Electrostatic Application of a Plant-Disease Biocontrol Agent for Prevention of Fungal Infection through the Stigmatic Surfaces of Blueberry Flowers

S. EDWARD LAW[1*] AND HARALD SCHERM[2]

UNIVERSITY OF GEORGIA, ATHENS, GA 30602-4435, USA
[1]Department of Biological and Agricultural Engineering
[2]Department of Plant Pathology

*Corresponding author: email edlaw@engr.uga.edu, tel. +706-542-0866, fax. +706-542-8806

Abstract

The long-term application of chemical pesticides for protecting agricultural crops has helped ensure increased food and fiber yields for the world's growing population, but not without certain disadvantages such as environmental impact and eventual development of pest resistance. Biologically derived pest-control agents offer significant promise as alternatives to chemical pesticides but may require precision delivery onto specific plant structures. This paper investigates the incorporation of electrostatic force to increase the mass transfer of the viable bacterial biocontrol agent *Bacillus subtilis* onto stigmatic surfaces of blueberry flowers for protection against a flower-infecting fungal pathogen. The population density of 1.52×10^5 colony-forming units (CFU) of biocontrol agent electrostatically deposited per ~0.7-mm-diameter stigma exceeded by 4.5-fold that deposited by conventional hydraulic spraying. Stated differently, one-eighth to one-quarter rate electrostatic-spray applications deposited at least as many CFU as did conventional full-rate application of *B. subtilis*. These deposition results, as well as measurements of stigma-style charge relaxation (τ = 44 ns), portent well for efficacious plant disease protection by electrostatic spraying of this bacterial biocontrol agent.

Keywords: Charged spray, Electrostatic deposition, Biological control, *Bacillus subtilis*, Electrostatic induction, Pesticide, Microbial fungicide, Blueberry

1. Introduction

Chemical pesticides have for decades been widely relied upon to protect agricultural crops against insect, disease, and weed pests. Such synthetically formulated chemical products present significant environmental challenges and can often be rendered ineffective over time by resistance acquired by the target pests. Pest-control agents of biological origin are becoming promising alternatives to certain chemical pesticides. Satisfactory protection, however, often depends upon precise placement of a given biocontrol agent onto specific plant structures. Thus, engineering considerations underlying spray application methods for delivery become critically important. In this paper we hypothesize that incorporation of electric force fields will increase the mass-transfer efficiency of viable bacterial biocontrol agents onto the stigmatic surfaces of plant flowers (*i.e.,* the reproductive tips of flower styles upon which pollen is deposited) for countering infection by specialized pathogens that invade *via* the stigma-style pathway.

Previous laboratory research by Scherm *et al.* [1] has confirmed the Gram-positive bacterium *Bacillus subtilis*, a well-known producer of natural antifungal compounds [2], to be a useful bacterial antagonist which suppresses floral infection of blueberry (*Vaccinium* spp.) by the fungal pathogen *Monilinia vaccinii-corymbosi*, the causal agent of the economically important mummy berry disease. Infection by this pathogen occurs *via* the stigma-style pathway during bloom of the plant host, leading to colonization of the ovary with fungal mycelium and eventual mummification of the developing fruit. Based on *in vitro* experiments, the biocontrol activity appeared to be due largely to antibiosis, primarily from anti-fungal compounds produced *in situ* by *B. subtilis* and secondarily from those pre-formed during fermentation and formulation of the commercial biocontrol product. Although application of *B. subtilis* was highly effective against infection by *M. vaccinii-corymbosi* on detached flowers in the laboratory [1], disease suppression was more variable following application of the biocontrol agent *via* a conventional hydraulic-atomizing

sprayer in the field [3]. It was hypothesized that lack of sufficient control was due to the difficulty

in targeting the stigma, a minute and ephemeral plant surface, by the conventional spraying method.

Prior work by Law *et al*. [4] used fluorometric tracers and scanning electron microscopy to show charged sprays of pollen deposited ~5-fold more pollen granules onto stigmatic surfaces than did uncharged sprays. They further verified very acceptable viability of the ~60-μm-diameter pollen bio-particulates following interaction with the intense electric fields and aerodynamic shear forces active for the 1-3-ms passage through a commercial pneumatic-atomizing, electrostatic-induction, spray-charging nozzle. This finding portended well regarding maintaining the viability of the disease biocontrol agent of current interest.

The objective of this present work was to investigate the beneficial usage of electrostatic attraction to increase the deposition of *Bacillus subtilis* onto typically 0.7-mm-diameter blueberry stigmas (Fig. 1) using charged sprays *vs*. conventionally applied hydraulic-atomized sprays. Also investigated were the electrical characteristics of flower components ensuring adequate charge transfer during the electrostatic-spraying event.

2. Methods and Materials

2.1. General

All spray applications of the biocontrol agent were made under rigorously controlled conditions in the laboratory (24°C) using a robotic arm (Fig.2) to sweep (average $\omega \sim 0.83$ rad/s) the selected nozzle past detached clusters of blueberry flowers mounted on a target arc. The typical travel of a field sprayer past target bushes was simulated by dual passes at 3.2 km/h (2 mile/h) in the laboratory as dictated by speed limitations of the robotic arm. To further simulate the slightly upward-directed field-spraying practice, the stems of pendant flower clusters were angled upward 15° toward the horizontal vector of the incoming spray. Volumetric rates of spray application were calculated assuming field planting of ~2.3-m (7.5-ft) tall bushes at 1.5-m (5-ft) intervals within rows spaced

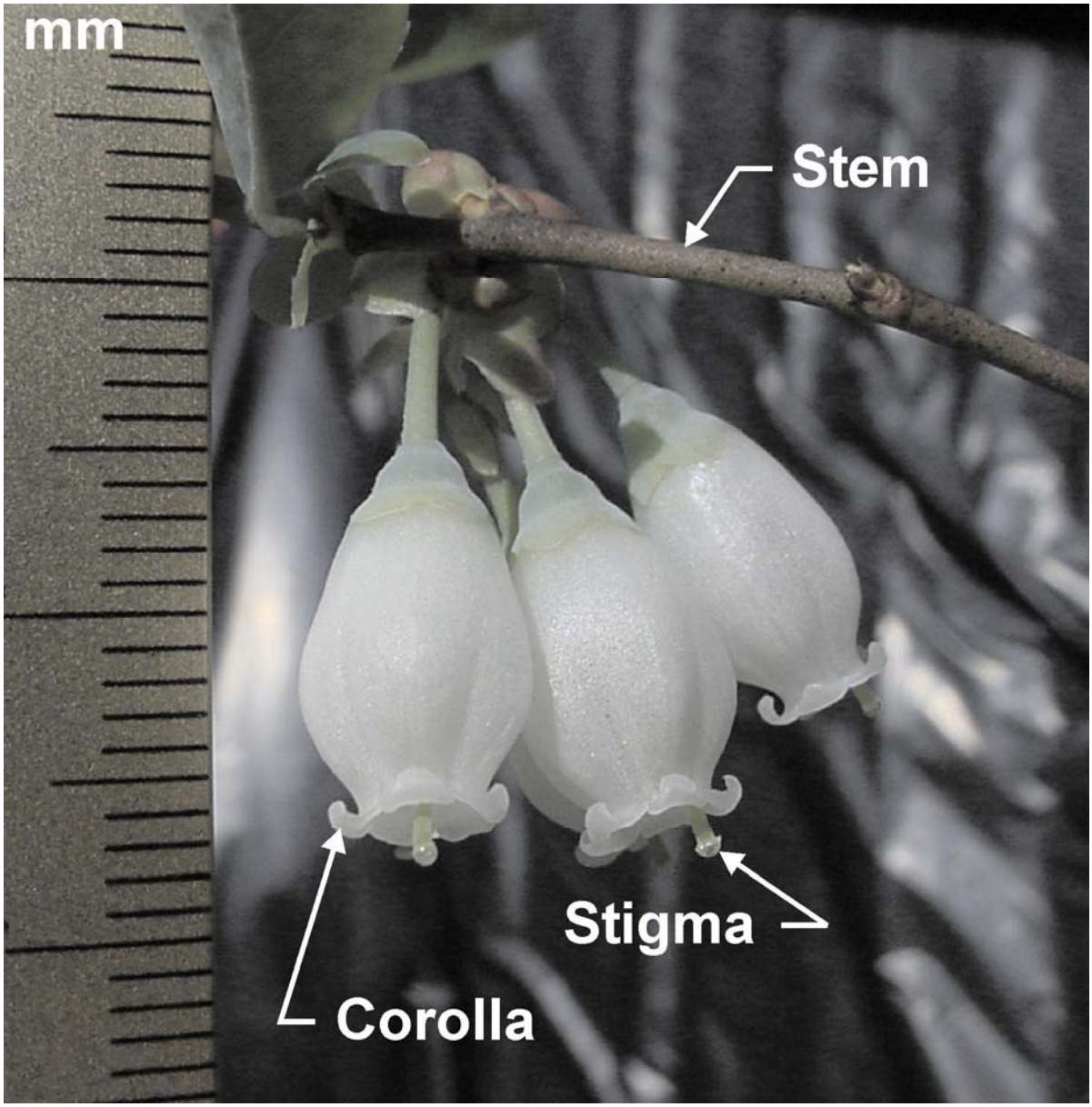

Fig. 1. Cluster of rabbiteye blueberry (*Vaccinium ashei*) flowers showing corolla and protruding stigmatic surface.

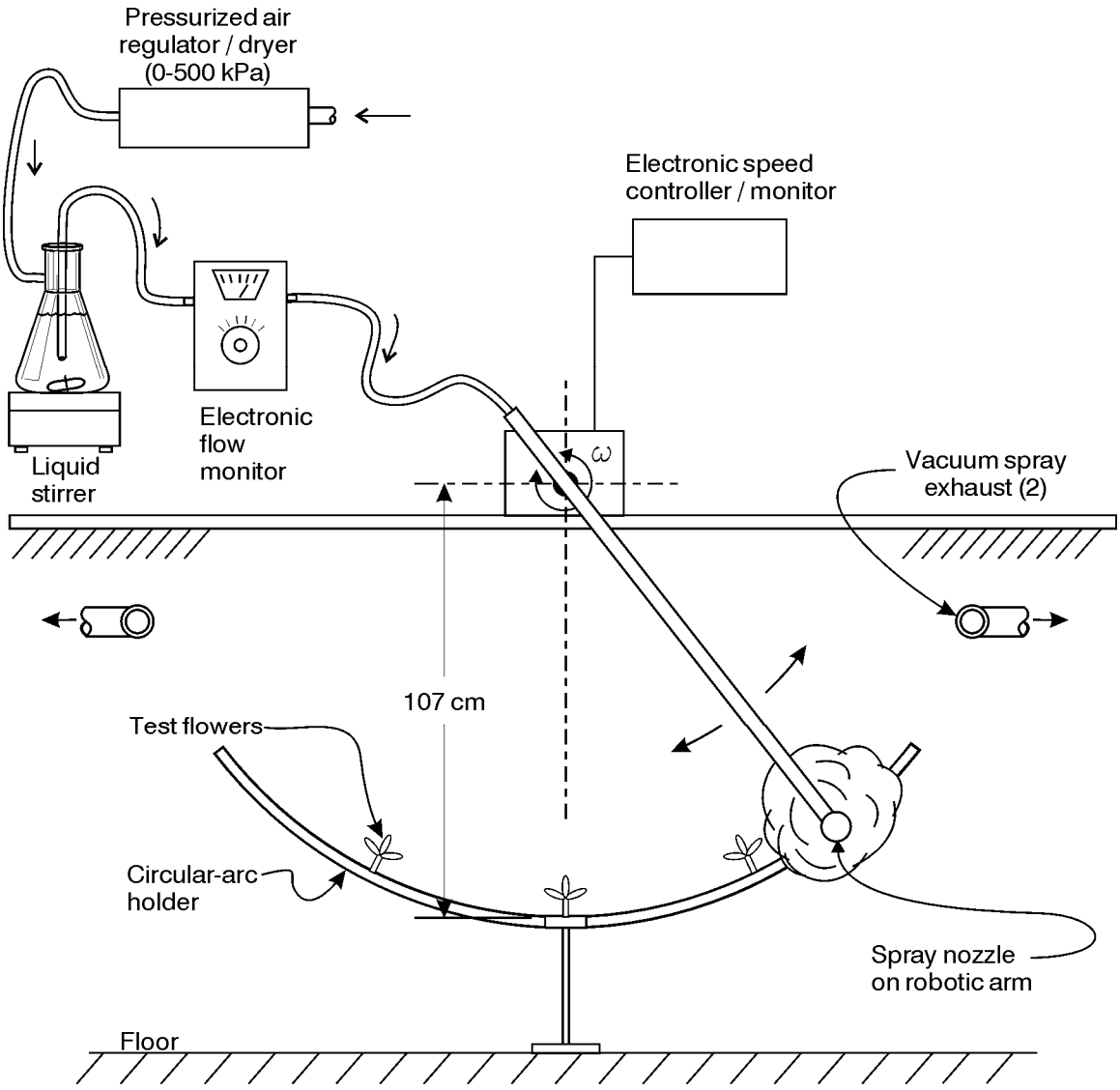

Fig. 2. Laboratory apparatus used to simulate field-sprayer applications of the bacterial biocontrol agent *Bacillus subtilis*.

3.0 m (10 ft) apart, and the spray intercepted by 75% of bush height. Subsequent to spray application, flowers were sampled, stigmas excised, and microbiological analyses conducted to determine the number of viable bacteria per stigma as deposited by the three spray-application methods tested: a) conventional hydraulic-atomizing nozzle; b) electrostatic nozzle having spray charging turned OFF; and c) electrostatic nozzle having spray charging turned ON. In a separate experiment, stigmatic deposition of the biocontrol agent applied electrostatically (*i.e.,* spray charging turned ON) was investigated as a function of varying application rate (*i.e.,* concentration) of the formulated biocontrol product.

2.2. Electrical characterization of target flowers

Electrostatic deposition onto a conductive target relies upon a displacement current to transfer electric charge onto or off of the target to a degree appropriate for maintaining it at earth potential in the presence of the approaching charged-particulate cloud [5,6]. The charge-relaxation time constant τ (s) of the target material characterizes this transient charge flux as

$$\tau = K\rho\varepsilon_0 \qquad (1)$$

where K is the target material's dielectric constant (unitless), ρ is its resistivity (ohm·m) and ε_0 is the electric permittivity of free space (8.854×10^{-12} F m^{-1}). Studies by Dai and Law [7] measured $K = 29$ for floral components of horticultural plants. To calculate the charge-relaxation time constant τ for the stigma-style pathway of blueberry, and hence theoretically confirm adequacy for electrodeposition, the electrically-shielded apparatus of Fig. 3 was used to measure the resistance R (ohms) between the stigmatic surface and the ovary at the base of flowers. With the style excised, the resistance through the outer corolla was also measured. Values were obtained for a number of flowers using a Keithley electrometer model 610C (Cleveland, OH). For the stigma-style geometry microscopically measured (*viz*., 0.7-mm diam. x 11-mm long), resistance values were converted to resistivity as $\rho = (3.50 \times 10^{-5}\ \text{m})\ R$. Experimental measurements gave values of $R = 4.9 \times 10^6$ ohm,

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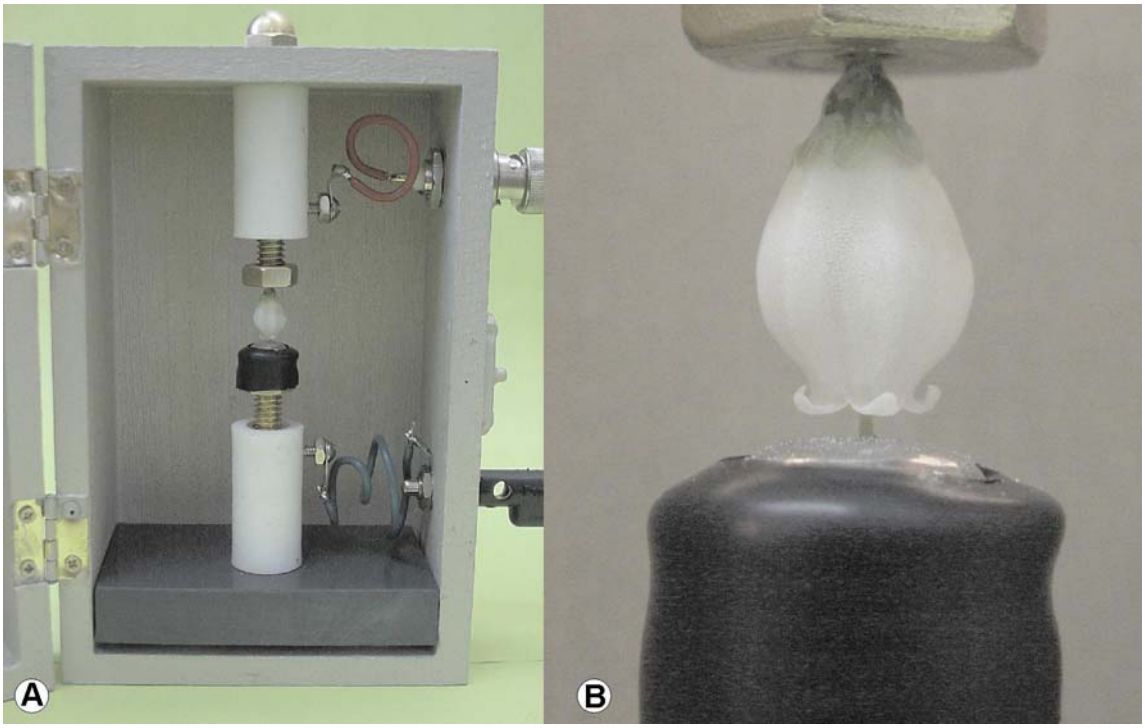

Fig. 3. A - Resistivity cell for measuring electrical properties of blueberry flowers; B - Stigma contacting conformable Hg electrode.

ρ = 171 ohm·m, and τ = 44 ns. The ~500-ms characterizing the electrostatic-deposition event as a field sprayer travels past a crop plant is ~10^7 times greater than this stigma-style charge relaxation time constant. Thus the grounding pathway through the stigma-style, in series with the ~48-ms *RC* time constant (~0.4 Mohm and ~120 pF [6]) of the blueberry plant measured from its uppermost branch to earth, is quite adequate to facilitate electrostatic deposition onto the stigmatic surface.

2.3. Biocontrol agent

Serenade® (AgraQuest, Inc., Davis, CA), a commercial formulation of the QST 713 strain of *B. subtilis*, was used as an aqueous suspension (USEPA Regist. No. 69592-8). The formulation has 1.34% active ingredient containing a minimum of 10^9 CFU/g in aqueous suspension (AS). In comparison with other bacterial biocontrol agents, this formulation's formation of endospores greatly enhances its survivability on treated surfaces. All three spray-application methods dispensed the same amount of Serenade® biocontrol formulation per equivalent land area, *i.e.*, 16.37 L/ha (7 qt/acre). The 0.48-S/m conductivity of the electrostatic tank mix of this material placed it well within the range for satisfactory induction charging.

2.4. Target flower preparation

For each experimental run, three replicate flower clusters, each containing 5 to 10 open flowers, were arbitrarily selected from greenhouse-maintained 'Tifblue' rabbiteye blueberry (*Vaccinium ashei*) plants growing in 3.8-L (1-gal) pots. Individual clusters were removed along with a 1-2-cm stem length at the point of attachment using a hand pruner. The three clusters' stems were affixed by metal clips positioned equidistantly around the earthed spray arc (Fig. 2) with the flower raceme perpendicular to the spray vector and angled ~15° upward toward the incoming spray. This positioning facilitated favorable targeting of stigmatic surfaces as commonly done in actual field spraying. Placement was 38.1 cm (15 in.) from the conventional nozzle or 76.2 cm (30 in.) from the electrostatic nozzle.

2.5. Spray applications

Conventional application of the biocontrol spray utilized a Spraying Systems, Inc. (Wheaton, IL) TeeJet® disc-core type D3-23 hollow-cone, hydraulic-atomizing nozzle. Electrostatic application utilized a MaxCharge® pneumatic-atomizing, electrostatic-induction, spray-charging nozzle [5,8] patent licensed by the University of Georgia Research Foundation to Electrostatic Spraying Systems,

Inc. (Watkinsville, GA). Its spray-charging performance for imparting convective spray-cloud current i_c (μA) onto the biocontrol liquid at 76 mL/min using 207 kPa (30 psi) atomizing-air pressure was experimentally measured to be

$$i_c = 0.48 - 9.58\ \Phi \qquad (2)$$

where Φ is the induction-electrode applied voltage (kV). Based upon this charging response exhibiting a 0.992 linear-regression correlation coefficient, all charged sprays were set at 1.09 kV to provide i_c = -10 μA and a corresponding spray charge-to-mass value of -7.8 mC/kg. For uncharged spray, the charging voltage of the electrostatic nozzle was set to zero, while its pneumatic-atomization feature was maintained ON, thus still providing ~5.4 m/s (17.8 ft/s) air-carrier velocity in the target vicinity. Table 1 specifies the nozzles' salient operational conditions.

Table 1. Operational conditions for spray-application nozzles

	Conventional	ES-Uncharged*	ES-Charged*
No. nozzles per side of plant row in field	4	3	3
Target spacing from nozzle	38 cm (15 in.)	76 cm (30 in.)	76 cm (30 in.)
Spray-mix application rate	468 L/ha (50 gal/acre)	56 L/ha (6 gal/acre)	56 L/ha (6 gal/acre)
Biocontrol agent application rate	16.37 L/ha (7 qt/acre)	16.37 L/ha (7 qt/acre)	16.37 L/ha (7 qt/acre)
Nozzle pressure	276 kPa (40 psi)	207 kPa (30 psi)	207 kPa (30 psi)
Spray charge-to-mass	0	0	-7.8 mC/kg

*ES = electrostatic nozzle

Between treatments a four-step process was utilized to disinfest the application system. The material reservoir, all system plumbing, and the nozzle were flushed first with tap water, then with a 10% bleach solution (0.6% NaOCl), followed by 70% ethanol, and finally with sterile tap water. The target arc was similarly disinfested.

2.6. Microbiological assessment of sprayed target stigmas

Following spray application, the flower clusters were removed from the spray arc and three arbitrarily selected flowers were excised from each cluster. Individual stigmas were removed from these flowers and placed in sterile microcentrifuge tubes – all stigmas associated with one cluster being placed in the same tube. One milliliter of potassium phosphate buffer (0.01 M, pH 6.7) was added to each tube prior to vortexing for 30 s and sonicating for 60 s to dislodge deposited bacteria. Appropriate dilutions were then made and plated in triplicate onto nutrient-yeast extract-dextrose agar [9]. Flowers treated with water as a control spray were similarly dilution-plated. Bacterial colonies were counted after 1 to 2 days, and population densities were expressed as CFU per stigma.

2.7. Statistical design of experiment and data analysis

The experiment investigating application method was set up as a split-plot statistical design with material (*i.e.*, Serenade® or water control) as the main-plot and application method (*i.e.*, hydraulic spray, electrostatic spray without charge, or charged electrostatic spray) as the sub-plots. There were four blocked replications carried out over time in separate experimental runs. Mean and standard error values were calculated from data across the four replications for each material and application method.

In the experiment investigating stigmatic deposition of *B. subtilis* in response to different application rates of Serenade® applied electrostatically, three blocked replications were carried out over time in separate experimental runs. Mean and standard error values were calculated from data across the replications for each rate and application method, and non-linear regression analysis (SigmaPlot v. 8.02; SPSS Inc., Chicago, IL) was applied to describe the relationship between

deposition (*i.e.*, CFU of *B. subtilis* per stigma) and Serenade® application rate for rates of 0x, ⅛x, ¼x, ½x and 1x of the 16.37-L/ha (7-qt/acre) recommended full-rate formulated product.

3. Results and Conclusions

Aqueous suspensions of the Serenade® formulation of *Bacillus subtilis* were satisfactorily atomized and sprayed by all application methods. Figure 4 presents treatment mean values for population densities showing 34 212, 48 611 and 152 129 CFU/stigma, respectively, deposited onto blueberry flowers by hydraulic sprays, electrostatic uncharged sprays, and electrostatic charged sprays. Electrostatic application of charged sprays differed significantly ($\alpha = 0.05$) from the other two methods; it deposited 4.5-times more *B. subtilis* than did conventional hydraulic spraying even though all application methods dispensed an equal 16.37-L/ha (7-qt/acre) amount of biocontrol agent. No statistically significant difference could be declared between population densities resulting from hydraulic *vs.* electrostatic uncharged spray applications.

For electrostatic charged spray application of *B. subtilis* onto blueberry flowers, Fig. 5 presents the effect which the relative rate of Serenade® (*i.e.*, spray-mix concentration) had upon the CFU/stigma population densities achieved. Results are based upon 56-L/ha (6-gal/acre) spray-mix application rate and full-rate (100%) Serenade® defined as 16.37 L/ha (7 qt/acre). Calculating a hemispherical stigmatic surface area (Fig. 1) of ~4 x 10^{-7} m^2, at the 100% rate each deposited CFU has an average ~2 μm^2 of nutrient-rich exudate-covered surface on which to grow, multiply, and provide biofungicidal protection. Additional study should elucidate the non-linear, saturation-implied, deposition response of Fig. 5.

In conclusion, this investigation showed that as compared with conventional hydraulic sprays, electrostatic forces can be incorporated to significantly increase by 4.5-fold the deposition of viable colony-forming units of the bacterial biocontrol agent *Bacillus subtilis* onto the small stigmatic surfaces of blueberry flowers. This experimental documentation, along with the rate-response

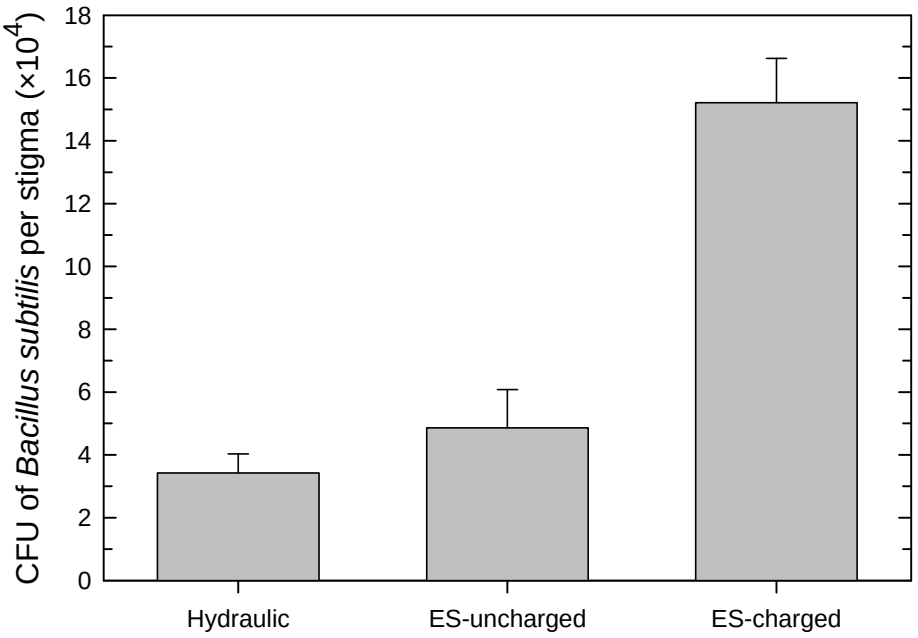

Fig. 4. Effect of spray application method of Serenade® biofungicide (*Bacillus subtilis* strain QST 713) on population densities of the biocontrol agent on detached blueberry flower stigmas in the laboratory. Values are means and standard errors of four independent experiments, each subsampled with three flower clusters per experiment and three flowers per cluster. CFU = colony-forming units, ES = electrostatic spray application.

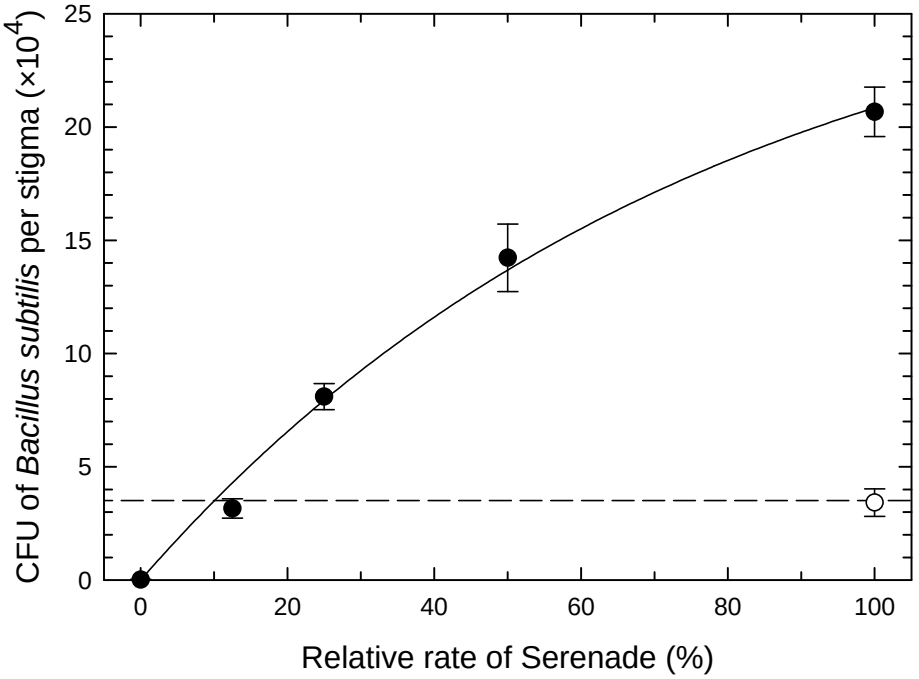

Fig. 5. Effect of relative rate of Serenade® biofungicide (*Bacillus subtilis* strain QST 713) on population densities of the biocontrol agent on detached blueberry flower stigmas when applied as an electrostatic spray in the laboratory. Values are means and standard errors of three independent experiments, each subsampled with three flower clusters per experiment and three flowers per cluster. The full rate (100%) corresponds to 16.37 L/ha (7 qt/acre) of formulated product. The regression equation is of the form $y = a\ (1 - e^{-bx})$, ($R = 0.997$, $P = 0.0002$). The dashed line and the open symbol with error bars indicate the mean population density of *B. subtilis* when applied as a hydraulic spray at full rate ($n = 4$). CFU = colony-forming units.

curve presented, illustrates the potential economic and environmental benefits offered by electrostatic crop-spraying technology. As has been demonstrated, as little as ⅛- to ¼-rates of

active ingredient dispensed by electrostatic charged sprays deposited equal amounts on target plant surfaces as did conventional hydraulic sprays dispensing full-rates of active ingredient. Inherent also is the logistical advantage of reduced total spray volumes with which to contend; for the field-sprayer applications modeled by this study, electrostatic sprays were successfully applied using only $\frac{1}{8}^{th}$ the volume of conventional methods.

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