Secondary Colonization of Pear Blossoms by Two Bacterial Antagonists of the Fire Blight Pathogen

R. L. Nucló, K. B. Johnson, and V. O. Stockwell, Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331-2902; and D. Sugar, Southern Oregon Research and Extension Center, Oregon State University, Medford 97502

ABSTRACT

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Dispersal of the bacteria *Pseudomonas fluorescens* strain A506 and *Erwinia herbicola* strain C9-1S from treated to nontreated pear blossoms, and the effect of their spread on fire blight, were investigated in an orchard block of 10 rows containing 4 trees per row. Center rows of trees were sprayed with a mixture of *P. fluorescens* A506 and *E. herbicola* C9-1S at 30, 15, and 50% bloom in 1994, 1995, and 1996, respectively. Immediately after spraying, antagonists were detected only on treated blossoms. In 1994 and 1996, as bloom progressed, both *P. fluorescens* A506 and *E. herbicola* C9-1S were detected on nontreated blossoms located up to 4 rows (10 m) from the treated rows. In 1995, establishment of the antagonists on treated blossoms was poor and spread to nontreated trees was limited, apparently because of cold temperatures. Each year, honey bees were used to inoculate all trees with *E. amylovora* at 80% bloom. After full bloom in 1994 and 1996, the proportion of blossoms with *E. amylovora* populations $>10^5$ CFU per flower were highest in the outermost rows, and decreased linearly ($P < 0.05$) with proximity to treated rows. In 1994, diseased blossom clusters decreased significantly ($P < 0.05$) from the outermost rows to the treated rows, but there was no significant effect of distance on disease incidence in 1995 or 1996. Secondary colonization of blossoms by *P. fluorescens* A506 and *E. herbicola* C9-1S can play a role in disease suppression, but, among seasons, rates of secondary colonization by *P. fluorescens* A506 and *E. herbicola* C9-1S were variable, indicating that multiple applications of antagonists may be necessary to optimize biological control.

Additional keywords: *Pyrus communis*

Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al., is a serious disease of pome fruits in many regions of the world (21), including the Pacific Northwest region of the United States. Damage to pear and apple caused by *E. amylovora* includes blight of flower clusters, shoots, and fruits, and the development of cankers that expand progressively, resulting in dieback of branches and limbs (2,13,21). In the Pacific Northwest, infections are initiated most commonly in blossoms. Warm temperatures during bloom ($>15^{\circ}\text{C}$) enable cells of *E. amylovora* to multiply epiphytically on the stigmatic surfaces of blossoms, on which population sizes of 10^5 to 10^6 CFU per blossom can be measured. Large epiphytic populations of *E. amylovora* provide the inoculum for blossom infections (3,18,20,27) and for dispersal of the patho-

gen to other blossoms via insects, especially bees (6–8,22), or rain (21).

Currently, fire blight is controlled, in part, by spraying antibiotics onto blossoms to prevent epiphytic growth and infection by *E. amylovora*. Over the past three decades, however, frequent use of streptomycin has selected for strains of the pathogen resistant to this chemical and, as a result, streptomycin-resistant populations of *E. amylovora* have become established in the major production regions of the western United States (11,14,17). Oxytetracycline is an alternative antibiotic for which resistance in *E. amylovora* has not yet been detected. Disease control with this material, however, is only partially effective (12), and thus there is interest in new methods for suppression of this disease.

Fire blight is controlled biologically when epiphytic, antagonistic bacteria (e.g., *E. herbicola* or *Pseudomonas fluorescens*) are applied to and become established on the stigmatic surfaces of pear and apple blossoms prior to colonization by *E. amylovora* (1,7,10,25,26). Strains of *E. herbicola* typically produce antibiotics that are thought to suppress growth of the pathogen (5,24,28,29). In contrast, *P. fluorescens* strain A506 is not known to produce any antibiotics and likely inhibits *E. amylovora* by outcompeting the pathogen for growth-

limiting resources (26). *P. fluorescens* A506 is registered and commercialized for suppression of fire blight (BliteBan A506, Plant Health Technologies, Boise, ID), and there is interest in registering *E. herbicola* strain C9-1S for the same purpose. Inconsistent results, however, after application of these bacteria in orchards are still a concern (7). Population dynamics of bacterial antagonists in orchard environments is not well understood. Earlier studies with *E. herbicola* C9-1S and *P. fluorescens* A506 have shown that these two strains are effective when used individually or in a mixture, and that there is potential for these antagonists to spread to nontreated blossoms after application (6,7,19). Rate of secondary colonization of blossoms by *E. herbicola* C9-1S and *P. fluorescens* A506 may be important in attaining effective control because these bacteria are often applied at stages of bloom when many blossoms are closed and not exposed directly to the sprayed antagonist suspension. Understanding the importance of secondary colonization of nontreated blossoms by antagonists of *E. amylovora* may help to explain variation in control efficacy, and provide information for the refinement of control strategies in commercial orchards. The objectives of this study were to investigate secondary movement and colonization by *P. fluorescens* A506 and *E. herbicola* C9-1S among blossoms in a pear orchard, and the effects of this spread on epiphytic populations of *E. amylovora* and on subsequent development of fire blight.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used were *P. fluorescens* strain A506, *E. herbicola* strain C9-1S, and *E. amylovora* strain 153N. *P. fluorescens* A506 was isolated originally from a pear tree in California by S. Lindow (University of California, Berkeley) and has suppressed fire blight in greenhouse and field studies (7,10,23,26). *P. fluorescens* A506 is resistant to streptomycin and rifampicin. *E. herbicola* C9-1S is a streptomycin- and rifampicin-resistant derivative of *E. herbicola* C9-1 (16), which was isolated originally from an apple tree in Michigan by C. Ishimaru (Colorado State University, Fort Collins; 5). *E. amylovora* 153N was isolated from an apple canker from Milton-Freewater, Oregon. *E. amylovora* 153N is a spontaneous, nalidixic acid-resistant mutant that has

Corresponding author: K. B. Johnson
E-mail: johnsonk@bcc.orst.edu

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shown pathogenicity similar to its parent in laboratory studies (V. Stockwell and K. Johnson, unpublished data).

Inoculum preparation. In 1994 and 1995, bacteria were prepared for use in the field by culturing lawns of each strain on autoclaved, 0.3- by 0.5-m cafeteria trays that contained 0.5 liter nutrient agar (Difco Laboratories, Detroit) amended with 1% (wt/vol) glycerol. Rifampicin (100 mg liter⁻¹) was added to the media to culture *P. fluorescens* A506 and *E. herbicola* C9-1S, and nalidixic acid (25 mg liter⁻¹) was added to the media for culture of *E. amylovora* 153N. Bacteria were spread over the trays and harvested with a spatula after 5 days of incubation at 20 to 24°C. Harvested bacteria were freeze-dried (6), ground with a mortar and pestle, and screened to a final mesh size of 0.3 mm². Preparations of freeze-dried bacteria were stored at -50°C; titers of these preparations ranged from 1 × 10¹⁰ to 1 × 10¹¹ CFU g⁻¹ and were re-evaluated periodically by serial dilution plating. Commercial preparations of freeze-dried *P. fluorescens* A506 and *E. herbicola* C9-1S (Plant Health Technologies, Boise, ID) were used in the 1996 experiment; these preparations also ranged from 1 × 10¹⁰ to 1 × 10¹¹ CFU g⁻¹. Freeze-dried *E. amylovora* 153N prepared in 1995 was used in the 1996 experiment.

Enclosure used to study antagonist spread. Secondary colonization of blossoms by *P. fluorescens* A506 and *E. herbicola* C9-1S was evaluated in the spring of 1994, 1995, and 1996 at the Southern Oregon Research and Extension Center near Medford. Each year, 40 19- to 21-year-old Bartlett pear trees, 4 m in height, were enclosed with 30% shade cloth (Nicolon Corp., Norcross, GA) in early March. The shade cloth was supported by a frame of metal pipe secured with metal cables; dimensions of the enclosure were 25 by 15 m and 4.5 m in height. Trees in the enclosure were arranged in 10 rows of 4 trees per row (Fig. 1); within-row spacing was 3.5 m, with 2.5 m between rows.

Fig. 1. Layout of trees used to evaluate secondary colonization of Bartlett pear blossoms by *Pseudomonas fluorescens* strain A506 and *Erwinia herbicola* strain C9-1S. Trees within the study area were enclosed with shade cloth. In 1994 and 1995, trees in rows 5 and 6 (black circles) were treated with a mixture of *P. fluorescens* A506 and *E. herbicola* C9-1S at 30 and 15% bloom, respectively. In 1996, trees in rows 5, 6, and 7 (dotted circles) were treated with the bacteria at 50% bloom. Trees in other rows were not treated. In all years, at 80% bloom, honey bees were used to disperse *Erwinia amylovora* throughout the study area.

A hive of honey bees (*Apis mellifera* L.) was placed inside the enclosure at 5% bloom and removed at petal fall.

Antagonist application. In 1994 and 1995, trees in rows 5 and 6 were treated with an equal mixture of *P. fluorescens* A506 and *E. herbicola* C9-1S at 30% (5 April) and 15% bloom (19 March), respectively. In 1996, trees in rows 5, 6, and 7 were treated with the antagonist suspension at 50% bloom (31 March). Suspensions of *P. fluorescens* A506 and *E. herbicola* C9-1S were prepared in the field by mixing pre-weighed packets of freeze-dried cells in 400 ml water in a 500-ml nalgene bottle. The bottles were agitated by hand until the bacteria became resuspended (10 min). Resuspended bacteria were then mixed with water in a 9-liter backpack sprayer to attain a 1:1 mixture of *P. fluorescens* A506 and *E. herbicola* C9-1S, each at a concentration of 5 × 10⁷ CFU ml⁻¹. Fluorescent carboxylate microspheres (Fluoresbrite YG microspheres, 1 µm diameter, Polysciences Inc., Warrington, PA) also were added to the suspension to obtain a concentration of 1 × 10⁷ spheres ml⁻¹. The purposes for the microspheres were to mark the blossoms that were open at time of application (15) and to indicate possible drift of the sprayed antagonist suspensions to blossoms on

nontreated trees. Suspensions of *P. fluorescens* A506 and *E. herbicola* C9-1S were applied with a hand-held wand attached to a backpack sprayer. Applications of antagonists were made shortly after sunrise (6:00 to 7:30 A.M.) under still wind conditions. Two to three liters of the antagonist suspension were applied to each treated tree; this amount was sufficient to treat blossoms to near run-off.

Pathogen inoculation. At 80% bloom (10 April 1994, 28 March 1995, and 3 April 1996), trees within the enclosure were inoculated with freeze-dried *E. amylovora* 153N using honey bees. Freeze-dried *E. amylovora* 153N was placed in a pollen insert (Antles Pollen Supplies, Wenatchee, WA) attached to the entry platform of the bee hive located inside the enclosure, and bees became contaminated with the pathogen as they exited the hive (6). The pollen insert was attached to the hive for 2 to 3 h beginning at approximately 11:00 A.M.; 10 to 15 g of freeze-dried *E. amylovora* 153N was dispensed by the bees during this period.

Bacterial incidence in blossoms. Dilution plating was used to estimate the incidence of recovery and population sizes of *P. fluorescens* A506, *E. herbicola* C9-1S, and *E. amylovora* 153N on blossoms sam-

Fig. 2. Incidence of recovery of *Pseudomonas fluorescens* strain A506 (clear bars) and *Erwinia herbicola* strain C9-1S (hatched bars) on blossoms sampled from Bartlett pear trees located at Medford, Oregon. In 1994, 1995, and 1996, trees in rows labeled "0" were treated with a suspension of *P. fluorescens* A506 and *E. herbicola* C9-1S at 30, 15, and 50% bloom, respectively. Blossoms were sampled at (A-C) 2 h after treatment, (D-F) 80% bloom, (G-I) full bloom, and (J-L) 3 to 8 days after full bloom.

pled periodically from trees within the enclosure. Sample dates were 5, 8, 10, 13, 18, and 21 April 1994; 19, 25, and 28 March and 1 and 4 April 1995; and 31 March and 3, 5, 8, and 11 April 1996. On each sample date, 8 mature blossoms (dark anthers) were sampled randomly from each tree. Blossoms were placed individually into wells of sterile microtiter plates and transported to the lab in a cooler. Blossoms were dissected using flamed forceps; sepals, petals, and stamens were discarded (these tissues do not support biologically significant bacterial growth; 3,18,25-27). The hypanthium with attached pistils of each blossom were placed individually into test tubes containing sterile potassium phosphate buffer (0.5 M, pH 6.5) and dilution-plated as described previously (7). The detection limit of the dilution plating procedure was 2.2×10^2 CFU per blossom. *Pseudomonas* Agar F (Difco Laboratories) amended with 100 mg/liter rifampicin was used for selective recovery of *P. fluorescens* A506 and *E. herbicola* C9-1S, and CCT medium (4) amended with 50 mg/liter nalidixic acid was used for selective recovery of *E. amylovora* 153N. Colonies of *P. fluorescens* A506, *E. herbicola* C9-1S, and *E. amylovora* 153N were

counted after 3 to 4 days incubation at 20 to 24°C. Colonies of *P. fluorescens* A506 and *E. herbicola* C9-1S were counted on the same plate and were differentiated based on colony size, color, and morphology. Sampled pistils and hypanthia also were examined for fluorescent microspheres after dilution plating. These floral parts were preserved by adding sodium azide (2 ng/ml) to each test tube and storing at 4°C until examination.

Bacterial incidence on honey bees. In 1996, honey bees foraging within the enclosure were sampled to determine if *E. herbicola* C9-1S and *P. fluorescens* A506 could be recovered from their bodies. Dates of sampling were 3, 5, and 8 April, which corresponded to 80% bloom, full bloom, and 3 days into full bloom, respectively. On each date, 18 foraging bees were captured individually in petri plates containing *Pseudomonas* Agar F with rifampicin. The plates containing the bees were agitated by hand to promote movement of the bees across the surface of the medium; the bee was released after 30 to 45 s. In addition, 18 bees were captured individually and drowned in sterilized glass jars containing 2 ml sterile phosphate buffer. In the laboratory, 1-ml aliquots of buffer from

the jars were spread on petri plates containing *Pseudomonas* Agar F with rifampicin. Plates for both capture methods were inspected for *P. fluorescens* A506 and *E. herbicola* C9-1S colonies after 3 days of incubation at 20 to 24°C.

Disease assessment. Prior to bloom, the number of blossom clusters on each experimental tree were counted. After bloom, the number of diseased blossom clusters were counted on each tree as symptoms developed over a 2- to 5-week period (4, 10, 19, 24, and 31 May 1994; 27 April, 2, 11, 18, and 25 May, and 1 June 1995; and 3 and 10 April 1996). Diseased clusters were pruned from trees on the date of detection. Cumulative disease incidence was determined for each tree by dividing the total number of diseased blossom clusters observed over all assessment dates by the prebloom count of total blossom clusters.

Microsphere detection. Estimates of fluorescent microspheres adhering to pistils of sampled blossoms were made with the aid of an epifluorescence microscope (485 nm excitation filter, 520 nm emission filter). In 1994, all microspheres observed on pistils were counted initially but, because of the large numbers of spheres on many treated blossoms, the majority of

Table 1. Mean population size^a of *Erwinia herbicola* strain C9-1S and *Pseudomonas fluorescens* strain A506 recovered from blossoms of Bartlett pear growing near Medford, Oregon in 1994

Tree row ^b	Sampling date in April											
	5		8		10		13		18		21	
	C9-1S	A506	C9-1S	A506	C9-1S	A506	C9-1S	A506	C9-1S	A506	C9-1S	A506
4 west	...	...	2.83	4.47	...	3.46	2.35	3.74 (0.73)	3.91	4.24 (0.73)	3.67 (0.53)	4.37 (0.64)
3 west	...	...	1 3.54 (0.35)	1 3.66 (0.61)	...	1 2.83	1 3.92 (1.26)	6 3.96 (0.85)	1 3.59 (0.07)	13 4.65 (0.69)	3 4.15 (0.77)	7 4.55 (0.65)
2 west	...	...	2 2.86 (0.17)	3 3.48 (0.12)	...	1 ...	2 3.03 (0.53)	7 4.02 (0.66)	2 3.67 (0.91)	15 4.32 (1.26)	3 3.72 (0.88)	13 4.55 (0.6)
1 west	...	...	4 3.46 (0.12)	4 3.32 (0.43)	3.20 (0.29)	3.89 (0.67)	4.27 (0.71)	4.56 (0.95)	4.61 (0.82)	5.00 (0.89)	4.41 (0.89)	5.19 (0.71)
0 west	4.56 (0.64)	4.74 (0.52)	2 3.78 (0.85)	3 4.27 (0.81)	4 4.44 (0.87)	7 4.86 (1.08)	13 4.79 (1.25)	24 5.24 (1.16)	17 4.60 (0.64)	25 5.33 (0.88)	15 4.99 (0.86)	25 5.53 (0.92)
0 east	32 4.57 (0.76)	32 4.81 (0.80)	25 3.33 (0.55)	30 4.25 (0.76)	26 4.37 (1.16)	31 4.91 (1.13)	23 5.56 (0.75)	32 5.71 (1.15)	26 4.97 (0.64)	32 5.67 (0.70)	22 5.30 (0.75)	30 5.72 (0.74)
1 east	...	...	27 3.04 (0.39)	30 3.22 (0.52)	25 3.61 (0.69)	31 4.56 (0.53)	22 4.25 (1.26)	31 4.66 (1.10)	23 4.71 (0.91)	31 5.23 (0.89)	21 4.16 (0.99)	31 4.82 (0.94)
2 east	...	...	2 ...	5 ...	7 ...	9 ...	14 4.26 (0.52)	27 4.20 (0.76)	13 3.93 (0.92)	26 4.48 (0.93)	21 4.83 (1.02)	29 4.69 (0.82)
3 east	...	...	...	...	...	2.65	4 3.30 (0.53)	13 3.69 (0.25)	7 3.40 (0.89)	15 4.66 (0.81)	4 3.51 (0.81)	17 4.73 (0.70)
4 east	...	...	2.65	3.33 (0.50)	...	1 3.52 (0.47)	1 ...	7 3.56 (0.43)	3 4.03	12 4.39 (0.70)	8 4.62 (0.38)	16 4.85 (0.83)
			1	2		2		4	1	12	2	14

^a Means are expressed as log₁₀(CFU per blossom) followed by the standard deviation in parentheses and the number of blossoms averaged. On each date, 32 blossoms were sampled from each row with the exception of row 2 east, which had 24 blossoms sampled on each date. Blossoms from which a bacterial strain was not recovered were excluded from the mean. The detection limit of the dilution plate assay was 2.2×10^2 CFU/ml.

^b Tree row indicates the number of rows from row 0 east or 0 west which were treated with *P. fluorescens* A506 and *E. herbicola* C9-1S.

^c Bacteria were not recovered on this date.

blossoms were categorized into one of the following classes: 0 = no microspheres, 1 = 1 to 10 spheres, 2 = 11 to 100 spheres, and 3 = >100 spheres. In 1995 and 1996, only the presence or absence of microspheres on pistillate surfaces of sampled blossoms was determined. All sampled blossoms from each treated tree and from the 16 nontreated trees most closely adjacent to the treated rows were examined for each sampling date in each year.

Data summary and analysis. Frequency of recovery and mean population size of *P. fluorescens* A506, *E. herbicola* C9-1S, and *E. amylovora* 153N were computed for each tree on each sample date. Prior to averaging, estimates of bacterial population size on individual blossoms were transformed to $\log_{10}$. Blossoms on which a bacterial strain was not detected were excluded from estimates of mean population size per tree. On sample dates after the pathogen introduction, the proportion of blossoms with populations of *E. amylovora* 153N $>1 \times 10^5$ CFU per blossom was computed for each tree.

For sample dates near full bloom, mean incidence and population size of *P. fluorescens* A506 and *E. herbicola* C9-1S on each nontreated tree was regressed on distance (tree row) from the row of trees treated with the antagonist suspensions, with separate models developed for nontreated trees on the east and west sides of the enclosure. Similarly, mean incidence and population size of *E. amylovora* 153N on blossoms, mean incidence of diseased blossom clusters, and the proportion of blossoms with populations of *E. amylovora* 153N $>1 \times 10^5$ CFU per blossom were regressed on distance from trees treated with the antagonist suspensions. Because of non-significance of models developed for 1995 and 1996 data concerned with *E. amylo-*

vor 153N and disease, these regressions analyses were repeated by adding the trees treated with *P. fluorescens* A506 and *E. herbicola* C9-1S to the data sets. Thus, in these latter analyses, separate regression models were developed for tree rows 1 to 5 (west) and 6 to 10 (east) in 1994 and 1995, and for tree rows 1 to 6 and 6 to 10 in 1996 (Fig. 1).

RESULTS

Establishment and secondary spread of bacterial antagonists. In 1994, at 2 h after application of the antagonist suspension, *P. fluorescens* A506 and *E. herbicola* C9-1S were recovered from 100% and 94%, respectively, of fully opened blossoms sampled from treated trees ($n = 64$), but they were not recovered from any of the 248 blossoms sampled from nontreated trees (Fig. 2A). At 80% bloom (3 days after treatment), incidence of recovery of *P. fluorescens* A506 and *E. herbicola* C9-1S on blossoms from treated trees had declined to 94 and 80%, respectively, but both of the antagonists began to be recovered from blossoms of nontreated trees adjacent to treated trees (Fig. 2D). During the remainder of the bloom period, mean incidence of recovery of *P. fluorescens* A506 and *E. herbicola* C9-1S from blossoms from treated trees ranged from 95 to 99% and 67 to 80%, respectively. By the last sample (21 April), mean incidence of detection of antagonist strains on blossoms of nontreated trees immediately adjacent to treated trees had increased to 84 and 56% for *P. fluorescens* A506 and *E. herbicola* C9-1S, respectively (Fig. 2G and J). In addition, on the last two sample dates (13 and 16 days after treatment), *P. fluorescens* A506, *E. herbicola* C9-1S, or both were

recovered from blossoms sampled from all trees within the enclosure (Fig. 2G and J). On 13 April, at full bloom, incidence of recovery of *P. fluorescens* A506 and *E. herbicola* C9-1S on nontreated trees decreased significantly with distance of a sampled tree from the row of treated trees (*P. fluorescens* A506 west: intercept = 0.87, slope = -0.18, $P < 0.001$, $r^2 = 0.68$; *P. fluorescens* A506 east: intercept = 1.08, slope = -0.26, $P < 0.001$, $r^2 = 0.88$; *E. herbicola* C9-1S west: intercept = 0.49, slope = -0.13, $P = 0.049$, $r^2 = 0.90$; *E. herbicola* C9-1S east: intercept = 0.63, slope = -0.18, $P = 0.016$, $r^2 = 0.59$).

In contrast, in 1995, establishment and detection of *P. fluorescens* A506 and *E. herbicola* C9-1S on blossoms was much reduced compared to 1994. *P. fluorescens* A506 and *E. herbicola* C9-1S were recovered from 98 and 97%, respectively, of the 64 blossoms sampled from treated trees at 2 h after application, but neither bacterium was detected on blossoms sampled from nontreated trees ($n = 248$; Fig. 2B). Mean incidence of *P. fluorescens* A506 and *E. herbicola* C9-1S on blossoms from treated trees declined to 40 and 29%, respectively, by 25 March, and to 16 and 8%, respectively, by 1 April (full bloom; Fig. 2H). For nontreated trees, *P. fluorescens* A506 was not detected on any blossoms sampled on 25 or 28 March or 1 April ($n = 744$), but was recovered from 1 of 248 blossoms sampled on 4 April (near petal fall; Fig. 2E, H, and K). *E. herbicola* C9-1S was not detected on any of the nontreated blossoms sampled on 25 or 28 March or 1 or 4 April ($n = 992$; Fig. 2E, H, and K).

In 1996, patterns of initial establishment and detection of *P. fluorescens* A506 and *E. herbicola* C9-1S on nontreated trees were similar to results from 1994 (Fig. 2). At 2 h after the antagonist treatment, *P. fluorescens* A506 and *E. herbicola* C9-1S were recovered from 98 and 95%, respectively, of 86 blossoms sampled from treated trees, and were not recovered from any of the 216 blossoms sampled from nontreated trees (Fig. 2C). At 80% bloom (3 April), incidence of *P. fluorescens* A506 and *E. herbicola* C9-1S on blossoms sampled from treated trees averaged 91 and 94%, respectively, and both antagonists began to be recovered in low frequencies from blossoms on nontreated trees (Fig. 2F). As bloom progressed, mean incidence of *P. fluorescens* A506 and *E. herbicola* C9-1S from blossoms of nontreated trees immediately adjacent to treated trees had increased to 70 and 78%, respectively; both strains were recovered from nearly all nontreated trees by the end of bloom (Fig. 2I and L). On 8 April, 3 days into full bloom, incidence of recovery of *P. fluorescens* A506 and of *E. herbicola*

Fig. 3. Percent of Bartlett pear blossoms with fluorescent microspheres adhering to pistillate surfaces at 0 to 16 days after treatment with a microsphere suspension. Data are from experiments conducted in Medford, Oregon during 1994 (diamonds), 1995 (squares), and 1996 (triangles). Suspensions applied to trees also contained *Pseudomonas fluorescens* A506 and *Erwinia herbicola* C9-1S. Estimated bloom stage at time of application was 35, 15, and 50% in 1994, 1995, and 1996, respectively.

Fig. 4. Incidence of recovery of *Pseudomonas fluorescens* strain A506 (clear and diagonal hatching) and *Erwinia herbicola* strain C9-1S (black and cross-hatched) on honey bees sampled during bloom of Bartlett pear at Medford, Oregon in 1996. Dates of sampling were 3, 5, and 8 April, which corresponded to 80% bloom, full bloom, and 3 days into full bloom, respectively. Clear and black bars represent bees captured in the orchard on petri plates containing selective media; diagonal and cross-hatched bars represent bees sampled in the field in jars containing 2 ml sterile buffer; a 1.0-ml aliquot of the buffer was spread onto a selective medium in the laboratory.

C9-1S decreased significantly with distance of a sampled tree from the row of treated trees (*P. fluorescens* A506 west: intercept = 0.86, slope = -0.19, $P = 0.002$, $r^2 = 0.52$; *P. fluorescens* A506 east: intercept = 1.07, slope = -0.24, $P < 0.001$, $r^2 = 0.66$; *E. herbicola* C9-1 west: intercept = 0.92, slope = -0.20, $P < 0.001$, $r^2 = 0.66$; *E. herbicola* C9-1 east, intercept = 1.04, slope = -0.18, $P = 0.019$, $r^2 = 0.41$).

In 1994 and 1996, mean population sizes of *P. fluorescens* A506 and *E. herbicola* C9-1S on blossoms from nontreated trees also were consistent with the hypothesis that these bacteria had dispersed from treated trees. Initial populations sizes of both antagonists on blossoms of treated trees were in the range of 4.5 to 5.7 log CFU per blossom. As bloom progressed, similar population sizes of *P. fluorescens* A506 and *E. herbicola* C9-1S were detected on blossoms located at progressively greater distances from treated rows (1994, Table 1; 1996, data not shown). At full bloom, for two of four regression models, mean population size of *P. fluorescens* A506 decreased significantly with distance of nontreated trees from the row of treated trees (1994 east: intercept = 5.38, slope =

-0.28, $P = 0.009$, $r^2 = 0.45$; 1996 east: intercept = 5.20, slope = -0.55, $P = 0.007$, $r^2 = 0.57$). Similarly, for four of four regression models, mean population size of *E. herbicola* C9-1S decreased significantly with distance of nontreated trees from the row of treated trees (1994 west: intercept = 5.31, slope = -0.64, $P = 0.006$, $r^2 = 0.55$; 1994 east, intercept = 5.49, slope = -0.75, $P = 0.006$, $r^2 = 0.69$; 1996 west: intercept = 4.63, slope = -0.42, $P < 0.001$, $r^2 = 0.81$; 1996 east, intercept = 5.58, slope = -0.56, $P = 0.007$, $r^2 = 0.50$).

In 1995, on 19 March, shortly after antagonist treatment, mean population size of *P. fluorescens* A506 and *E. herbicola* C9-1S on treated trees was in the range of 5.5 to 5.7 log CFU per blossom (data not shown). On subsequent dates, the mean population sizes of both antagonists on blossoms from treated trees were smaller, ranging from 2.5 to 4.6 log CFU per blossom. Regression analyses were not conducted because these organisms were not detected on nontreated trees.

Detection of fluorescent microspheres.

Each year, fluorescent microspheres were detected on pistillate surfaces of 100% of fully opened blossoms sampled from

treated trees at 2 h after application. Subsequently, as more blossoms opened, the proportion of open blossoms with microspheres on pistillate surfaces declined (Fig. 3). On the last sample dates of each year, microspheres were detected on the pistils of 38, 10, and 53% of blossoms sampled in 1994, 1995, and 1996, respectively, which corresponded approximately with the estimated bloom stages at the time of treatment in each year. No microspheres were detected on blossoms from nontreated trees on any sampling date in any of the three seasons ($n = 1872$). For blossoms with microspheres in 1994 ($n = 196$), 6% of blossoms were classified with 1 to 10 microspheres on pistillate surfaces, 16% with 11 to 100 spheres, and 78% with >100 spheres; the relative size of each class of microsphere number remained constant among sampling dates.

Bacterial incidence on honey bees.

Colonies of *P. fluorescens* A506 and *E. herbicola* C9-1S developed on the selective medium in petri dishes used to capture foraging honey bees at 3 to 8 days after the antagonist application. Similarly, dilution plating of buffer in jars used to capture foraging bees resulted in the recovery of *P. fluorescens* A506 and *E. herbicola* C9-1S on the selective medium. Frequency of recovery of *P. fluorescens* A506 and *E. herbicola* C9-1S averaged 48 and 55%, respectively, for bees captured on petri plates, and 63 and 64%, respectively, for bees captured in jars (Fig. 4).

Establishment of *E. amylovora* 153N on blossoms.

Each year, incidence of recovery of *E. amylovora* 153N in individual pear blossoms increased from 0% in early bloom to an average of 76 to 82% at petal fall (21 April 1994, 4 April 1995, and 11 April 1996). In 1994, mean population size of *E. amylovora* 153N ranged from 2.4 to 3.9 log CFU per blossom shortly after introduction of *E. amylovora* 153N and increased to 3.9 to 6.1 log CFU per blossom for all subsequent sample dates (Table 2). A similar increase in population size was observed in both 1995 and 1996 (data not shown). Incidence of recovery of *E. amylovora* 153N was not affected significantly ($P > 0.05$) by tree row on any of the sample dates in any year. In 1994, however, at full bloom, incidence of blossoms with *E. amylovora* 153N population sizes $>1 \times 10^5$ CFU per blossom increased significantly with distance from the rows treated with the antagonistic bacteria (west: intercept = 0.29, slope = 0.08, $P < 0.015$, $r^2 = 0.37$; east: intercept = 0.34, slope = 0.08, $P < 0.025$, $r^2 = 0.33$). Similarly, at full bloom in both 1994 and 1996, incidence of blossoms with *E. amylovora* 153N population sizes $>1 \times 10^5$ CFU per blossom increased significantly with distance from the treated rows if data from the treated trees were included in the regression analysis (1994 west: intercept = 0.26, slope = 0.084, $P < 0.001$, $r^2 = 0.59$; 1994

Table 2. Mean population size^a of *Erwinia amylovora* strain 153N recovered from blossoms of Bartlett pear growing near Medford, Oregon in 1994

Tree row ^b	Sampling date in April				
	5	10	13	18	21
4 west	... ^c	3.23 (0.35)	4.53 (0.73)	5.24 (1.18)	5.79 (1.17)
3 west	...	4 2.65	15 4.36 (0.90)	26 5.25 (1.24)	26 6.06 (0.96)
2 west	...	1 3.87 (0.08)	15 4.42 (1.08)	28 5.25 (1.29)	27 5.56 (1.07)
1 west	...	2 2.66 (0.30)	17 4.28 (1.23)	24 4.72 (1.26)	26 5.33 (1.08)
0 west	...	2 2.43 (0.13)	14 4.65 (0.87)	21 4.82 (1.08)	19 5.43 (1.22)
0 east	...	4 2.81 (0.63)	13 4.15 (0.95)	23 4.50 (0.99)	20 5.02 (1.37)
1 east	...	5 2.82 (0.47)	9 4.80 (0.86)	21 5.09 (1.25)	22 5.16 (1.61)
2 east	...	3 ...	12 4.19 (0.79)	24 5.05 (1.22)	24 5.39 (1.27)
3 east	...	11 2.88 (0.43)	18 4.62 (0.94)	17 5.52 (1.30)	17 5.74 (1.05)
4 east	...	5 ...	16 3.88 (1.33)	28 5.73 (1.04)	27 5.97 (1.23)
			16	25	30

^a Means are expressed as log₁₀ (CFU per blossom) followed by the standard deviation in parentheses and the number of blossoms averaged. On each date, 32 blossoms were sampled from each row with the exception of row 3 east, which had 24 blossoms sampled on each date. Blossoms from which a bacterial strain was not recovered were excluded from the mean. The detection limit of the dilution plate assay was 2.2×10^2 CFU/ml.

^b Tree row indicates the number of rows from row 0 east or 0 west which were treated with *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S.

^c Bacteria were not recovered on this date.

east: intercept = 0.29, slope = 0.097, $P < 0.001$, $r^2 = 0.52$; 1996 west: intercept = 0.35, slope = 0.035, $P = 0.003$, $r^2 = 0.35$; 1996 east: intercept = 0.51, slope = 0.043, $P < 0.001$, $r^2 = 0.50$; Fig. 5A and C). In 1995, when secondary spread of *P. fluorescens* A506 and *E. herbicola* C9-1S did not occur, the incidence of recovery of *E. amylovora* 153 population sizes $>1 \times 10^5$ CFU per blossom was not affected significantly by distance of a tree from the row of treated trees ($P > 0.05$) on any of the sample dates (Fig. 5B).

Disease incidence. In 1994, incidence of diseased blossom clusters averaged 2.9% in the tree rows treated with *P. fluorescens* A506 and *E. herbicola* C9-1S, and 4.5% in the rows most distal to the tree treated with the antagonist suspension (Fig. 5D). Incidence of blighted blossom clusters in nontreated trees increased significantly with distance from the row treated with *P. fluorescens* A506 and *E. herbicola* C9-1S (west: intercept = .017, slope = 0.05, $P < 0.004$, $r^2 = 46\%$; east: intercept = 0.027, slope = 0.08, $P < 0.001$, $r^2 = 73\%$).

In 1995 and 1996, the incidence of blighted blossom clusters averaged 16 and 43%, respectively, with no difference occurring among trees that received the antagonist suspension compared to those that did not. Models for disease incidence regressed on tree row were not significant ($P > 0.05$) in either season (Fig. 5E and F).

DISCUSSION

In two of three experiments (1994 and 1996), *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S developed significant populations on blossoms sampled up to four rows (10 m) from trees treated with a suspension of these bacteria. For these experiments, the overall dynamics of secondary spread of *P. fluorescens* A506 and *E. herbicola* C9-1S from treated trees to

nontreated trees resembled a diffusion process, which was typified by a gradual and symmetrical increase in the incidence of recovery and population size of both *P. fluorescens* A506 and *E. herbicola* C9-1S on blossoms from nontreated trees over time (Fig. 2). For 1994 and 1996, we also concluded that secondary colonization of blossoms by *P. fluorescens* A506 and *E. herbicola* C9-1S occurred within treated trees. The antagonist suspensions were applied when only 30 to 50% of blossoms had opened. The low proportion of blossoms with fluorescent microspheres attached to pistillate surfaces after full bloom on treated trees indicated that blossoms closed at the time of application were not inoculated directly with the antagonist suspension. Thus, after full bloom, the high incidence of *P. fluorescens* A506 and *E. herbicola* C9-1S in blossoms of treated trees ($>90\%$) was likely the result of within-canopy dispersal and colonization by the antagonists from a previous site of establishment.

In contrast, in 1995, little evidence was obtained for significant secondary colonization by *P. fluorescens* A506 or *E. herbicola* C9-1S within canopies of treated or nontreated trees. For example, for the 992 blossoms sampled from nontreated trees during this season, *P. fluorescens* A506 was detected only once and *E. herbicola* C9-1S not at all. Moreover, within treated trees, the average incidence of recovery of the antagonist strains declined to less than 8% by the last sample, which contrasted sharply with the results from other experiments. In comparing this experiment to the others, we concluded that secondary colonization of *P. fluorescens* A506 and *E. herbicola* C9-1S within and between trees is a variable process and, as discussed below, was probably influenced by several factors including weather, insect activity,

and the bloom stage at the time of treatment.

Among seasons, temperatures varied significantly during pear bloom, and was likely the most important factor affecting epiphytic growth and dispersal of *P. fluorescens* A506 and *E. herbicola* C9-1S. During the experiments, the mean daily temperature for the first 7 days after the antagonist application varied from 12.1°C in 1996 to 4.1°C in 1995 (Fig. 6). For 1994, the only year in which significant disease control was observed, temperatures during bloom were intermediate to the other years, averaging 8°C for the week following antagonist application. Growth rates of epiphytic bacteria, including growth of *E. amylovora* on blossoms, are greatly influenced by temperature (20). The lack of secondary colonization in the coolest year (1995) contrasted against the significant growth and dispersal observed in the warmer years (1994 and 1996) is an indication that secondary colonization by *P. fluorescens* A506 and *E. herbicola* C9-1S was favored by conditions that promote bacterial growth. Foraging by honey bees, however, also was reduced greatly during the cooler weather in 1995.

Honey bees are the principle pollinator for pears and apples grown in the western United States, and most commercial growers place hives of bees in their orchards during bloom. This insect (as well as others) is considered an important vector for blossom-to-blossom transmission of *E. amylovora* (6,8,22) and, in two studies, marked strains of *P. fluorescens* or *E. herbicola* were recovered from apple or pear blossoms after bees were infested with these strains as they exited a hive (6,19). Based on this knowledge and the rates of recovery of *P. fluorescens* A506 and *E. herbicola* C9-1S from the bees sampled in 1996 (Fig. 4), we speculate that honey bees contributed to the bacterial dispersal detected in the 1994 and 1996 experiments. Potentially, other insects could have been involved in the dispersal of bacteria from blossom to blossom, but, within the shade cloth enclosure, few insects other than honey bees were observed on blossoms

Fig. 5. Percent of blossoms of Bartlett pear with populations of *Erwinia amylovora* strain 153N that exceeded 1×10^5 CFU per blossoms at full bloom in (A) 1994, (B) 1995, and (C) 1996, and percent of blossom clusters diseased with fire blight in (D) 1994, (E) 1995, and (F) 1996 in field experiments at Medford, Oregon. In all figures, trees in the rows labeled "0" were sprayed with a mixture of *Pseudomonas fluorescens* strain A506 and *Erwinia herbicola* strain C9-1S at 30, 15, and 50% bloom in 1994, 1995, and 1996, respectively. Points represent means of individual trees; position of lines drawn through were determined by regression analysis. Note the different y-axis scales used to present the data in D-F.

Fig. 6. Mean daily temperatures at Medford, Oregon, during bloom of pear trees used in experiments conducted in 1994 (diamonds), 1995 (squares), and 1996 (triangles).

during the morning and midday hours when samples were collected. Moreover, in a recent experiment conducted in an open orchard (no shade cloth), we observed similar gradients of detection of *P. fluorescens* A506 and *E. herbicola* C9-1S on both blossoms and bees sampled at various distances from a row of apple trees treated with these bacteria (K. Johnson, unpublished data). Rain also is an important mechanism of dispersal of epiphytic populations of *E. amylovora* (2,13,21) and, by analogy, *P. fluorescens* A506 and *E. herbicola* C9-1S are probably spread by rain. Rain, however, was prevalent during the 1995 experiment, when little secondary colonization was detected, and did not occur in 1996 from 50% to full bloom, when significant populations of *P. fluorescens* A506 and *E. herbicola* C9-1S developed on nontreated trees.

Bloom stage at the time of antagonist application was an additional factor that may have contributed to the variation in secondary colonization by *P. fluorescens* A506 and *E. herbicola* C9-1S observed among seasons. In order for secondary colonization to occur, a source population of the antagonists had to be established in treated trees. Because stigmatic surfaces are the principle site of growth of *P. fluorescens* A506 and *E. herbicola* C9-1S (no expanded leaves were on the trees at the time of application; 3,25,26), the number of sites available for primary colonization on treated trees at the time of spraying varied from year to year owing to differences in the number of open blossoms (35, 15, and 50% in 1994, 1995, and 1996, respectively). The greater proportion of open blossoms on trees at the time of treatment in 1994 and 1996 compared to 1995 may have resulted in a larger source population from which the bacteria could spread. We delayed the antagonist treatment and added a third row of treated trees to the experiment in 1996 to ensure a robust primary population of the antagonistic bacteria. More recently, we have compared incidence of recovery of *P. fluorescens* A506 and *E. herbicola* C9-1S at full bloom as affected by the bloom stage at the time of application (20 to 90%); relatively poorer recovery was obtained when the bacteria were applied to trees before 33 to 40% of the blossoms had opened (K. Johnson, unpublished data).

To suppress populations of *E. amylovora* effectively, a large population of *P. fluorescens* A506 or *E. herbicola* C9-1S must develop preemptively on stigmatic surfaces of pome fruit blossoms (3,7,25,26). Dilution plating measures these large populations adequately, but has a drawback in that populations below the detection limit cannot be separated from blossoms on which the bacteria were completely absent. Therefore, in spite of the consistent gradients of secondary colonization measured in 1994 and 1996, we cannot conclude with

complete certainty that the incidence of bacterial populations on nontreated blossoms was entirely the result of secondary spread from treated trees. Drift of the applied antagonist suspensions was minimized by spraying the bacteria at a low pressure and when wind conditions were still. Visual observations during application indicated that drift of the sprayed inoculum was negligible, and neither *P. fluorescens* A506 nor *E. herbicola* C9-1S were recovered from any nontreated blossom sampled 2 h after the spray treatment (712 blossoms over three seasons). Furthermore, over all sampling dates, fluorescent microspheres were not detected on any blossoms sampled from nontreated trees in the four rows most closely adjacent to the treated trees, whereas all blossoms from treated trees sampled 2 h after spraying had microspheres adhering to pistillate surfaces. Overwintering of *P. fluorescens* A506 and *E. herbicola* C9-1S in orchards is another potential source of antagonist inoculum that we have considered; however, neither strain survived at detectable levels from one season to the next (V. Stockwell, unpublished data).

As reported by Stockwell et al. (15), the fluorescent microspheres persisted once applied onto blossom surfaces (even after sonication), and their addition to the antagonist suspension effectively marked treated blossoms. The incidence of microspheres on pistillate surfaces provided another means to estimate the bloom stage at the time when *P. fluorescens* A506 and *E. herbicola* C9-1S were applied and, because blossoms were sampled every few days, a record of the relative rate of bloom development. For example, for 1995 data in Figure 3, the high proportion of sampled blossoms (77%) that had detectable microspheres on pistillate surfaces at 7 days after the antagonist application is an indication of how cold temperatures in this experiment delayed the opening (and consequently, sampling) of blossoms that were closed when the bacterial suspension containing the microspheres was applied. In the warmer seasons (1994 and 1996), blossoms opened more rapidly and, correspondingly, the proportion of blossoms with microspheres on pistillate surfaces declined more rapidly (Fig. 3).

This study provided some evidence that secondary colonization by epiphytic bacterial antagonists of *E. amylovora* can influence the population size of the pathogen that develops in flowers and the development of disease. In 1994, significant relationships were obtained for the incidence of *E. amylovora* populations greater than 1×10^5 CFU per blossom regressed on distance from trees treated with the antagonist suspension. These regressions had an opposite relationship to analogous regression models that described the incidence of *P. fluorescens* A506 and *E. herbicola* C9-1S in blossoms at full bloom. By contrast,

regression of the incidence of detection of *E. amylovora* (i.e., pathogen populations of any size) was not significant in 1994. Johnson et al. (7) reported that a major effect of a mixture of *P. fluorescens* A506 and *E. herbicola* C9-1S was to suppress the size of *E. amylovora* populations in blossoms; they also reported that incidence of disease corresponded closely to the number of blossoms that developed high populations of the pathogen (i.e., $>1 \times 10^5$ CFU per blossom). Results of this study from 1994 showed a similar correspondence (i.e., significant gradients were detected for both high populations of *E. amylovora* and for disease incidence, but not for incidence of recovery of *E. amylovora* populations of any size).

A number of factors contributed to the lack of fire blight control observed in the 1995 and 1996 experiments. In 1995, low populations of *P. fluorescens* A506 and *E. herbicola* C9-1S resulted in no measurable effect of the antagonist treatment on *E. amylovora* populations or on incidence of disease. In 1996, establishment and spread of *P. fluorescens* A506 and *E. herbicola* C9-1S was comparable to the results from 1994, and the incidence of high populations of *E. amylovora* was greatest on trees distal to those treated with antagonists. Unlike 1994, however, disease incidence in 1996 was not affected significantly by the antagonist treatment. Temperature may have contributed to this difference. In 1996, conditions favorable for epiphytic growth of *E. amylovora* occurred early in bloom, with the mean daily temperature exceeding 15°C (a common threshold criterion used to indicate high risk of a fire blight epidemic; 20) at 6 days after the antagonist application (Fig. 6). In 1994, this threshold was not exceeded until 12 days after the antagonist treatment. In 1996, the high temperatures early in bloom may have allowed *E. amylovora* to become established in blossoms before populations of *P. fluorescens* A506 and *E. herbicola* C9-1S had developed to sizes that could suppress epiphytic growth of the pathogen. Additionally, the size of the bee hive placed within the enclosure in 1996 was considerably larger than that in 1994. More bees in 1996 may have resulted in an earlier introduction of the pathogen to blossoms, and subsequently, a higher rate of secondary colonization of blossoms by *E. amylovora*. Pollination and fruit set also were enhanced in 1996 compared to 1994. High rates of pollination increases the proportion of flowers (fruitlets) that survive the first drop (dehiscence) period, which in turn increases that probability that a blossom infected by *E. amylovora* will remain attached to the blossom cluster. We and others (9) have observed that, if infected blossoms dehisce rapidly, the blossom cluster may escape disease. These differences among season in the timing of warm temperatures during bloom and in pollina-

tion efficiency also may have contributed to the large difference in disease incidence between the 1994 and 1996 experiments (3.3 versus 43%, respectively).

The rate of secondary colonization of blossoms by *P. fluorescens* A506 or *E. herbicola* C9-1S is important for commercial use of biological control because it affects the frequency at which these organisms should be sprayed to obtain optimal fire blight suppression. Owing to the requirement for preemptive colonization of blossoms by bacterial antagonists, early bloom applications of antagonists are considered desirable because they allow the most time for establishment of the bacteria before epiphytic populations of the pathogen have begun to develop. By contrast, if applications are delayed to later in bloom, the number of open blossoms and temperature are likely to become more favorable for the establishment of a large primary population of the antagonists. Application of *P. fluorescens* A506 and *E. herbicola* C9-1S at more than one bloom stage is an approach that addresses this paradox, but this also adds to the costs of control. Based on the results of this and other studies (7,10, K. Johnson unpublished data), a recommendation for use of *P. fluorescens* A506 and *E. herbicola* C9-1S is to apply the organisms at least twice during bloom. In field trials over the past 6 years, an application of *P. fluorescens* A506, *E. herbicola* C9-1S, or both between 25 and 40% bloom followed by a second application between 60 and 90% bloom has suppressed fire blight by an average of 50 to 60% (7,10, V. Stockwell and K. Johnson unpublished data).

We conclude that dispersal and secondary colonization by *P. fluorescens* A506 and *E. herbicola* C9-1S from sites of initial establishment to noncolonized blossoms is an important process that can affect the ability of these bacteria to suppress populations of *E. amylovora* and to reduce fire blight development. Dispersal and secondary colonization by *P. fluorescens* A506 and *E. herbicola* C9-1S allows populations of these bacteria established at mid-bloom to interact with pathogen populations up through the time of petal fall. Among seasons, however, rates of establishment and of secondary colonization by *P. fluorescens* A506 and *E. herbicola* C9-1S varied, indicating that environmental conditions influencing establishment and dispersal of the

antagonists requires further research. This variation also may indicate that multiple applications of these bacteria are necessary to attain the full benefits of this control strategy.

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