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Effect of flower-applied Serenade biofungicide (*Bacillus subtilis*) on pollination-related variables in rabbiteye blueberry

H.K. Ngugi[a], S. Dedej[b], K.S. Delaplane[b], A.T. Savelle[a], H. Scherm[a,*]

[a] *Department of Plant Pathology, University of Georgia, Athens, GA 30602, USA*
[b] *Department of Entomology, University of Georgia, Athens, GA 30602, USA*

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Abstract

Application of Serenade, a commercial biofungicide formulation containing the bacterium *Bacillus subtilis*, to the stigmatic surface of open blueberry flowers suppresses floral infection by the mummy berry fungus *Monilinia vaccinii-corymbosi*. The deliberate targeting of the stigma with the biocontrol agent in this pathosystem prompted us to evaluate potential negative impacts on pollination and pollination-related fruit characteristics. Application of Serenade to the stigmatic surface of detached blueberry flowers in the laboratory had no effect ($P > 0.05$) on the number of pollen tubes entering the style or their growth rates within the stylar canal. There was also no reciprocal effect, i.e., population dynamics of *B. subtilis* were unaltered by the presence of pollen. Application of the biocontrol product to open flowers, regardless of whether it was done 1 day before or immediately prior to pollination, did not impact fruit set or the number of seeds per berry, but marginally ($P = 0.048$) affected fruit weight in one of two experimental runs in the greenhouse; fruit weights in the two Serenade timing treatments were significantly different from each other but neither was different from that of the control that received pollen only. In a field experiment in which honey bees were utilized to vector the biocontrol product to open flowers, application of Serenade did not affect fruit weight but significantly reduced fruit set from 49.1 to 38.1% ($P = 0.0382$) and seed number to about half of that of the untreated control ($P = 0.0109$). However, fruit weights and seed numbers in the experiment were low even in treatments receiving no Serenade, indicative of poor pollination overall. Taken together, these results indicate that application of Serenade has no inherently adverse effects on pollination and associated fruit characteristics, but caution should be exercised in applying this product in conditions otherwise unfavorable for adequate pollination.

Keywords: *Bacillus subtilis*; Biocontrol risk assessment; Pollination; Fruit set; Rabbiteye blueberry; *Vaccinium ashei*

1. Introduction

The risk of resistance development in pathogen populations as well as environmental and human health concerns have fostered interest in identifying and assessing alternatives to synthetic fungicides for managing plant disease. Use of biological control agents is one such alternative that has been proposed and evaluated in numerous pathosystems with varying degrees of success (Cook, 1993).

Recently, we identified the biofungicide Serenade, a commercial formulation of the bacterium *Bacillus subtilis* (Ehrenberg) Cohn, as a promising product for managing mummy berry disease of blueberry, caused by the fungus *Monilinia vaccinii-corymbosi* (Reade) Honey (Scherm et al., 2004). In Georgia and other states in the southeastern US, where predominantly rabbiteye blueberries (*Vaccinium ashei* Reade) are grown, fruit mummification by the pathogen, which results from conidial infection of open flowers via the gynoecial pathway (Batra, 1983; Milholland, 1977; Ngugi et al., 2002; Shinners and Olson, 1996), can cause substantial yield and quality reductions (Scherm and Copes, 1999).

* Corresponding author. Fax: +1 706 542 1262.
E-mail address: scherm@uga.edu (H. Scherm).

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Since conidial infection by *M. vaccinii-corymbosi* occurs exclusively during bloom, protection is needed only during a short period of host development, increasing the likelihood of successful biological control. Furthermore, the presence of copious amounts of exudate on blueberry flower stigmas (Heslop-Harrison and Shivannah, 1977; Parrie and Lang, 1992) has been shown to sustain populations of *B. subtilis* adequate to inhibit hyphal ingress of the pathogen into the style (Scherm et al., 2004). When applied using standard orchard spray equipment, Serenade has not been as effective against mummy berry disease as the commonly used synthetic fungicides (Scherm and Stanaland, 2001), presumably owing to inadequate deposition of the biofungicide on the flower stigmas. However, improved field efficacy based on a more targeted delivery of the product to newly open flowers using honey bees (*Apis mellifera* L.) as vectors has recently been documented (Dedej et al., 2004). In the latter study, which was carried out using caged blueberry bushes in the field, population densities of *B. subtilis* on flower stigmas reached a carrying capacity of $<10^3$ colony forming units (CFUs) after 2 days of plant exposure to honey bee hives with dispensers containing Serenade. These population levels reduced fruit disease incidence by between 34.6 and 68.7% (Dedej et al., 2004), demonstrating the effectiveness of using bees to deliver the biocontrol agent in the field to suppress the disease. Based on this result, efforts are underway to develop a honey bee-based application strategy for Serenade for commercial use.

Before any new biocontrol strategy can be recommended for large-scale implementation, a thorough assessment of its potential risks is essential (Cook et al., 1996; Thomas and Willis, 1998). Although there appears to be no documented case of adverse effects of Serenade or *B. subtilis* on crops, the intentional delivery of the biocontrol product to the flower stigma to control mummy berry disease necessitates an evaluation of its potential impact on pollination-related variables. Similar risk assessments to ensure absence of adverse effects on pollination have been carried out previously for synthetic fungicides for which the time of application coincides with the bloom period. Ziram and triforine, for example, were reported to inhibit pollen germination in lowbush and highbush blueberry, respectively (Bristow, 1981; Lockhart, 1967). In other fruit crops, inhibitory effects of synthetic fungicides on pollen function also have been documented (e.g., Butt et al., 1985; Church and Williams, 1977; Mayer and Lunden, 1998).

In the case of flower-applied *B. subtilis*, adverse effects on pollination could be brought about in various ways. For example, large numbers of bacterial cells on the stigmatic surface and/or any antibiotics or other metabolites produced by them could interfere with pollen germination and pollen tube growth, resulting in poor fruit set and/or low seed numbers. Low seed numbers, in turn, would lead to reduced fruit weight and increased time to fruit ripening (Darnell and Lyrene, 1989; Garvey and Lyrene, 1987; Gupton and Spiers, 1994). Conversely, pollen present on the stigma may inhibit the multiplication of the biocontrol agent, thereby reducing its efficacy. This could occur, for example, if pollen grains occupy the limited space on the stigmatic surface or if they deplete the stigmatic exudate, leaving the biocontrol agent without a nutrient base and thus compromising biocontrol efficacy.

Based on the above considerations, the primary objective of this study was to evaluate the effects of floral application of Serenade on pollination-related variables in rabbiteye blueberry. As a secondary objective, we considered the potential reciprocal effects of the presence of pollen on the population dynamics of *B. subtilis*, the product's active ingredient, on the flower stigma.

2. Materials and methods

2.1. Effect of pollination on population dynamics of *B. subtilis* on stigmas of detached flowers

Three-year-old potted rabbiteye blueberry plants (cv. 'Tifblue') were maintained in a greenhouse following natural or artificial chilling as described previously (Ngugi et al., 2002) to induce flowering. Individual flowers were detached 1 day after they opened and placed, with the stigma facing upward, in 96-well microtiter plates containing 150 μl sterile deionized water per well. Commercially formulated Serenade QRD 132WP (AgraQuest, Davis, CA) was applied directly to the stigmas with a transfer needle (Scherm et al., 2004). The population density of *B. subtilis* in the formulation was determined as 1.2×10^{10} CFU/g based on dilution-plating of an aqueous suspension of the product on nutrient yeast dextrose agar (NYDA) medium (Lelliott and Stead, 1987). Treated flowers were then separated into two groups; one set was pollinated as described previously (Ngugi et al., 2002) with 50–70 pollen grains per stigma collected from greenhouse-grown 'Powderblue' plants, whereas the other set remained without pollen. Microtiter plates were incubated in a moist chamber at 23 °C with 12 h light (34–55 μmol/m^2/s) for 0, 1, 2, 3, or 4 days before 12 flowers in each group were assayed for population densities of *B. subtilis* by dilution-plating on NYDA (Scherm et al., 2004). Flowers were processed in groups of two, resulting in six replicates per pollination treatment and sampling date. Colonies were counted after 36–48 h at 23 °C, and population densities were expressed as CFU per stigma. Data were analyzed by repeated-measures ANOVA (GENSTAT, Lawes Agricultural Trust, Rothamsted, UK) after expressing CFU per stigma as a percentage of the mean initial population density of the biocontrol agent for each of the two pollination treatments. The

experiment was carried out twice, and the two experimental runs were analyzed separately.

2.2. Effect of Serenade on pollen tube growth in detached flowers

Flowers of greenhouse-maintained 'Tifblue' plants were detached 1 day after they opened, placed in microtiter plates, and incubated as described above. The same day, Serenade was applied to stigmas of 8 flowers as described previously, whereas the remaining 16 flowers were not treated. One day later, Serenade was applied to 8 of the 16 flowers, with the remaining 8 left as untreated controls. All flowers were then immediately pollinated with 50–70 grains of 'Powderblue' pollen. Thirty-six hours after pollination, corollas were removed from the flowers and the pistils were prepared and observed for pollen tube growth using fluorescence microscopy as described by Ngugi et al. (2002). The number of pollen tubes that had entered each style was determined, and the lengths of the eight longest pollen tubes in each style were measured with an ocular micrometer. Pollen tube lengths were converted to growth rates (mm per day), and an average was computed for each flower. Data were subjected to a one-way ANOVA with Serenade application (1 day before pollination, immediately prior to pollination, or untreated) as the treatment factor and individual flowers as replicates. The experiment was carried out three times, and the three experimental runs were analyzed separately.

2.3. Effect of Serenade on pollination-related fruit characteristics in greenhouse-maintained plants

Flower clusters on greenhouse-maintained 'Tifblue' plants were monitored daily, and individual flowers were marked the day they opened. In one set of clusters, Serenade was applied to stigmas of individual flowers on the day they opened, and 'Powderblue' pollen was applied the following day. In a second set of clusters, both Serenade and pollen were applied 1 day after the flowers opened, starting first with the biocontrol product and followed by pollen. A third set of flower clusters was assigned to a control treatment to which only pollen was applied 1 day after the flowers opened. Twelve clusters, comprising at least 70 flowers, were used for each of the three treatments. The experiment was replicated four times and repeated once. For each treatment, fruit were harvested as they matured, counted to determine the percentage of flowers setting fruit, and weighed individually. Within each replicate, fruit from a given treatment harvested on separate days were pooled, a random sample of 20 fruit was drawn, and each fruit was processed individually to determine the number of seeds present. Partially developed and fully mature seeds were included in the counts. Data on fruit set, fruit weight, and number of seeds per berry were subjected to a one-way ANOVA with Serenade application (1 day before pollination, immediately prior to pollination, or untreated) as the treatment factor and four replicates. Additionally, regression analysis was applied to a subsample of 16 fruit per treatment taken across all replicates to determine the relationship between fruit weight and number of seeds per berry.

2.4. Effect of bee-vectored Serenade on pollination-related fruit characteristics in the field

The experiment was carried out in a mature rabbiteye blueberry planting at the University of Georgia Horticulture Farm in 2002 and 2003. Prior to bloom, plants of cv. 'Climax' were enclosed, together with potted 2- or 3-year-old 'Tifblue' plants that served as pollenizers, in 1.8-m × 1.8-m × 1.8-m insect-proof cages made of Lumite screen (Bioquip, Gardena, CA); there were two plants of each cultivar per cage. Each cage also contained one standard Langstroth beehive equipped with a biocontrol product dispenser (Gross et al., 1994). Dispensers were filled with Serenade to a depth of 0.5 cm, and the biocontrol product was replenished regularly. In this set-up, bees exited the hive through a small aperture, crawling through the Serenade-filled dispenser before visiting flowers. Bees exiting from hives with dispensers containing Serenade typically carried 5.8 ± 10^5 CFU of *B. subtilis*, whereas no colonies were obtained from bees exiting through empty control dispensers (Dedej et al., 2004). The experiment involved a factorial arrangement of bee density (0, 1600, or 6400 honey bees per cage) and presence or absence of Serenade. Specific details of bee handling and maintenance were as described by Dedej and Delaplane (2003).

Each year, seven randomly selected lateral shoots, comprising several hundred individual flowers, were tagged in each cage prior to bloom and assessed for subsequent fruit set at the full-sized green fruit stage. Fruit were harvested as they matured, and at least 15 fruits from each cage were weighed individually and processed to determine seed numbers. Data from the 2 years were combined and subjected to a two-way ANOVA with years as blocks and bee density and presence/absence of Serenade as treatment factors; data from cages without bees were omitted from the analysis because of extremely low fruit set and because no Serenade was vectored in these cages. Treatment means were compared with Fisher's protected LSD ($\alpha = 0.05$).

3. Results and discussion

3.1. Effect of pollination on population dynamics of B. subtilis on stigmas of detached flowers

Mean population densities of *B. subtilis* per stigma ranged from 1.5×10^4 to 1.2×10^5 CFU for flowers

assayed immediately after application of Serenade (Fig. 1); based on these values, we estimated that between 1.25 and 10.0 μg of the biocontrol product was applied per stigma. Similar population densities were found to be adequate for inhibiting penetration of *M. vaccinii-corymbosi* into blueberry styles in a previous study (Scherm et al., 2004).

Population dynamics observed in this study were consistent with those reported previously (Scherm et al., 2004) and were unaffected by the presence or absence of pollen ($P = 0.248$ and 0.099 in runs 1 and 2 of the experiment, respectively). Populations of the biocontrol agent decreased significantly over time in both runs ($0.0001 < P \leqslant 0.013$) to between 4.2×10^3 and 2.1×10^4 CFU per stigma for flowers assayed 4 days after application (Fig. 1). There was no pollination × time interaction ($0.275 \leqslant P \leqslant 0.633$).

Since neither the presence nor absence of pollen altered actual bacterial populations or their rate of decrease over time, it appears that competition for space on the stigma or the resources attributable to the exudate did not exist or was very limited. Because flower receptivity to infection by *M. vaccinii-corymbosi* declines rapidly with flower age, only a short period of protection lasting a few days is required to prevent infection (Ngugi et al., 2002); thus, the population decline of the biocontrol agent is of little consequence for management of mummy berry disease (Scherm et al., 2004). Moreover, in field conditions, multiple visits by bee pollinators (Dedej and Delaplane, 2003) would ensure that newly opened flowers receive repeated applications of the biocontrol agent, provided an adequate bee population density is maintained and the biocontrol product in the hive-based dispensers is replenished regularly.

3.2. Effect of Serenade on pollen tube growth in detached flowers

Application of Serenade had no effect ($0.148 \leqslant P \leqslant 0.953$) on the number of pollen tubes that entered the stylar canal (Fig. 2A). Similarly, ANOVA results showed that the biocontrol product, applied 1

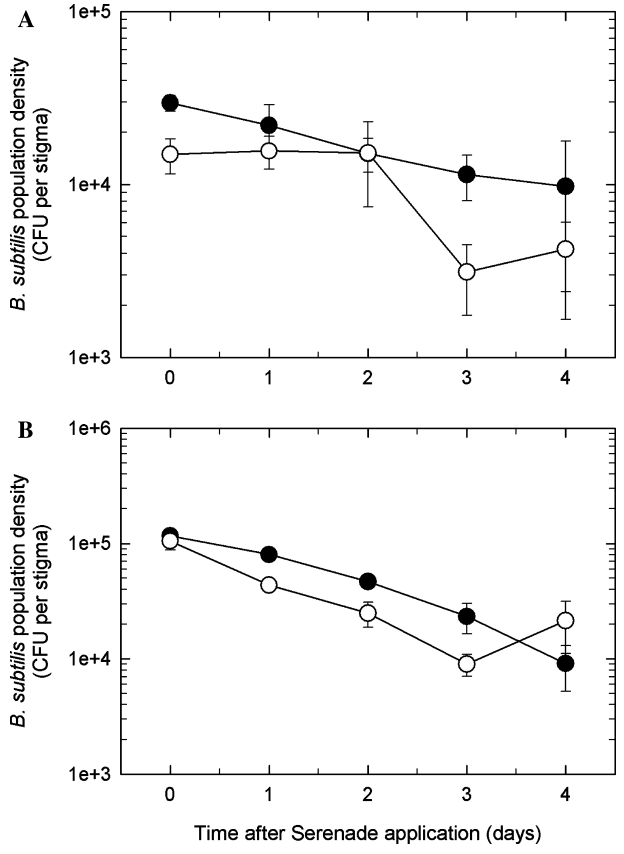

Fig. 1. Population dynamics of *B. subtilis* QRD 132 on the stigmatic surface after application of the commercially formulated biocontrol product Serenade to detached blueberry flowers 1 day after the flowers opened; flowers were either pollinated (○) or not pollinated (●) immediately after application of Serenade. Values are means and standard errors of 12 flowers per sampling date, processed in groups of two, from two experimental runs (A and B). CFU, colony-forming units.

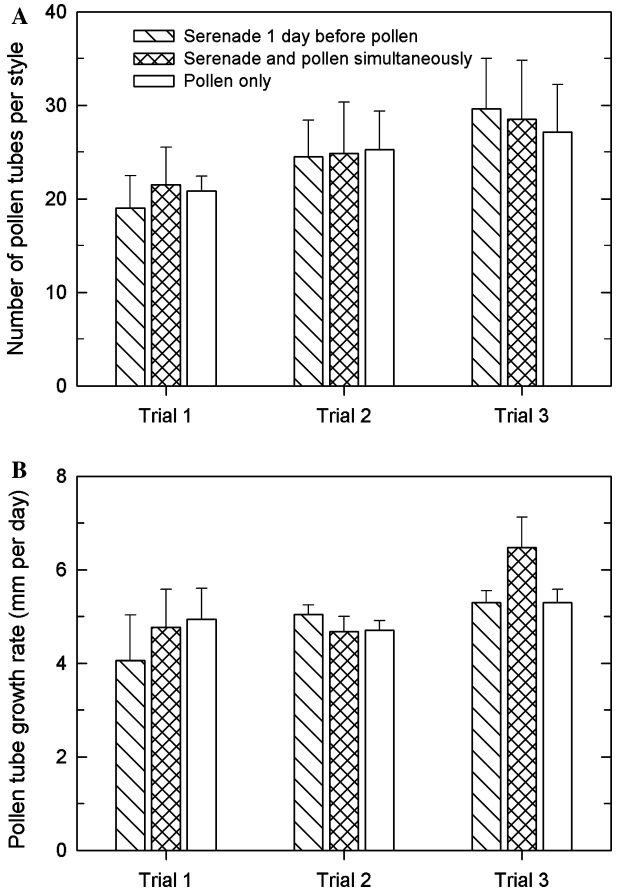

Fig. 2. Effect of application of Serenade (*B. subtilis* QRD132) to the stigmatic surface on numbers (A) and growth rates (B) of pollen tubes entering the stylar canal of detached blueberry flowers. Styles were fixed for microscopic observation 36 h after pollination. Values are means and standard errors of eight styles per treatment. Growth rates were measured for the eight longest pollen tubes per style.

day before or immediately prior to pollination, did not affect the rate of pollen tube growth in any of the three experimental runs compared with flowers receiving no Serenade ($0.104 \leqslant P \leqslant 0.826$; Fig. 2B). On average, pollen grew at a rate of 5.2 ± 0.55 mm per day within styles of detached flowers. Based on these results, it can be concluded that early events in the pollination process are not inherently affected by the presence of Serenade applied to newly opened flowers.

3.3. Effect of Serenade on pollination-related fruit characteristics in greenhouse-maintained plants

Across the three Serenade treatments and the two experimental runs, fruit set, fruit weight, and seed number averaged 58.8%, 1.83 g, and 44 seeds per berry, respectively. The presence of Serenade did not consistently affect fruit set, fruit weight, or seed number in controlled greenhouse conditions (Table 1). However, a marginally significant ($P = 0.048$) difference among treatments in fruit weight was noted in run 2 of the experiment (Table 1), in which fruit weights in the two Serenade treatments were significantly different from each other but neither was different from that of the control that received pollen only. The average fruit weight in this experimental run was 1.79 g for flowers treated with Serenade 1 day before pollination, 1.97 g for flowers receiving the biocontrol product and pollen on the same day, and 1.93 g for flowers that were pollinated but not exposed to Serenade.

Fruit weight was significantly correlated with the number of seeds per berry (Fig. 3), and the relationship between weight (y, in grams) and seed number (x) for this cultivar in these conditions was described by the equation $\log_{10}(y) = 0.0698 + 0.0044\ x$ ($r = 0.779$; $P < 0.0001$; $n = 48$). Based on this equation, the average weight of fruit formed parthenocarpically (i.e., without seeds) was estimated by extrapolation as 1.17 g. A positive association between seed number and fruit weight is well documented in *Vaccinium* spp. (Dogterom et al., 2000; Garvey and Lyrene, 1987; Gupton and Spiers, 1994), although previous studies have not expressed this relationship quantitatively.

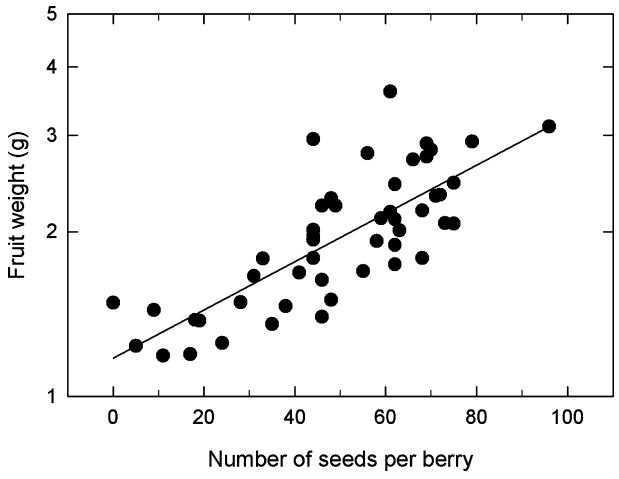

Fig. 3. Relationship between fruit weight (y) and seed number (x) in greenhouse-maintained 'Tifblue' rabbiteye blueberry plants. The regression equation corresponding to the solid line is $\log_{10}(y) = 0.0698 + 0.0044\ x$ ($r = 0.779$; $P < 0.0001$; $n = 48$).

3.4. Effect of bee-vectored Serenade on pollination-related fruit characteristics in the field

Increasing bee density per cage significantly increased fruit set, fruit weight, and seed number (Tables 2 and 3). Whereas application of Serenade did not affect fruit weight, it significantly reduced fruit set from 49.1 to 38.1% and seed number to about half of that of the control (Tables 2 and 3). There was no significant bee density × Serenade application interaction for any of the three fruit characteristics (Table 2). Fruit weights and seed numbers were lower in this experiment than in the greenhouse trial described above, indicating that conditions for pollination were less favorable in the field than in the greenhouse.

Table 1
Analysis of variance to determine the effects of Serenade biofungicide on pollination-related fruit characteristics of 'Tifblue' rabbiteye blueberry in the greenhouse

Source	*df*	Fruit set			Fruit weight			Seed number		
		Mean square	*F*	*P* > *F*	Mean square	*F*	*P* > *F*	Mean square	*F*	*P* > *F*
Run 1										
Replicate	3	85.8	0.77	0.550	0.097	8.38	0.014	264.5	6.43	0.026
Serenade treatment[a]	2	62.3	0.56	0.598	0.007	0.63	0.565	17.61	0.43	0.670
Error	6	111.1	—	—	0.012	—	—	41.14	—	—
Run 2										
Replicate	3	175.7	4.42	0.058	0.125	14.16	0.004	127.56	3.82	0.076
Serenade treatment[a]	2	107.6	2.71	0.145	0.046	5.24	0.048	37.56	1.13	0.385
Error	6	39.7	—	—	0.009	—	—	33.38	—	—

[a] Serenade (*B. subtilis* strain QRD 132) was applied manually to individual flowers either 1 day before or immediately prior to pollination. A third treatment consisted of control flowers that received pollen only.

Table 2
Analysis of variance to determine the effects of year, honey bee density, presence or absence of Serenade biofungicide, and their interaction on fruit set, fruit weight, and seed number of 'Climax' rabbiteye blueberry in screen cages in the field

Source	*df*	Fruit set			Fruit weight			Seed number		
		Mean square	*F*	*P* > *F*	Mean square	*F*	*P* > *F*	Mean square	*F*	*P* > *F*
Year[a]	1	1347.8	15.4	0.0024	0.163	9.47	0.0105	35.7	2.84	0.1202
Bee density[b]	1	6076.9	69.4	<0.0001	0.267	15.5	0.0023	361.9	28.8	0.0002
Serenade treatment[c]	1	484.9	5.54	0.0382	0.014	0.82	0.3850	117.4	9.34	0.0109
Bee density × Serenade treatment	1	139.6	1.59	0.2328	0.010	0.58	0.4612	37.9	3.02	0.1102
Error	11	963.1	—	—	0.189	—	—	138.2	—	—

[a] 2002 and 2003.
[b] Bee densities were 1600 or 6400 individuals per 1.8-m × 1.8-m × 1.8-m cage. Control cages without bees were not included in the analysis of variance.
[c] Serenade (*B. subtilis* strain QRD 132) was applied via beehive-based dispensers.

Table 3
Effects of honey bee density and presence or absence of Serenade biofungicide on fruit set, fruit weight, and seed number of 'Climax' rabbiteye blueberry in screen cages in the field

Factor	Fruit set (%)[a]	Fruit weight (g)[a]	Seed number[a]
Bee density per cage[b]			
1600 bees	24.1 ± 4.54 b	1.09 ± 0.048 b	2.54 ± 0.623 b
6400 bees	63.1 ± 5.64 a	1.34 ± 0.066 a	12.0 ± 2.34 a
Serenade[c]			
Absent	49.1 ± 9.15 A	1.18 ± 0.091 A	10.0 ± 2.91 A
Present	38.1 ± 8.28 B	1.24 ± 0.055 A	4.58 ± 1.33 B

[a] Values are means and standard errors ($n = 8$). Values within the same column followed by the same letter are not significantly different according to Fisher's protected LSD ($\alpha = 0.05$).
[b] Cages were 1.8-m × 1.8-m × 1.8-m in size. Control cages without bees were not included in the analysis.
[c] Serenade (*B. subtilis* strain QRD 132) was applied via beehive-based dispensers.

Discrepancies in the effects of Serenade in controlled experiments in the laboratory and greenhouse (where application had no effect on pollen tube growth or resultant fruit characteristics) compared with those carried out in the field (where the product reduced fruit and seed numbers) may have been due to overall unfavorable conditions for pollination in the field. Low seed numbers, in general, are a good indicator of poor pollination and—in our field experiments—may have been due to the lack of sufficient non-self pollen owing to the difference in size and flower numbers between the mature 'Climax' test bushes and the potted 'Tifblue' pollenizer plants. Indeed, it is very likely that primarily self-pollen, which results in poor pollination in rabbiteye blueberry (Garvey and Lyrene, 1987; Gupton and Spiers, 1994), rather than non-self pollen was supplied to 'Climax' flowers in the cages. By contrast, only non-self pollen was applied to flowers in the greenhouse, providing more favorable conditions for successful pollination. Our hypothesis of inadequate pollen supply in the field is supported by the low seed numbers observed in 'Climax' fruit harvested from the cages (Table 3), and by the low fruit weight which is comparable to the value of 1.17 g predicted for 'Tifblue' fruit formed parthenocarpically based on our regression model. In fact, even in cages with 6400 bees and no Serenade application, seed numbers were only about half of those reported previously for uncaged 'Climax' bushes exposed to ambient bee activity at the same site (Dedej and Delaplane, 2003). Especially in 2003, weather conditions during bloom in the field were characterized by periods of high relative humidity and rain, which may have affected both pollen release and effectiveness of the pollinators negatively (Lyrene, 2004).

4. Conclusions

Although Serenade has been available in the US since 2000 and is labeled for use in numerous crops, there appear to be no reports of risk assessments for this product in commercial conditions. Because the biofungicide must be applied to flower stigmas during bloom to be effective against mummy berry disease, we hypothesized that it could interfere with pollen germination and pollen tube growth, with potentially adverse consequences for rabbiteye blueberry production in which factors affecting pollination can limit productivity (Scherm et al., 2001). Here, we show that application of Serenade to newly opened flowers does not negatively affect pollen germination, pollen tube growth, or resultant pollination-related fruit characteristics when evaluated in controlled conditions in the laboratory or greenhouse. However, field data indicated a negative impact on both fruit set and seed numbers, implying that caution should be exercised in applying this product in circumstances or conditions otherwise unfavorable for adequate pollination. This study also revealed that pollination did not alter population dynamics of *B. subtilis* on the stigmatic surface, indicating that the presence of pollen would not negatively impact the biocontrol activity of Serenade on the stigmatic surface.

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