	—	117.3	—	—

^a Bee densities were 1600 or 6400 individuals per 5.8-m³ screen cage containing two mature test plants along with two potted pollinizer plants. Control cages without bees were not included in the analysis of variance.

^b Applied via bee hive-mounted dispensers.

bee-vectored Serenade significantly and consistently reduced the incidence of mummy berry disease. Taken together, results of this study suggest that application of Serenade via hive-mounted dispensers is a promising strategy to reduce the potential for transmission of mummy berry disease when honey bees are used as blueberry pollinators.

An active role of honey bees and other pollinating insects in vectoring of conidia of *M. vaccinii-corymbosi* to open blueberry flowers has been documented previously. Indeed, pollinators are attracted to infected, conidia-bearing shoots by a sweet, almond-like odor (Batra and Batra, 1985; Woronin, 1888) and by a UV reflection pattern that mimics that of blueberry flowers (Batra and Batra, 1985). These earlier studies also documented the presence of conidia of *M. vaccinii-corymbosi* on the body parts of pollinating insects and a considerable reduction in the incidence of fruit mummification when pollinators were excluded from flowers (Batra and Batra, 1985). The highly significant effect of bee density on disease incidence in our study is congruent with these reports.

In the present study, individual honey bees carried around 5×10^5 CFU of *B. subtilis* when exiting hive-mounted dispensers containing Serenade. This is similar to values reported for other formulated biocontrol agents such as *Trichoderma harzianum* (Kovach et al., 2000), *Gliocladium roseum* (Yu and Sutton, 1997), and *Pseudomonas fluorescens* or *Pantoea agglomerans* (Thomson et al., 1992) when vectored by honey bees or bumble bees. In a previous study with *B. subtilis*, honey bees acquired $2.1\text{--}3.2 \times 10^3$ spores of the bacterium in an aerosol wind tunnel experiment (Prier et al., 2001). It was hypothesized that the ability of bees to adsorb and retain the bacteria was related to the bees' electrostatic charge (Prier et al., 2001).

As shown previously for other pathosystems (Johnson and Stockwell, 1998; Kovach et al., 2000; Maccagnani et al., 1999; Peng et al., 1992; Thomson et al., 1992; Yu and Sutton, 1997), this study documented the ability of honey bees to vector a commercially formulated biocontrol agent to open flowers of a target crop in the field. Population densities of *B. subtilis* on

blueberry flower stigmas reached a carrying capacity of $<10^3$ CFU following bee transmission of Serenade. This is similar in magnitude to population sizes of $2.9\text{--}3.2 \times 10^2$ CFU for *P. fluorescens* on apple flowers (Thomson et al., 1992) and $0.7\text{--}1.7 \times 10^3$ CFU for *G. roseum* on raspberry flowers (Yu and Sutton, 1997) when honey bees were used to vector these biocontrol agents. Population densities of *B. subtilis* in our study were determined on flowers within 2 days of anthesis. This relatively short exposure period was chosen because blueberry flowers are susceptible to infection by *M. vaccinii-corymbosi* for only a few days after they open (Nguigi et al., 2002); thus, reaching an adequate population density of the biocontrol agent within the first few days after anthesis is critical to provide effective control of the disease.

The ability of honey bees to vector *B. subtilis* was positively (but not linearly) related to bee activity. An average of 10 legitimate bee visits per cage per 2-min period was required to reach the apparent carrying capacity of the biocontrol agent on flower stigmas. This level of bee activity is realistic for field conditions on uncaged blueberry bushes (Dedej, unpublished), although with progressing bloom period in rabbiteye blueberry, the proportion of legitimate visits decreases relative to that of illegitimate visits (Dedej and Delaplane, 2004). During illegitimate visits, honey bees rob nectar through lateral perforations in the corolla (created by carpenter bees, *Xylocopa virginica* L.), thereby avoiding contact with the stigma during visitation. Such change of behavior is likely to reduce the ability of honey bees to vector *B. subtilis* to the stigmatic surface in the field. Nectar-robbing behavior in the presence of carpenter bees is common in the southeastern United States (Cane and Payne, 1990; Delaplane, 1995).

As expected (Batra and Batra, 1985), higher honey bee densities increased the incidence of fruit infection by *M. vaccinii-corymbosi* in control cages without Serenade. When bee hives were supplied with the biocontrol product, however, disease incidence was reduced not only relative to levels measured in the control cages, but also to those in the open plots where bee numbers

were not manipulated and where no Serenade was used. Thus, given the interest in increasing bee density in blueberry plantings to improve pollination (Dedej and Delaplane, 2003), use of a hive-dispersed biocontrol product such as Serenade as a supplement during pollination can reduce the risk of mummy berry disease. This may be a prudent practice that optimizes the benefits to pollination of high bee densities while reducing the associated disease-vectoring risk.

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