Establishment of Bacterial Antagonists of *Erwinia amylovora* on Pear and Apple Blossoms as Influenced by Inoculum Preparation

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ABSTRACT

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The influence of inoculum preparation on the establishment of bacterial antagonists that suppress fire blight and *Erwinia amylovora* on blossoms was evaluated. Aqueous suspensions of *Pseudomonas fluorescens* A506, *E. herbicola* C9-1R, or *E. amylovora* 153N were prepared from cells harvested from the surface of an agar medium or from cells that were lyophilized after culture under similar conditions. Bacterial suspensions (1×10^8 CFU/ml) were sprayed on pear and apple trees at 50% bloom near midday. The incidence of recovery (proportion of blossoms containing detectable populations) and the population sizes of the bacteria on individual blossoms with detectable populations were followed over a period of several days. Fluorescent microspheres (1 μ m in diame-

ter) were added to sprays at a concentration of 1×10^7 microspheres per ml to mark blossoms that were open during application of bacteria. After dilution-plating, the stigmas and styles of each blossom were examined for the presence of microspheres with an epifluorescence microscope. In three of five trials, bacteria applied as suspensions of lyophilized cells were recovered from a greater proportion of blossoms than bacterial cells harvested directly from culture media. Every blossom harvested within 6 days after spraying had microspheres present on the surfaces of the styles and stigmas; thus, lack of establishment of detectable populations, rather than escape of blossoms from spray inoculation, accounted for the differences in proportion of blossoms colonized by the different preparations of bacteria. The use of lyophilized cells in field trials decreased variability in the establishment of bacteria on blossoms.

Additional keywords: biological control.

Fire blight, caused by *Erwinia amylovora*, is a serious disease of pear trees and is of increasing economic importance on apple, because the spectrum of commercial cultivars has expanded from the relatively resistant 'Red Delicious' to cultivars more susceptible to fire blight (e.g., 'Braeburn', 'Fuji', 'Gala', and 'Jonagold') (22,29). Severe epidemics of fire blight occur when rain or heavy dew and temperatures exceeding 15°C are coincident with full bloom of pear or apple (31). Under these conditions, *E. amylovora* colonizes the stigmatic surfaces of blossoms, where it establishes large population sizes before infecting the host (6,30,36). Pear and apple growers in the United States apply antibiotics during bloom to suppress growth and infection by the pathogen. Streptomycin has been the most effective antibiotic to control fire blight, but streptomycin-resistant isolates of *E. amylovora* are now prevalent in many regions of the United States (19–21). Streptomycin resistance in indigenous populations of *E. amylovora* has increased the risk of fire blight epidemics, thereby increasing the need for alternative control measures.

If applied to blossoms at early to mid-bloom, the biological control agents *Pseudomonas fluorescens* A506 (PfA506) and *E. herbicola* C9-1 (EhC9-1) can proliferate on pear and apple stigmas (11, 12,15,17,23,28,33) and exclude the pathogen from infection sites (6, 12,15,32,33). The incidence of fire blight on blossoms was reduced by about 60% with two applications of bacterial antagonists in experimental plots in the Pacific Northwest (12,23) and California (15,17). The efficacy of biological control approached or equaled levels obtained with chemical control in many of the field trials (17).

Biological control is most effective against fire blight if the antagonists are established in high populations prior to the introduction of *E. amylovora* to blossoms (23,33). Under field conditions, Johnson et al. (12) found that early establishment of populations exceeding 10^5 CFU per blossom of PfA506 and EhC9-1 on pear blossoms suppressed establishment and growth of *E. amylovora*, thereby decreasing disease incidence. In field plots, population sizes established by PfA506 or EhC9-1 on blossoms averaged 10^5 to 10^6 CFU per blossom; however, only 50 to 70% of the treated blossoms had detectable populations of the antagonists (28). The percentage of blossoms with detectable populations of PfA506 or EhC9-1 correlated inversely with the percentage of diseased blossoms (12,23). Because consistent and early colonization of stigmas by an antagonist is required to suppress fire blight, improvements in the initial establishment of *P. fluorescens* or *E. herbicola* on blossoms should enhance biological control of this disease.

Establishment of bacterial antagonists on aerial plant surfaces is a challenge in biological control. Antagonists that suppress disease in laboratory or greenhouse experiments may fail to establish threshold populations required for biological control in the field. In field trials, inoculum often consists of bacterial cells grown on solid media, harvested, resuspended, and sprayed onto plant surfaces. Aerial surfaces of plants can be inhospitable to bacterial growth, because they are exposed to UV light, rapid changes in temperature and water availability, and coincident fluctuations in osmotic potential and nutrient availability (3). For example, populations of *Pseudomonas* spp. applied to aerial surfaces of plants under hot and dry conditions may decrease rapidly to below the detection limit (1,2,8,13,14,24,34,35). Bacterial cells unable to survive the transition from a growth medium to an exposed plant surface do not establish population sizes needed to suppress disease. Current formulation technology may allow for the development of commercial preparations that optimize the capacity of bacterial antagonists to survive the conditions encountered immediately up-

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on inoculation of aerial plant surfaces. For example, the process of lyophilization imposes both temperature and desiccation stresses on bacterial cells (4) and could select for cells within a population that are tolerant to those stresses. The purpose of this investigation was to compare bacterial cells harvested directly from a culture medium with lyophilized cells for their establishment on blossoms of pear and apple.

MATERIALS AND METHODS

Experimental plot design. All experiments were conducted at the Oregon State University Department of Botany and Plant Pathology Field Laboratory near Corvallis on pear and apple trees spaced 6 m apart. In 1993, the experiments were conducted in a block of 50 34-year-old 'Rome Beauty' apple trees, in a block of 70 17-year-old 'Golden Delicious' apple trees, and in a block of 75 17-year-old 'Bosc' pear trees. In 1994, experiments were conducted in the block of 'Rome Beauty' apple trees used in 1993 and in a block of 90 30-year-old 'Bartlett' pear trees. Treatments were applied in a completely randomized block design with at least three single-tree replications of each treatment.

Bacterial strains. *PfA506*, obtained from S. E. Lindow (Department of Plant and Microbial Biology, University of California, Berkeley), is a rifampicin-resistant (100 µg/ml) derivative of an epiphyte recovered from pear in California (15). *EhC9-1*, obtained from C. A. Ishimaru (Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins), was isolated from an apple tree in Michigan (10). *EhC9-1R* is a spontaneous rifampicin-resistant (100 µg/ml) derivative that is similar to the parental strain in growth rate and herbicolidin production (10). *E. amylovora* strain 153 was isolated from a fire blight canker on apple in eastern Oregon. *E. amylovora* strain 153N (*Ea153N*) is a spontaneous nalidixic acid-resistant (100 µg/ml) derivative of strain *Ea153*. Pathogenicity of *Ea153N* was verified in previous field experiments (12,23). All bacterial strains were stored in nutrient broth containing 15% glycerol at -80°C until use.

Inoculum preparation. Bacteria were cultured for 5 days at 20°C on nutrient agar (Difco Laboratories, Detroit) amended with 1% (wt/vol) glycerol. To prevent contamination, rifampicin (50 µg/ml) was added to the medium for the culture of *PfA506* or *EhC9-1R*, and nalidixic acid (50 µg/ml) was added for the culture of *Ea153N*. Lyophilized inocula were prepared from bacterial cells harvested from the agar surface. The bacterial mass was weighed and mixed 2:1 (wt/vol) with a cryoprotectant slurry of 38% powdered milk and 2% xanthan gum before lyophilization as described by Johnson et al. (11). Lyophilized bacterial preparations were ground to a fine powder, passed through a 0.3-mm mesh screen, and stored at -20°C. Titer of the lyophilized preparations was determined by dilution-plating and ranged from 1×10^{10} to 1×10^{11} CFU/g. Fresh-cell inocula consisted of bacteria that were harvested from 5-day-old lawns and suspended in 10 mM potassium phosphate buffer, pH 7.0. The bacterial suspensions were adjusted to 0.1 optical density at 600 nm using a spectrophotometer (Spectronic 20; Milton Roy Co., Rochester NY) to approximate a cell density of 1×10^8 CFU/ml.

Application of bacteria. Bacteria were applied to trees at 50% bloom on 12 April 1993 ('Bosc' pear), 20 April 1993 ('Golden Delicious' apple), 29 April 1993 ('Rome Beauty' apple), 10 April 1994 ('Bartlett' pear), and 24 April 1994 ('Rome Beauty' apple). All treatments were made at noon, except in the 'Golden Delicious' plot, in which they were applied at 9:30 a.m. Fresh-cell bacterial suspensions were made within 1 h before inoculation. At the same time, lyophilized antagonists were suspended in 10 mM potassium phosphate buffer, pH 7.0, to obtain a cell density of 1×10^8 CFU/ml, which was confirmed by dilution-plating of the spray mixture. In the 1994 trials, *PfA506* and *EhC9-1R* also were sprayed at a lower dose of 1×10^7 CFU/ml. *Ea153N* was applied at three different inoculum doses: 1×10^8 CFU/ml on apples in 1993; 1×10^7 CFU/ml

on pears in 1993 and 1994 and on 'Rome Beauty' apple in 1994; and 1×10^6 CFU/ml in other 1994 trials. Bacterial suspensions were sprayed until near runoff to blossom clusters with a hand-held, 1-liter spray bottle. At least 75 blossom clusters on branches marked with plastic ribbon were inoculated on each tree.

Recovery of bacteria from blossoms. Open blossoms with black, mature anthers were collected 3 to 4 h after application of bacteria and periodically during the following week. Twenty-four ('Rome Beauty' apple, 'Golden Delicious' apple, and 'Bartlett' pear) or thirty blossoms ('Bosc' pear) were sampled from each treatment (six to eight blossoms per replicate tree) on each sampling date and placed immediately into individual wells of surface-disinfected, plastic, 12-well microtiter plates (Corning Inc., Corning, NY) to avoid cross-contamination during transport to the laboratory. The stigmas and styles of each apple blossom or the pistil and nectary of each pear blossom were excised and placed into test tubes containing 1 ml of sterile 10 mM potassium phosphate buffer, pH 7.1, and sonicated for 3 min. After sonication, a 10-µl sample of the buffer in the test tube containing pistillate tissue and 10-µl samples of two sequential 100-fold dilutions of the buffer were spread onto *Pseudomonas* agar F (Difco Laboratories) with 50 µg of cycloheximide per ml and 100 µg of rifampicin per ml for selective recovery of the bacterial antagonists or CCT medium (9) containing 50 µg of nalidixic acid per ml for recovery of the pathogen. The detection limit was 1×10^2 CFU per blossom.

Fluorescent microspheres. Fluorescent carboxylate microspheres (Fluoresbrite YG microspheres, 1 µm in diameter; Polysciences Inc., Warrington, PA) were added to bacterial suspensions at a concentration of 1×10^7 spheres per ml to mark blossoms that were open during spraying. After dilution-plating for recovery of bacteria from blossoms, 0.03% sodium azide was added to the buffer containing the blossom sample. Preserved tissue samples were stored at 4°C. The surface of each pistil was examined with a Zeiss standard epifluorescence microscope (485-nm excitation filter, 520-nm emission filter; Carl Zeiss, Inc., Thornwood, NY) for the presence of microspheres. The Fluoresbrite YG microspheres fluoresce a yellow-green color with that filter combination. Autofluorescent microspheres are available in several colors; however, we found that microspheres containing a blue-fluorescing dye (Fluoresbrite BB microspheres, Polysciences Inc.) faded after several days of exposure to the environment and could not be seen on plant tissues.

Weather data. Weather data were recorded during each of the experiments. Relative humidity, solar radiation, and wind speed data were obtained from an AgriMet weather station (Northwest Cooperative Agricultural Weather Network, Bonneville Power Administration, and the U.S. Bureau of Reclamation, Boise, ID) located near Corvallis. Moisture events and canopy temperatures were monitored with a CR-21X micrologger (Campbell Scientific, Inc., Logan, UT). Eight leaf wetness and four temperature sensors were placed on the periphery of the canopy at a height of 1.5 m in a tree within the plot. Four of the leaf wetness sensors were sprayed until near runoff with a bacterial suspension, and the duration of wetness (minutes) resulting from spray inoculation was recorded. Recordings from these four sensors were averaged.

Desiccation tolerance. Aqueous suspensions of lyophilized and fresh cells of *PfA506* and *EhC9-1R* (1×10^8 CFU/ml) were prepared as described above. Ten-microliter samples were placed onto 1-cm² pieces of sterile filter paper (Whatman No. 1; Whatman International, Ltd., Maidstone, England). Filter papers were incubated at 20°C suspended over distilled water or a saturated solution of NaCl in sealed plastic chambers to maintain a relative humidity of 100 or 75%, respectively (16). Five to seven times during a 24-h period, five replicate paper squares of each preparation were removed from the chambers, placed in 1 ml of phosphate buffer, sonicated, and 10-fold dilutions were spread on *Pseudomonas* agar F. Plates were incubated for 3 days at 27°C, and the colonies were counted. The experiment was repeated.

Data analysis. The population size of inoculated bacterial strains on blossom surfaces in some treatments was commonly below the minimum value (100 CFU per blossom) detectable by dilution-plating. Therefore, population data were presented as two values: the incidence of recovery, indicating the proportion of blossoms from which a bacterial strain was detected, and the size of detectable populations, indicating the mean population size only on those blossoms on which bacteria were detected (11,12,23,28). Taken together, the two values accurately represent the population data. Nevertheless, estimates of detectable population size presented here are not always directly comparable to population size estimates from data sets in which

values from replicates below the detection limit are set at zero (17), near the detection limit (7), or at values extrapolated from the distribution of populations on replicates in which bacteria were detected (7,26).

The SAS (Statistical Analysis Systems Institute, Cary, NC) General Linear Model procedure was used for statistical analysis of bacterial population data. Incidence data were arcsine square root-transformed before analysis. Fisher's protected least significant difference test ($P = 0.05$) was used to separate means. The analyzed incidence data were back-transformed and presented as percentages. Mean detectable population size and standard error of bacterial strains on individual blossoms were calculated by

Fig. 1. Establishment of lyophilized (●) and fresh cell (○) preparations of *Pseudomonas fluorescens* A506 (PfA506) applied to near runoff at a concentration of 1×10^8 CFU/ml on blossoms of **A and F**, 'Bosc' pear in 1993; **B and G**, 'Rome Beauty' apple in 1993; **C and H**, 'Golden Delicious' apple in 1993; **D and I**, 'Bartlett' pear in 1994; and **E and J**, 'Rome Beauty' apple in 1994. **A to E**, Incidence of recovery of PfA506 from blossoms (i.e., the proportion of blossoms with detectable populations). **A, D, and E**, The asterisks indicate that treatment means of transformed incidence of recovery of PfA506 differed significantly on a sampling date according to Fisher's least significant difference procedure ($P = 0.05$). **F to J**, The mean detectable population size of PfA506 on blossoms. Vertical bars, often obscured by the symbol, represent the standard error of the mean detectable population size.

Fig. 2. Establishment of lyophilized (●) and fresh cell (○) preparations of *Erwinia herbicola* C9-1R (EhC9-1R) applied to near runoff at a concentration of 1×10^8 CFU/ml on blossoms of **A and F**, 'Bosc' pear in 1993; **B and G**, 'Rome Beauty' apple in 1993; **C and H**, 'Golden Delicious' apple in 1993; **D and I**, 'Bartlett' pear in 1994; and **E and J**, 'Rome Beauty' apple in 1994. **A to E**, Incidence of recovery of EhC9-1R from blossoms (i.e., the proportion of blossoms with detectable populations). **D and E**, The asterisks indicate that treatment means of transformed incidence of recovery of EhC9-1R differed significantly on a sampling date according to Fisher's least significant difference procedure ($P = 0.05$). **F to J**, The mean detectable population size of EhC9-1R on blossoms. Vertical bars, often obscured by the symbol, represent the standard error of the mean detectable population size.

averaging the logarithm (base 10) of values obtained for blossoms on which bacteria were recovered.

In the desiccation tolerance studies, bacterial strains were recovered at detectable populations from all of the filter papers sampled. The populations were log-transformed, and Fisher's protected least significant difference test ($P = 0.05$) was used to separate treatment means.

RESULTS

Establishment of *PfA506*. Spray inoculation of pear and apple trees at 50% bloom with a suspension of lyophilized cells of *PfA506* (1×10^8 CFU/ml) resulted in the establishment of detectable populations on 70 to 100% of apple and pear blossoms in 1993 and 1994 (Fig. 1A to E). On those blossoms on which *PfA506* was detected, the mean detectable population size varied from 10^3 to 10^6 CFU per blossom following inoculation with lyophilized cells (Fig. 1F to J). In contrast, establishment of *PfA506* applied as fresh cells (1×10^8 CFU/ml) onto blossoms was more variable. In two trials ('Rome Beauty' and 'Golden Delicious' apple in 1993), *PfA506* established detectable populations on a similar proportion of blossoms when applied as fresh or lyophilized cells (Fig. 1B and C). In three trials, however, the incidence of establishment of fresh cells of *PfA506* on blossoms was significantly lower compared with the lyophilized preparation (Fig. 1A, D, and E). For example, at 4 h after application on 'Bosc' pear in 1993, 'Bartlett' pear in 1994, and 'Rome Beauty' apple in 1994, the incidence of recovery of fresh cells of *PfA506* was reduced 47, 92, and 83%, respectively, compared with the incidence of recovery of lyophilized cells. By late bloom in each of these trials, *PfA506* was recovered from a majority (70 to 88%) of blossoms inoculated with lyophilized cells, but from a significantly lower proportion of blossoms inoculated with fresh cells of the bacterium. In these trials, the mean detectable population size of *PfA506* on blossoms commonly was lower when inoculated as fresh cells compared with lyophilized cells of the antagonist (Fig. 1F, I, and J). In two trials in which the incidence of recovery of *PfA506* was similar between inoculum preparations (Fig. 1B and C), the mean detectable population sizes also were not significantly different between treatments (Fig. 1G and H).

In 1994 trials with 'Bartlett' pear and 'Rome Beauty' apple, *PfA506* was applied at both 1×10^8 CFU/ml and 1×10^7 CFU/ml. *PfA506* was recovered rarely from blossoms inoculated with fresh cells at 1×10^7 CFU/ml (data not shown). When comparing the two inoculum-dose treatments on the establishment of lyophilized cells of *PfA506*, the mean detectable population size recovered from blossoms differed significantly for 2 days after inoculation, but incidences of recovery (85 to 100% of blossoms) were not significantly different (data not shown). One week after applying lyophilized cells of *PfA506*, the incidence of recovery and the mean detectable population sizes on blossoms were not significantly different between the two inoculum doses.

Establishment of *EhC9-1R*. Similar to the results obtained with *PfA506*, application of suspensions of lyophilized cells of *EhC9-1R* (1×10^8 CFU/ml) on pear and apple trees at 50% bloom resulted in the establishment of detectable populations on 70 to 100% of blossoms sampled (Fig. 2A to E). At the first sample time, the bacterium was recovered at mean detectable population sizes of 10^4 to 10^5 CFU per blossom from nearly all blossoms sprayed with lyophilized cells (Fig. 2F to J). In all trials except the 'Bosc' pear trial, the mean detectable population size of *EhC9-1R* from lyophilized inocula increased 10- to 100-fold during bloom.

The establishment of *EhC9-1R* on blossoms inoculated with fresh cells at 1×10^8 CFU/ml was variable. In 1993 trials, there was no significant difference in the incidence of recovery of *EhC9-1R* from blossoms treated with fresh or lyophilized inocula (Fig. 2A to C). The mean detectable population size of *EhC9-1R* applied as fresh-cell inoculum was not significantly different than the mean detectable population sizes recovered from 'Rome Beauty' and 'Golden Delicious' apple blossoms treated with lyophilized preparations of the bacterium in 1993 (Fig. 2G and H). The mean detectable population size of *EhC9-1R* recovered during the first three sample times from 'Bosc' pear blossoms in 1993 treated with the fresh cells, however, was significantly lower than the mean detectable population size recovered from blossoms treated with the lyophilized cells (Fig. 2F), although mean detectable population sizes were not significantly different in later samples. Inoculum preparation had a greater effect on the establishment of *EhC9-1R* on blossoms in 1994 trials than in 1993 trials. Although similar numbers of bacterial cells were sprayed onto blossoms regardless

Fig. 3. Establishment of lyophilized (●) and fresh cell (○) preparations of *Erwinia amylovora* 153N (*Ea153N*) applied to near runoff at a concentration of 1×10^8 CFU/ml on blossoms of **A and F**, 'Golden Delicious' apple in 1993; **B and G**, 'Rome Beauty' apple in 1993; or at a concentration of 1×10^7 CFU/ml on blossoms of **C and H**, 'Bosc' pear in 1993; **D and I**, 'Bartlett' pear in 1994; and **E and J**, 'Rome Beauty' apple in 1994. **A to E**, Incidence of recovery of *Ea153N* from blossoms (i.e., the proportion of blossoms with detectable populations). **C, D, and E**, The asterisks indicate that treatment means of transformed incidence of recovery of *Ea153N* differed significantly on a sampling date according to Fisher's least significant difference procedure ($P = 0.05$). **F to J**, The mean detectable population size of *Ea153N* on blossoms. Vertical bars, often obscured by the symbol, represent the standard error of the mean detectable population size.

of the inoculum preparation method, few fresh cells survived on blossom surfaces until the first sampling time (4 h after application) in 1994 trials. *EhC9-1R* was recovered at population sizes near the detection limit (1×10^2 CFU per blossom) from only 5 to 35% of the blossoms sampled within 2 days after application of the fresh-cell preparation (Fig. 2D, E, I, and J). By late bloom in 1994, detectable fresh-cell populations averaged 10^4 CFU per blossom on 20 to 45% of the blossoms sampled.

Lyophilized cells of *EhC9-1R* applied in 1994 at 1×10^7 CFU/ml established mean detectable populations of 10^2 to 10^3 CFU per blossom (data not shown), which was significantly lower than mean detectable populations established when blossoms were sprayed with lyophilized cells at 1×10^8 CFU/ml (Fig. 2I and J). One week after inoculation, the mean detectable population size of *EhC9-1R* applied as lyophilized cells at 1×10^7 CFU/ml increased from 10^3 CFU per blossom to 10^5 CFU per blossom, which was not significantly different than the mean detectable populations on blossoms treated with *EhC9-1R* applied at 1×10^8 CFU/ml. The incidence of recovery of *EhC9-1R* applied as lyophilized cells at 1×10^7 CFU/ml and 1×10^8 CFU/ml was similar (95 to 100% of blossoms sampled) 4 h after spraying. One week after inoculation, *EhC9-1R* was recovered from significantly fewer blossoms treated with 1×10^7 CFU/ml (33% 'Bartlett' pear and 62% 'Rome Beauty' apple blossoms) compared with 1×10^8 CFU/ml (79% 'Bartlett' pear and 75% 'Rome Beauty' apple blossoms). When *EhC9-1R* was sprayed as a fresh-cell inoculum preparation at a concentration of 1×10^7 CFU/ml, the incidence of recovery of *EhC9-1R* ranged from 4% on 'Bartlett' pear blossoms and up to 50% on 'Rome Beauty' blossoms (data not shown).

Establishment of *Ea153N*. In 1993, inoculum preparation did not effect the incidence of recovery or the mean detectable popu-

lation size of *Ea153N* when applied at 1×10^8 CFU/ml to apple blossoms (Fig. 3A, B, F, and G). Four hours after spraying, mean detectable populations of 10^5 CFU per blossom were recovered from nearly every blossom inoculated. During the sampling period, *Ea153N* maintained statistically similar mean detectable population sizes of 10^5 to 10^6 CFU per blossom (Fig. 3F and G) and was recovered from 95 to 100% of the blossoms sampled (Fig. 3A and B), regardless of the preparation of the cells sprayed onto the blossoms.

Differential establishment of fresh or lyophilized cells of *Ea153N* applied at an inoculum dose of 1×10^7 CFU/ml was observed in blossoms sampled from 'Bosc' pear (1993), 'Bartlett' pear (1994), and 'Rome Beauty' apple (1994). The incidence of recovery on blossoms inoculated with lyophilized cells ranged from 43 to 96%, whereas the bacterium applied as fresh cells was recovered from significantly fewer blossoms (Fig. 3C to E). The mean detectable population size of *Ea153N* on 'Bartlett' pear and 'Rome Beauty' apple blossoms sprayed with fresh cells was significantly lower than populations established by lyophilized cells (Fig. 3I and J). On 'Bosc' pear blossoms, the mean detectable population size of *Ea153N* did not differ significantly between treatments 8 days after application (Fig. 3H).

Ea153N also was applied to 'Bartlett' pear and 'Rome Beauty' apple blossoms in 1994 at a concentration of 1×10^6 CFU/ml. The bacterium was not recovered from blossoms treated with a fresh-cell preparation at 1×10^6 CFU/ml in either trial (data not shown). *Ea153N* also was not recovered from blossoms of 'Bartlett' pear that were inoculated with lyophilized cells at 1×10^6 CFU/ml. In the 'Rome Beauty' apple trial (1994), *Ea153N* was recovered from 4 to 10% of the blossoms treated with lyophilized cells at 1×10^6 CFU/ml (data not shown), a significantly lower incidence of recovery compared with application of lyophilized cells at a concentration of 1×10^7 CFU/ml (Fig. 3E). The mean detectable population size of *Ea153N* recovered from 'Rome Beauty' apple blossoms inoculated with lyophilized cells at 1×10^6 CFU/ml ranged from 2×10^2 to 1×10^4 CFU per blossom during the sampling period (data not shown), which was significantly lower than the mean detectable populations recovered from blossoms inoculated with the lyophilized pathogen at 1×10^7 CFU/ml (Fig. 3J).

Detection of fluorescent microspheres on blossoms. Even after sonication, vortexing, and prolonged storage (>1 year) in buffer at 4°C, fluorescent microspheres applied to blossoms with the bacterial sprays were detected readily on floral tissues with an epifluorescence microscope (Fig. 4A). The majority of the microspheres remained attached to floral tissues under these storage conditions, but some microspheres also could be detected in the washing buffer. We did not observe samples in which microspheres were present in the wash buffer and not present in high numbers on the surfaces of the floral tissues. The relative density of microspheres from tissues harvested 4 h after application of bacteria was fairly uniform among individual blossom samples. The nectary surface of pear blossoms retained numerous microspheres. Within individual blossoms, each of the five styles and stigmas were coated with microspheres, although the relative density of microspheres varied from style to style.

In each of the trials, microspheres were detected on all of the treated blossoms harvested within 6 days after spraying. Microspheres were not detected on the styles or nectaries of blossoms harvested from nontreated branches (Fig. 4B). Detection of microspheres on the floral tissues confirms that all of the blossoms sampled within this time were sprayed directly with the bacterial suspensions. In three trials, blossoms were harvested 8 days after inoculation. Occasionally, one or two blossoms of the eight harvested from a replication in late bloom did not have microspheres, indicating that the blossoms were not sprayed directly with the bacterial suspension, probably because the blossoms were closed when bacteria were sprayed onto the cluster.

Environmental conditions during field experiments. From the time of inoculation to the first sample taken 4 h later, mean

Fig. 4. A, Epifluorescence micrographs of autofluorescent microspheres (1 μ m in diameter) on an apple stigma. B, Spheres were not visible on floral tissues of nontreated blossoms of apple. Bar represents 20 μ m.

relative humidity ranged from 43 to 61% among field trials (Table 1). Solar radiation varied from 400 to 650 J/m²/s during application of treatments (Table 1). Wind speed recorded in four of the plots was moderate, about 10 km/h. Little wind (0.4 km/h) was recorded in the 1993 'Rome Beauty' apple plot between the time of inoculation and harvest of the first sample (Table 1). Average canopy temperatures from the time of application of bacteria to the first harvest ranged from 14 to 18°C (Table 1). No rain or dew was recorded for at least 8 h after bacterial sprays in all plots, except in the trial with 'Golden Delicious' apple in 1993 (Table 1). Approximately 1.5 h after application of bacteria in that trial, a light rain fell for 2.3 h. The duration of wetness resulting from spraying was the least (9 min) in the 1994 'Bartlett' pear experiment (Table 1). The sensors remained wet for 30 min after inoculation in the 1993 'Rome Beauty' apple plot. In the 1993 'Golden Delicious' apple plot, the sensors did not dry before additional sensors in the canopy recorded rainfall.

Desiccation tolerance of *PfA506* and *EhC9-1R*. At 100% relative humidity, the mean population sizes of lyophilized and fresh cells of *PfA506* or *EhC9-1R* did not differ significantly and remained stable on filter papers, which remained damp throughout the experiment (Fig. 5A and B). The mean population size of fresh cells of *PfA506* was significantly lower than the population size of lyophilized cells after 3 h of incubation at 75% relative humidity (Fig. 5A). After 4 h of incubation at 75% relative humidity, the mean population size of lyophilized cells of *PfA506* was significantly lower than the mean populations maintained at 100% relative humidity. By this time, the mean population size of fresh cells of *PfA506* incubated at 75% relative humidity decreased by nearly 3 log units in repeated experiments (Fig. 5A). After an incubation period of 20 h at 75% relative humidity, the mean population sizes of fresh and lyophilized cells of *PfA506* did not differ significantly (Fig. 5A). Similar to *PfA506*, the mean population size of fresh cells of *EhC9-1R* was significantly lower than that of lyophilized cells after 3 h of incubation at 75% relative humidity (Fig. 5B). After 6 h of incubation at 75% relative humidity, the mean populations of fresh and lyophilized cells of *EhC9-1R* were significantly lower than the mean populations maintained at 100% relative humidity. The mean population size of fresh cells of *EhC9-1R* decreased by approximately 3 log units after 6 h of incubation at 75% relative humidity, which was a significantly greater decrease in the mean population size compared with that of lyophilized cells maintained under similar conditions (Fig. 5B).

DISCUSSION

Establishment of bacterial antagonists on plant surfaces is a critical phase in disease control. Populations of antagonists established on plant surfaces are necessary for competition with pathogens for sites and nutrients and to provide a reservoir for secondary spread to tissues not colonized by the initial treatment (11,12,15,23,33). We demonstrated that inoculum preparation can influence the es-

tablishment of bacteria on aerial plant surfaces. The lyophilized inoculum of two bacterial antagonists and a bacterial pathogen became established on a greater proportion of blossoms than did cells harvested directly from solid media under conditions in which blossoms dried within 15 min after spraying. Differences in establishment frequently were evident at the time of the first sample, 4 h after treatment. When blossoms remained wet for longer periods, cells from both inoculum preparations established well.

Under conditions conducive for bacterial survival and establishment, the mean population size of bacteria recovered from blossoms for both inoculum preparations were similar, and populations of bacteria incubated at 100% relative humidity remained stable for 20 h. This indicates that differences in nutritional composition of the inocula, i.e., skim milk and xanthan gum in the lyophilized preparation that were not present in the fresh inoculum, did not lead to detectable differences in establishment or growth of the bacteria. Prolonged moisture on the plant surface may have enhanced establishment by allowing bacteria to migrate in the water film to sites protected from desiccation and UV radiation. Others have demonstrated that mutants of *P. syringae* that lack motility establish smaller population sizes than do wild-type strains on leaves (5), presumably because they cannot move to protected sites. Furthermore, free moisture also may have allowed cell division and population size increase before conditions became nonconductive for growth and survival. In previous research on biological control of fire blight, the antagonists have, in general, established

Fig. 5. Mean population sizes of **A**, *Pseudomonas fluorescens* A506 and **B**, *Erwinia herbicola* C9-1R applied as fresh cells (■) or resuspended lyophilized cells (▲) to filter paper and incubated at 100% (open symbols) or 75% relative humidity (solid symbols). Lower case letters represent significant differences ($P = 0.05$) in the population size among treatment means at a sample time according to Fisher's least significant difference procedure. Results from repeated experiments were similar, so representative data from one experiment for each strain is presented.

TABLE 1. Summary of environmental conditions in Corvallis, OR, over a 4-h period from the time when bacterial suspensions were applied to pear and apple blossoms to the time of the first blossom sample

Cultivar and species	Year	Duration of wetness (min) ^a	Hours until natural moisture event ^b	Mean temperature (°C) ^c	Mean relative humidity (%) ^d	Mean wind speed (km/h) ^d	Mean solar radiation (J/m ² /s) ^d
'Bosc' pear	1993	12	12	14.3	44	10.6	651
'Rome Beauty' apple	1993	30	14	17.9	61	0.4	435
'Golden Delicious' apple	1993	>90 ^e	1.5	16.2	54	9.6	485
'Bartlett' pear	1994	9	10	16.3	43	10.6	402
'Rome Beauty' apple	1994	16	8	17.0	54	14.3	628

^a Duration of wetness represents the average measurement of four leaf wetness sensors to which a bacterial suspension was applied to near runoff at the time of inoculation.

^b Number of hours elapsed after inoculation before unsprayed leaf wetness sensors recorded a rainfall or dew event.

^c Average of four temperature probes placed in the periphery of the tree canopy.

^d Data obtained from a weather station located 4 km to the north of the experimental site.

^e The leaf wetness sensors did not dry between the time that bacteria were applied and the next rainfall event.

high populations on blossoms (11,12,15,17,23,28). In some of those experiments, the bacteria were harvested from solid media and applied under calm weather at sunrise, environmental conditions that would favor slow drying of blossoms after spraying.

On the whole, aerial surfaces of plants are considered to be inhospitable to bacterial growth, because they are exposed to UV radiation, rapid changes in temperature and water availability, and coincident fluctuations in osmotic potential and nutrient availability (3,13,14,16,24). As a consequence, bacteria are not distributed randomly on aerial plant surfaces, but instead are localized in cell junctures or are associated with stomates and trichomes, which provide habitats protected from full exposure to environmental stresses (1,14). Bacterial cells that persist after exposure to stress are most likely those that are established in protected microsites on plant surfaces and, thus, are able to rebound when conditions become favorable for growth (35). The capacity to survive desiccation and osmotic stress are epiphytic fitness traits of *P. syringae* (16). In *Escherichia coli*, such phenotypic traits and others of probable significance to epiphytic survival (i.e., tolerance of UV light) are expressed preferentially by stationary-phase cells (18). Compared with actively growing cells, stationary-phase cells are relatively tolerant of certain stresses imposed under laboratory experiments (18,25,27). The process of lyophilization exposes cells to stresses imposed by freezing and desiccation (4). We suggest that a stress-tolerant subset of a bacterial population survives these stresses. Alternatively, the whole population of lyophilized cells may differ physiologically from fresh cells, because of alterations imposed by the process of lyophilization. Therefore, viable cells present in lyophilized formulations may be more capable than the average bacterial cell residing on the surface of an artificial medium to survive the stresses encountered immediately upon application to plant surfaces. We examined tolerance of fresh and lyophilized cells to desiccation stress and found that lyophilized cells of *EhC9-1R* and *PfA506* were more tolerant to drying at 75% relative humidity than were fresh cells. In the orchard, the average relative humidity ranged from 43 to 61%, so differential tolerance to desiccation stress may have influenced establishment of the bacteria applied as fresh and lyophilized cells.

Enhanced survival of lyophilized cells of bacteria on plant surfaces also could be due to the presence of nonviable cells or protective materials in the inoculum preparation. Wilson and Lindow (35) showed that the addition of heat-killed cells of *P. syringae* to a bacterial suspension increased the established population size of the bacteria applied to plants maintained under arid conditions. Lyophilized preparations contain killed cells, which may contribute in part to the successful establishment of viable cells in the inoculum under stressful conditions on plant surfaces, although these killed cells and inoculant materials become relatively dilute in the spray suspension.

Cell concentration in inocula has been shown to be important in the establishment of bacteria on aerial surfaces. Wilson and Lindow (35) demonstrated in a growth chamber experiment that populations of *P. syringae* increase to the carrying capacity of leaves if the inoculum concentration is sufficient for the establishment of a single bacterium in each protected site present. We found that the incidence of establishment of lyophilized cells of bacteria on blossoms was similar when applied at concentrations of 1×10^8 CFU/ml or 1×10^7 CFU/ml and eventually achieved similar mean detectable population sizes on blossoms. Decreasing the inoculum dose of lyophilized cells of *E. amylovora* to 1×10^6 CFU/ml led to poor establishment, presumably because too few cells established in sites available on blossoms. When the inoculum dose of fresh cells of bacteria was decreased to 1×10^7 CFU/ml, the bacteria were only recovered rarely, which was expected because the higher inoculum dose of fresh cells also established poorly. Because lyophilized cells exhibited a greater tolerance of stressful conditions on plant surfaces, the applied inoculum dose was less critical to successful colonization.

Amending inoculants with fluorescent microspheres provided a simple, qualitative control to determine if the sprayed suspension was deposited on the stigmatic surface of sampled blossoms. For example, the observed differences in the establishment of bacteria on stigmatic surfaces could be due to lack of application of the inoculum onto the stigmatic surfaces or lack of survival of the inoculant. Lack of application could result from a blossom being closed during spraying or being turned at an angle that made it less assessable to the spray inoculum. In contrast, lack of survival could be concluded because the microspheres were easily visualized on the surfaces of treated tissues with an epifluorescence microscope. Mean detectable population sizes of bacteria recovered from blossoms treated with inocula containing microspheres were similar to those obtained from previous studies in which microspheres were not included (12,28); thus, the microspheres apparently do not affect establishment or growth of bacteria on stigmatic surfaces.

The bacterial antagonists *EhC9-1* and *PfA506* suppress fire blight of pear and apple (12,15,17,23), but, like many biocontrol agents, they have not been uniformly effective in all field conditions in which they have been tested. The proportion of blossoms that support populations of these bacterial antagonists is correlated positively to their success in suppressing fire blight (12,23). Therefore, methods that improve the consistent establishment of bacterial antagonists on blossoms are expected to reduce variations in the efficacy of biological control. In this study, lyophilized bacterial cells exhibited less variation in colonization of blossom surfaces under the spectrum of environmental conditions encountered in different field trials. Thus, the use of lyophilized cells of bacterial antagonists should mitigate one source of variability that has impeded the success of biological control for management of plant disease.

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