Effect of Antagonistic Bacteria on Establishment of Honey Bee-Dispersed *Erwinia amylovora* in Pear Blossoms and on Fire Blight Control

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ABSTRACT

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In field trials conducted in 1991 and 1992 at Medford, OR, and in 1992 at Wenatchee, WA, *Pseudomonas fluorescens* strain A506 and *Erwinia herbicola* strain C9-1 established epiphytic populations on pear blossoms and were effective antagonists for the biological control of fire blight. Both bacterial antagonists, water, or streptomycin sulfate were applied to trees at 30% and full bloom. Pear trees were challenged-inoculated with freeze-dried cells of *E. amylovora* vectored to blossoms by honey bees. One week after full bloom, the antagonists were established in more than 95% of treated blossoms in Oregon in 1991 and Washington in 1992, but in less than 50% of blossoms in Oregon in 1992. At the same bloom stage, 41% (Oregon, 1991), 27% (Oregon, 1992), and 49% (Washington, 1992) of water-treated blossoms had detectable populations of *E. amylovora,* whereas trees treated with bacterial antagonists always had a significantly lower ($P < 0.05$) percentage of blossoms with detectable *E. amylovora* populations: 18–20% (Oregon, 1991), 9–15% (Oregon, 1992), and 8–17% (Washington, 1992). In Oregon in 1991, only 4% of blossoms treated with bacterial antagonists supported populations of *E. amylovora* that exceeded 10^5 cfu per blossom compared with 19% of blossoms treated with water; however, suppression of population size of *E. amylovora* by bacterial antagonists was not apparent in 1992. In 1991, fire blight symptoms developed in 8, 0.1, and 1% of blossom clusters treated with water, streptomycin, or bacterial antagonists, respectively. In 1992, the percentage of diseased blossom clusters in these same treatments in Oregon averaged 44, 2, and 22%, respectively, and 9, 2.5, and 4%, respectively, in Washington.

The bacterial disease fire blight, caused by *Erwinia amylovora*, is an important constraint to the production of pears (*Pyrus communis* L.). Under the dry climatic conditions typical of the western United States, fire blight is most commonly initiated by epiphytic populations of *E. amylovora* that develop on blossoms (2,14). Antibiotic sprays are applied during bloom to control the disease; however, resistance of *E. amylovora* to streptomycin sulfate is widespread in California (11) and Washington (8), and also has been detected in Oregon (V. O. Stockwell, *unpublished data*).

Development of streptomycin resistance in *E. amylovora* in the United States and recent spread of this pathogen to countries in Europe where antibiotics are not registered for application on pome fruits have prompted increased research efforts on alternative control methods. Previous studies conducted in greenhouse and growth chamber environments (3,16–18) have demonstrated that the blossom blight phase of fire blight can be reduced by introducing bacteria antagonistic to *E. amylovora* onto floral surfaces. These antagonists (e.g., *Erwinia herbicola, Pseudomonas fluorescens*) colonize the nutrient-rich surfaces of stigmas and nectaries (3,12,16–18), and are thought to preemptively or competitively inhibit epiphytic *E. amylovora* populations from reaching levels required for disease development (3,13, 16–18). Several studies (1,9,15) have also demonstrated that spray applications of antagonistic bacteria can reduce fire blight in the field. However, the extent to which applied antagonists colonize a population of blossoms in an orchard and the degree to which the antagonists influence establishment and epiphytic growth of *E. amylovora* in blossom have not been described.

Because of the sporadic nature of fire blight epidemics in the field, researchers commonly spray-inoculate trees with *E. amylovora* to establish uniform populations of the pathogen in experimental field plots. Spray applications of *E. amylovora* onto blossoms, however, do not always result in disease. When disease does occur, the resulting epidemic may be so severe that even proven chemicals for control of fire blight appear ineffective. In response to this problem, we have employed honey bees (*Apis mellifera* L.), a natural vector of *E. amylovora* (7,19), to deliver the pathogen to pome-fruit blossoms. Freeze-dried cells of *E. amylovora* are placed into a dispenser attached to the entry of a beehive. Bees become infested with the pathogen as they exit and begin foraging activity (6). Foraging bees inoculate blossoms with freeze-dried *E. amylovora* as they move from flower to flower (6).

The purpose of this study was to evaluate the establishment of two spray-applied antagonistic bacteria in pear blossoms and their effect on establishment and epiphytic growth of honey bee-dispersed *E. amylovora* in blossoms and on fire blight development. The study was conducted within orchards located in two pear production areas of the western United States.

MATERIALS AND METHODS

Bacterial strains. Bacteria used in the studies were a spontaneous mutant of *E. amylovora* strain 153 resistant to nalidixic acid (100 mg/L)(*Ea*153nal^R), rifampicin-resistant (100 mg/L) *Pseudomonas fluorescens* strain A506 (*Pf*A506), and a spontaneous rifampicin-resistant mutant of *Erwinia herbicola* strain C9-1 (*Eh*C9-1rif^R). *Ea*153 was isolated in 1989 from fire blight cankers on Gala apple at Milton-Freewater, OR. Pathogenicity of *Ea*153nal^R was verified in preliminary inoculations of detached pear blossoms. *Pf*A506 was obtained from S. Lindow, Dept. of Plant Pathology, University of California, Berkeley. This bacterium has controlled fire blight in field and greenhouse studies (9,17). *Eh*C9-1 was obtained from C. Ishimaru, Dept. of Plant Pathology and Weed Science, Colorado State University, Fort Collins. *Eh*C9-1 produces at least two antibiotics, herbicolins O and I, that are inhibitory to *E. amylovora* (5). In culture, inhibition of *E. amylovora* by *Eh*C9-1rif^R was similar to that shown by *Eh*C9-1.

Experimental design. The same experiment was conducted three times; once in each of the springs of 1991 and 1992 in a 16-yr-old planting of pear cv. Bartlett on the Southern Oregon Experiment Station near Medford and once in the spring of 1992 in a 14-yr-old block of pear cv. d'Anjou at Washington State University's Columbia View Experimental Orchard near Wenatchee, WA. At each site, three or four rows of 10–15 trees each were enclosed with 30% polypropylene shade cloth (2.2 mm mesh, Nicolon Corp., Norcross, GA) in order to confine bee flight activity to the test plot during bloom. Trees were spaced approximately 2.4 m apart within rows with 3.7 m between rows. Dimensions of the shade cloth enclosure were 38 or 25 m in length by 18 m wide by 4.5 m in height. Support for the enclosure was provided by a frame constructed of 3.8 cm diameter galvanized steel conduit and 0.9 cm diameter braided steel cable. For each experiment, the shade cloth enclosure was assembled in mid-March and dismantled in early to mid-May.

Twenty trees within each enclosure were assigned treatments and the remaining trees served as buffers between treated trees. Treatments were arranged in a randomized block design with four replications: 1) water control, 2) *Pf*A506 plus *Eh*C9-1rif^R (each at 10^8 cfu/ml) applied twice during bloom, 3) *Pf*A506 plus *Eh*C9-1rif^R (each at 10^8 cfu/ml) applied three to four times during bloom, 4) streptomycin sulfate (Agristrep 21% 0.54 g/L) applied twice during bloom, and 5) nalidixic acid (0.05 g/L) applied twice during bloom. At Medford, treatments 1 and 3 were applied on 11, 13, 15, and 17 April in 1991 (5, 35, 75, and 95% bloom, respectively) and on 18, 20, 22, and 24 March 1992 (3, 25, 65, and 90% bloom, respectively). Treatments 2, 4, and 5 were applied on the second and forth application dates in each season, and trees in these treatments were sprayed with water on other dates when treatments 1 and 3 were applied. At Wenatchee, treatments 1 and 3 were applied on 1, 3, and 5 April 1992 (5, 75, and 95% bloom, respectively); treatments 2, 4, and 5 were applied on the first and third application dates and sprayed with water on the second application date.

Treatment preparation and application. *Pf*A506 and *Eh*C9-1rif^R were prepared for use in the field by separately growing lawns of each bacterium on Difco nutrient agar plus 1% glycerol for 3–4 days. Just before application to trees, each bacterium was harvested by flooding the agar surface with distilled water. Aqueous suspensions of each bacterium were adjusted to a concentration of 10^8 cfu/ml by measuring optical density at 450 nm. Suspensions of *Pf*A506 and *Eh*C9-1rif^R were combined in the tank of a back-pack sprayer and 3–4 L of the suspension was applied to each tree with a hand-directed spray wand. Other treatments were similarly applied with a different sprayer to avoid cross-contamination of chemical and biological treatments. Applications were made either shortly after sunrise or near sunset to enhance survival of applied bacteria.

Inoculum dispersal by honey bees. A single beehive was placed near the center of each shade cloth enclosure at 10–15% bloom (Medford: 11 April 1991 and 19 March 1992; Wenatchee: 3 April 1992) and a pollen insert (Antles Pollen Supplies, Inc., Wenatchee, WA) was attached to the entry platform of the hive. On days in early bloom when bees were expected to forage on blossoms, the pollen insert was filled with freeze-dried *Ea*153nal^R, which had a concentration of approximately 1×10^{11} cfu per gram. Methods for inoculum preparation have been described previously (6). Honey bees were infested with *Ea*153nal^R on 14–17 April 1991 and 21–24 March 1992 at Medford, and on 4 days between 4 and 11 April 1992 at Wenatchee. In general, 8–10 g of inoculum was placed into a pollen insert to begin an inoculation period and supplemented with an additional 3–5 g of inoculum every 1–2 hr. As bees exited the hive through the pollen insert, they carried an average of 10^5 to 10^6 cfu of *Ea*153nal^R (6). Most inoculation periods were about 4 hr long (6). The bee hive was removed from the enclosure area after 9 and 4 days at Medford in 1991 and 1992, respectively, and after 12 days at Wenatchee.

To determine if biological or chemical treatments influenced activity of the honey bee vectors, bee activity within individual trees at Medford was quantified. Hourly counts of the number of bees foraging in each treated tree were made on days when bees were infested with the pathogen. The time required to count the number of bees foraging on a tree was about 30 s. The number of blossoms per tree was estimated once during each experiment. These values were obtained by counting the number of blossom clusters per tree and multiplying by the average number of blossoms per cluster. Cumulative foraging activity of honey bees for each experimental tree was computed by summing the hourly counts of bees per tree over all inoculation periods and dividing by the number of blossoms per tree (units of this ratio were bee hours per blossom).

Bacterial incidence in blossoms. Blossoms were sampled three to six times during bloom in each experiment to determine incidence and population size of bacterial antagonists and *Ea*153nal^R on the pistilate surfaces. At Medford, 22–33 blossoms with mature (dark-colored) anthers were removed in a random pattern from each tree on each sampling date and placed into individual wells of ethanol-disinfected Styrofoam egg cartons or plastic microtiter plates to avoid cross contamination in transport to the laboratory. Similar sampling methods were used at Wenatchee but the sample size was 12 blossoms per tree. At Medford, blossom sampling dates were 11 (before application of treatments), 14, 16, 18, 21, and 24 April in 1991, and 18 (before application of treatments), 23, 26, and 29 March and 2 April in 1992. At Wenatchee, blossoms were sampled on 4, 9, 13, and 16, April 1992.

Blossoms were processed individually. The pistil and hypanthium were excised from each blossom with a sterile scalpel and placed in a test tube that contained 2.24 ml of sterile potassium phosphate buffer (0.05 M, pH 6.5). Racks of test tubes that contained the excised floral parts were agitated in a bath-type sonicator for 60 s. After sonication, a 0.01-ml aliquot of the wash and of a 1:224 dilution were plated on separate halves of an agar surface in a petri dish. Culture media used were Difco Pseudomonas Agar F plus rifampicin (100 mg/L)(PFR) for selective recovery of *Pf*A506 and *Eh*C9-1rif^R, PFR plus streptomycin sulfate (50 mg/L) for selective recovery of *Pf*A506 (Wenatchee only), and CCT medium (4) amended with nalidixic acid (50 mg/L) for selective recovery of *Ea*153nal^R. Minimum detection limits for bacteria in the wash and the 1:224 dilution were 2.24×10^2 and 5.0×10^4, respectively. A pair of test tubes that contained only sterile buffer were processed as controls after every 11 or 12 blossoms.

In the Medford experiments, colonies of *Pf*A506 and *Eh*C9-1rif^R on PFR were counted on the same plates after 2 days of incubation at 20–24 C. Differentiation of the two strains was based on characteristic differences in colony morphology, size, color, and fluorescent pigment production. At Wenatchee, media and cultural conditions were used to separate the bacteria for quantification. Colonies of *Pf*A506 appeared on PFR plus streptomycin after 2 days of incubation at 20–24 C; *Eh*C9-1rif^R is sensitive to streptomycin and did not grow on this medium. Colonies of *Eh*C9-1rif^R appeared on PFR after 1 day of incubation at 37 C; growth of *Pf*A506 was inhibited at this temperature. At both locations, characteristic colonies of *Ea*153nal^R were counted on CCT-nal after 3–4 days of incubation at 20–24 C. Pathogenicity of a subset of 25 isolates of *Ea*153nal^R recovered on CCT-nal was verified each year by stab inoculation of immature pear fruit (19) followed by incubation in a high humidity chamber at 20–24 C for 4–6 days (6).

Disease assessment. Effects of treatments on fire blight were assessed by counting the number of blossom clusters that developed characteristic symptoms of the disease for up to 9 wk after full bloom. Disease assessment dates for the Medford experiments were 8 and 23 May and 26 June in 1991 and 3, 10, and 22 April and 7 May in 1992. Disease was assessed on 30 April; 11 and 20 May, and 4 June 1992 at Wenatchee. Blighted blossom clusters were pruned from the trees on the day they were first observed. Fire blight incidence on a tree was computed as number of blighted blossom clusters divided by the total number of blossom clusters. Each year, an attempt was made to isolate *Ea*153nal^R from 25 of these blighted clusters on CCT-nal with methods described previously (6).

Data analysis. The SAS (Statistical Analysis Systems, Cary, NC) analysis of variance procedure (ANOVA) was used to test if the imposed treatments significantly affected cumulative bee foraging activity, frequency of recovery of *Pf*A506, *Eh*C9-1rif^R, and *Ea*153nal^R in blossom washes for each sampling date, and cumulative incidence of blighted blossom clusters. The proportion of blossoms with detectable populations of *Ea*153nal^R greater than 10^5 cfu per blossom also was subjected to ANOVA. All proportional frequency and incidence data were arcsine square root-transformed before analysis; Fisher's protected least significant difference was used as the mean separation procedure ($P = 0.05$). Mean population size and standard deviation of bacteria and bacterial strains in individual pear blossoms were calculated by averaging the logarithm (base 10) of values obtained for blossoms on which bacteria or a bacterial strain were detected; i.e., blossoms with bacterial populations below the detection limit

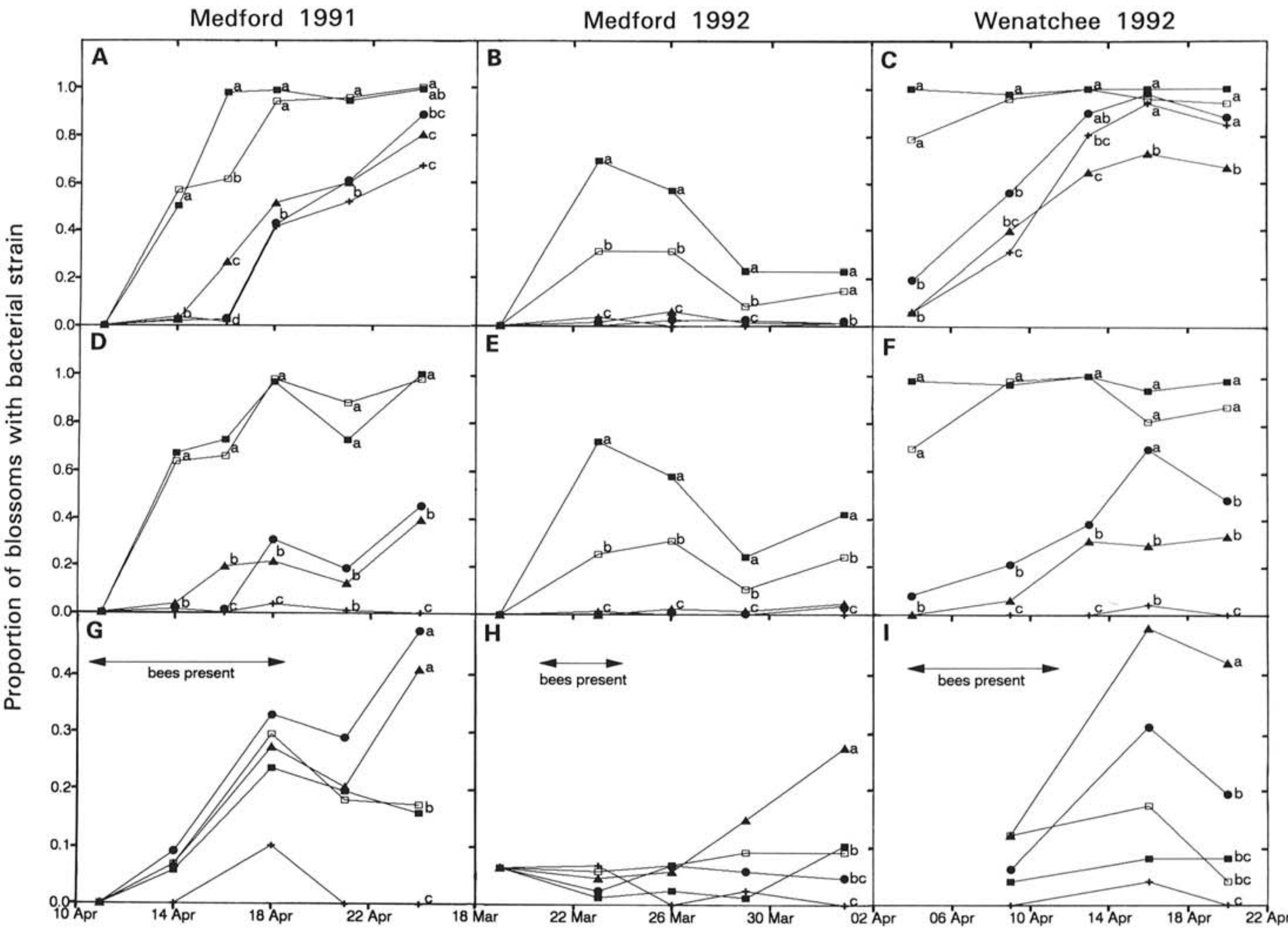

Fig. 1. Frequency of recovery of **A–C**, *Pseudomonas fluorescens* strain A506, **D–F**, *Erwinia herbicola* strain C9-1rif^R, and **G–I**, honey bee-dispersed *Erwinia amylovora* strain 153nal^R in blossoms of pear cv. Bartlett sampled in Medford, OR, in 1991 and 1992, and in blossoms of pear cv. d'Anjou in Wenatchee, WA, in 1992. Symbols: water control (▲), biological I (□), biological II (■), streptomycin +, nalidixic acid (●). The treatment 'biological I' was sprayed with a combined suspension of *P. fluorescens* strain A506 (10^8 cfu/ml) and *E. herbicola* strain C9-1rif^R (10^8 cfu/ml) twice during bloom; the treatment 'biological II' was the same combination of bacteria applied three (Wenatchee) or four (Medford) times during bloom. Within a panel, letters positioned near data points indicate significant differences ($P = 0.05$) among means sampled on the same date according to Fisher's protected least significant difference test.

were not included in the mean. A correlation matrix of logarithm of population sizes of *Pf*A506, *Eh*C9-1rif^{R}, and *Ea*153nal^{R} in individual blossoms was computed for each sampling date at each location. In the correlation analysis, a zero value was entered for a bacterial strain not recovered from a blossom.

RESULTS

Establishment of antagonists in blossoms. At Medford in 1991 and at Wenatchee in 1992, shortly after full bloom (18 April and 9 April, respectively), *Pf*A506 and *Eh*C9-1rif^{R} were recovered in washes of 77–100% of blossoms treated with these bacteria (Fig. 1A, C,D,F). Mean population sizes of *Pf*A506 and *Eh*C9-1rif^{R} in these blossoms ranged from 10^4 to 10^6 cfu per flower (Tables 1 and 2). Initially, few blossoms treated with water, nalidixic acid, or streptomycin had detectable populations of the applied antagonistic bacteria (Fig. 1A, C, D, F) but late in bloom *Pf*A506 was recovered from 64–95% of blossoms sampled from water-, nalidixic acid-, and streptomycin-treated trees (Fig. 1A and C). Similarly, on the last sampling date in the two trials,

TABLE 1. Mean population size[a] of *Pseudomonas fluorescens* strain A506, *Erwinia herbicola* strain C9-1rif^{R}, and *Erwinia amylovora* strain 153nal^{R} recovered from flowers of pear cv. Bartlett sampled in Medford, OR, in 1991

	Bacterial strain													
	P. fluorescens A506					*E. herbicola* C9-1rif^{R}					*E. amylovora* 153nal^{R}			
Treatment	14 Apr	16 Apr	18 Apr	21 Apr	24 Apr	14 Apr	16 Apr	18 Apr	21 Apr	24 Apr	14 Apr	18 Apr	21 Apr	24 Apr
Water	2.7	5.2	3.5	4.3	5.2	2.9	4.0	2.7	3.5	4.5	3.3	3.5	3.8	4.9
	...	(1.3)	(0.8)	(1.0)	(1.2)	(0.3)	(1.0)	(0.4)	(0.9)	(1.1)	(0.5)	(0.8)	(0.9)	(1.3)
	2	22	45	79	106	3	17	11	16	52	6	24	27	54
Biological I[b]	3.3	3.8	4.8	5.1	5.5	3.8	4.2	5.0	4.6	5.1	3.2	3.3	3.6	4.3
	(0.8)	(1.0)	(0.7)	(0.9)	(0.8)	(0.7)	(1.0)	(0.9)	(0.9)	(0.9)	(0.6)	(0.8)	(1.3)	(1.2)
	49	54	82	126	132	56	58	86	115	128	6	26	24	23
Biological II[c]	4.1	4.8	5.5	5.4	5.9	4.5	4.2	5.0	4.7	5.1	3.3	3.1	3.6	3.7
	(1.2)	(0.8)	(0.6)	(0.8)	(0.7)	(1.2)	(1.1)	(0.8)	(1.0)	(0.9)	(0.6)	(0.6)	(1.0)	(0.8)
	44	85	87	125	131	58	64	85	96	132	5	21	26	21
Streptomycin	3.0	2.7	3.7	4.4	5.0	x[e]	x	3.6	5.4	x	x	3.4	x	x
sulfate[d]	(0.1)	...	(0.8)	(0.8)	(1.1)			(0.3)	...			(0.8)		
	3	1	36	68	89			3	1			9		
Nalidixic	3.1	3.1	3.3	4.1	4.8	3.0	x	3.0	3.5	4.1	3.6	3.5	4.3	5.1
acid[f]	(0.7)	(0.4)	(0.8)	(0.9)	(1.1)	...		(0.5)	(0.9)	(0.9)	(0.6)	(0.7)	(1.2)	(1.2)
	8	2	37	80	117	1		27	24	60	8	29	38	68

[a] Means are expressed as $\log_{10}$ (cfu) per blossom followed by the standard deviation in parentheses and the number of blossoms averaged. Eighty-eight blossoms per treatment per date were individually processed on 14, 16, and 18 April; 132 blossoms per treatment per date were individually processed on 21 and 24 April. Blossoms from which a bacterial strain was not detected were excluded from the mean calculations. The detection limit of the blossom washing process was 2.24×10^2 cfu per blossom.
[b] Treatment was a combination of *P. fluorescens* A506 (10^8 cfu/ml) and *E. herbicola* C9-1rif^{R} (10^8 cfu/ml) spray applied (3 L per tree) twice during bloom.
[c] Treatment was a combination of *P. fluorescens* A506 (10^8 cfu/ml) and *E. herbicola* C9-1rif^{R} (10^8 cfu/ml) spray applied (3 L per tree) four times during bloom.
[d] Rate was 0.11 g/L spray applied (3 L per tree) twice during bloom.
[e] Bacterium was not recovered on this date.
[f] Rate was 0.05 g/L spray applied (3 L per tree) twice during bloom.

TABLE 2. Mean population size[a] of *Pseudomonas fluorescens* strain A506, *Erwinia herbicola* strain C9-1rif^{R}, and *Erwinia amylovora* strain 153nal^{R} recovered from flowers of pear cv. d'Anjou sampled in Wenatchee, WA, in 1992

	Bacterial strain												
	P. fluorescens A506					*E. herbicola* C9-1rif^{R}					*E. amylovora* 153nal^{R}		
Treatment	4 Apr	9 Apr	13 Apr	16 Apr	20 Apr	4 Apr	9 Apr	13 Apr	16 Apr	20 Apr	13 Apr	16 Apr	20 Apr
Water	2.5	3.2	4.1	4.8	4.5	x[b]	2.3	3.0	3.8	3.2	3.1	3.8	4.0
	(0.8)	(1.0)	(1.0)	(1.0)	(1.1)		(0.3)	(0.9)	(0.8)	(1.0)	(1.2)	(1.0)	(1.2)
	3	19	3	34	32		3	15	14	16	6	23	20
Biological I[c]	4.8	4.6	6.2	5.6	5.2	4.6	4.5	5.7	5.4	4.8	2.7	3.1	4.0
	(1.0)	(1.0)	(0.8)	(1.1)	(1.2)	(1.1)	(0.9)	(0.7)	(0.6)	(0.8)	(1.0)	(1.3)	(0.8)
	37	47	48	44	45	33	47	48	39	42	6	8	2
Biological II[d]	5.3	5.5	6.3	6.3	5.8	4.9	4.8	5.4	5.6	4.8	3.3	4.2	3.3
	(0.6)	(0.7)	(0.7)	(0.4)	(0.5)	(1.0)	(0.9)	(0.7)	(0.5)	(0.6)	(0.9)	(1.6)	(1.6)
	45	47	48	48	48	47	47	48	45	47	2	4	4
Streptomycin	4.8	2.7	4.0	5.3	4.9	x	x	x	2.7	x	x	2.7	x
sulfate[e]	(0.5)	(0.7)	(1.1)	(0.8)	(0.9)				(0.3)			(0.8)	
	3	15	39	45	41				2			2	
Nalidixic	3.8	3.3	4.9	5.5	4.8	2.4	3.1	3.6	4.0	3.6	2.9	3.3	3.4
acid[f]	(1.2)	(0.9)	(1.1)	(0.8)	(0.9)	(0.5)	(0.6)	(1.1)	(0.9)	(0.8)	(1.2)	(1.3)	(1.2)
	9	27	43	45	42	4	10	18	33	23	3	15	9

[a] Means are expressed as $\log_{10}$ (cfu) per blossom followed by the standard deviation in parentheses and the number of blossoms averaged. Forty-eight blossoms per treatment were individually processed on each sampling date. Blossoms from which a bacterial train was not recovered were excluded from the mean calculations. The detection limit of the blossom washing process was 2.24×10^2 cfu per blossom.
[b] Bacterium was not recovered on this date.
[c] Treatment was a combination of *P. fluorescens* A506 (10^8 cfu/ml) and *E. herbicola* C9-1rif^{r} (10^8 cfu/ml) spray applied (4 L per tree) twice during bloom.
[d] Treatment was a combination of *P. fluorescens* A506 (10^8 cfu/ml) and *E. herbicola* C9-1rif^{r} (10^8 cfu/ml) spray applied (4 L per tree) four times during bloom.
[e] Rate was 0.11 g/L spray applied (4 L per tree) twice during bloom.
[f] Rate was 0.05 g/L spray applied (4 L per tree) twice during bloom.

TABLE 3. Mean population size[a] of *Pseudomonas fluorescens* strain A506, *Erwinia herbicola* strain C9-1rif^R, and *Erwinia amylovora* strain 153nal^R recovered from flowers of pear cv. Bartlett sampled in Medford, OR, in 1992

	Bacterial strain											
	P. fluorescens A506				*E. herbicola* C9-1rif^R				*E. amylovora* 153nal^R			
Treatment	23 Mar	26 Mar	29 Mar	2 Apr	23 Mar	26 Mar	29 Mar	2 Apr	23 Mar	26 Mar	29 Mar	2 Apr
Water	3.0	3.3	2.9	2.7	x[b]	1.6	x	3.6	3.2	3.0	4.7	5.4
	...	(0.5)	(0.1)	...		(1.1)	...	(0.5)	(0.6)	(1.4)	(1.2)	(1.6)
	1	5	2	1		2	...	4	5	5	13	24
Biological I[c]	3.3	3.1	3.3	3.3	3.3	3.0	3.0	3.6	2.4	3.3	5.3	6.0
	(0.6)	(0.7)	(0.8)	(0.8)	(0.9)	(0.5)	(1.1)	(1.1)	(1.0)	(0.3)	(0.9)	(1.5)
	27	27	7	13	22	27	9	21	5	6	9	8
Biological II[d]	3.8	3.6	3.4	3.3	4.2	3.1	3.6	3.8	3.0	2.4	3.5	5.1
	(1.1)	(1.1)	(0.8)	(0.8)	(1.1)	(1.4)	(1.0)	(1.0)	...	(0.0)	...	(1.8)
	61	50	20	20	64	56	21	37	1	2	1	9
Streptomycin sulfate[e]	3.7	x	x	3.5	x	x	x	x	2.9	x	3.2	x
	(1.3)			...					(0.9)		(0.5)	
	3			1					9		2	
Nalidixic acid[f]	x	2.9	5.4	4.2	x	x	x	4.3	4.3	3.0	5.5	3.7
		(0.6)	(1.3)	...				(0.9)	...	(0.8)	(0.7)	(2.0)
		2	2	1				3	1	6	5	6

[a] Means are expressed as $\log_{10}$ (cfu) per blossom followed by the standard deviation in parentheses and the number of blossoms averaged. Eighty-eight blossoms per treatment were individually processed on each sampling date. Blossoms from which a bacterial strain was not recovered were excluded from the mean calculations. The detection limit of the blossom washing process was 2.24×10^2 cfu per blossom.
[b] Bacterium was not recovered on this date.
[c] Treatment was a combination of *P. fluorescens* A506 (10^8 cfu/ml) and *E. herbicola* C9-1rif^R (10^8 cfu/ml) spray applied (3 L per tree) twice during bloom.
[d] Treatment was a combination of *P. fluorescens* A506 (10^8 cfu/ml) and *E. herbicola* C9-1rif^R (10^8 cfu/ml) spray applied (3 L per tree) four times during bloom.
[e] Rate was 0.11 g/L spray applied (3 L per tree) twice during bloom.
[f] Rate was 0.05 g/L spray applied (3 L per tree) twice during bloom.

*Eh*C9-1rif^R was detected in 37–47% of blossoms treated with water or nalidixic acid but not in blossoms treated with streptomycin (Fig. 1D and F). With the exception of 16 April at Medford, mean population sizes of *Pf*A506 recovered from blossoms not treated with this bacterium were 0.3–2.4 log units lower than populations measured in blossoms to which the antagonist was applied directly (Tables 1 and 2). Blossoms treated with water or nalidixic acid to which *Eh*C9-1rif^R had spread also had smaller populations of this bacterium compared to blossoms treated with *Eh*C9-1rif^R directly (Tables 1 and 2).

At Medford in 1992, the frequency of recovery of both *Pf*A506 and *Eh*C9-1rif^R in blossoms peaked before full bloom (23 March) then declined through petal fall (Fig. 1B and E). Maximum recovery of *Pf*A506 and *Eh*C9-1rif^R was 69 and 73%, respectively, of blossoms that received four sprays of bacteria, and 31% for each bacterium from blossoms that received two bacterial sprays (Fig. 1B and E). Mean population sizes of *Pf*A506 and *Eh*C9-1rif^R in blossoms treated with these bacteria ranged from 10^3 to 10^4 cfu per blossom (Table 3), which were 0.7–2 log units lower than mean populations in the same trees in 1991 (Table 1). Spread of the bacteria to other trees in the plot in 1992 was limited, as neither antagonist was recovered from a high proportion of blossoms that received water or chemical treatments (Fig. 1B and E).

Over all sampling dates, the measured population sizes of *Pf*A506 and of *Eh*C9-1rif^R in individual blossoms treated with these bacteria were always positively correlated. At Medford in 1991, the correlation coefficients ranged from 0.24 to 0.65 (Fig. 2), of which values > 0.25 were significant ($P < 0.01$). In 1992, correlation coefficients for the population size of *Pf*A506 and *Eh*C9-1rif^R in the bacterial antagonist treatments ranged from 0.28 to 0.81 at Medford and from 0.44 to 0.80 in blossoms treated twice with the antagonists at Wenatchee, all of which were significant ($P < 0.01$). For blossoms treated with water, correlation coefficients determined for the population sizes of *Pf*A506 and *Eh*C9-1rif^R in blossoms were significant ($P < 0.01$) on at least one sampling date in each experiment (values obtained at Medford 1991 are shown in Fig. 2). Correlation coefficients for population size of *Ea*153nal^R with *Pf*A506 or *Eh*C9-1rif^R within blossoms treated with both antagonistic bacteria were neutral, ranging from −0.14 to 0.28.

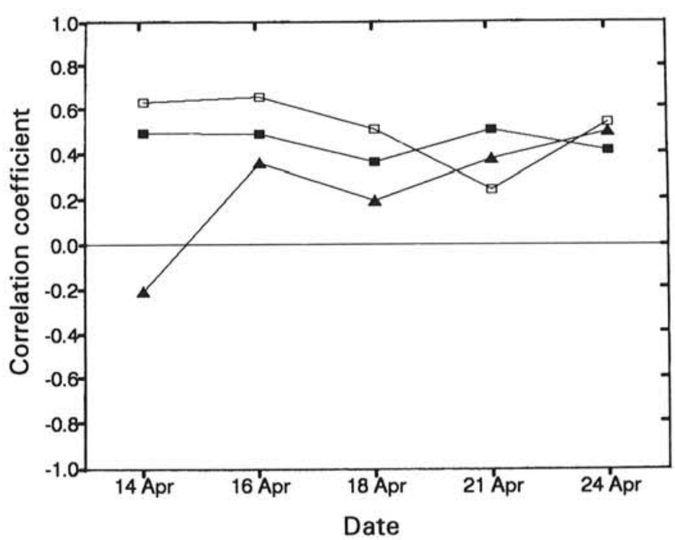

Fig. 2. Correlation of the population size of *Pseudomonas fluorescens* strain A506 with the population size *Erwinia herbicola* strain C9-1rif^R in treated pear blossoms sampled throughout bloom at Medford, OR, in 1991. Symbols: water control (▲), biological I (□), and biological II (■). The treatments 'biological I' and 'biological II' were sprayed with combined suspension of *Pseudomonas fluorescens* strain A506 (10^8 cfu/ml) and *Erwinia herbicola* strain C9-1rif^R (10^8 cfu/ml) twice or four times during bloom, respectively.

Establishment of the pathogen in blossoms. Cumulative bee foraging activity at Medford in 1991 and 1992 averaged 0.03 and 0.05 bee hours per blossom, respectively. Biological or chemical treatments did not significantly affect ($P > 0.05$) cumulative bee foraging activity in either year.

At Medford in 1991, recovery of *Ea*153nal^R from blossoms increased over time from 0% before placement of the beehive in the enclosure to 41% of water-treated blossoms sampled shortly after full bloom (24 April) (Fig. 1G). On 24 April, blossoms treated with *Pf*A506 and *Eh*C9-1rif^R had a significantly lower ($P < 0.05$) proportion of blossoms with detectable populations of *Ea*153nal^R (18%) compared to those treated with water (41%) (Fig. 1G).

Also, on this date, the proportion of blossoms with detectable populations of *Ea*153nal^R greater than 10^5 cfu per blossom was significantly affected by treatment ($P < 0.05$). This proportion averaged 0.49 + 0.07 (S.E.) and 0.50 + 0.10 for the water and nalidixic acid treatments, respectively, and 0.24 + 0.05 and 0.06 + 0.07 for blossoms that received two and four applications of the bacterial antagonists, respectively (data from which these proportions were derived are shown in Fig. 3). Populations of *Ea*153nal^R were recovered from blossoms of trees treated with streptomycin on only one of the five sampling dates (Fig. 1G).

In 1992, at both locations, the pattern of establishment of honey bee-dispersed *Ea*153nal^R in blossoms was similar to 1991. The proportion of water-treated blossoms with detectable populations of *Ea*153nal^R increased throughout the bloom period and was highest on the last or second to last sampling date (Fig. 1H and I). In addition, near the end of the bloom period, incidence of detection of *Ea*153nal^R was significantly lower ($P = 0.05$) in blossoms treated with bacterial antagonists than in the water control (Fig. 1H and I). For both locations, however, the proportion of blossoms with detectable populations of *Ea*153nal^R greater than 10^5 cfu per blossom was not significantly affected by treatment on any sampling date. On the last sampling date, this proportion averaged across treatment was 0.52 at Medford and 0.04 at Wenatchee. Treatment with streptomycin resulted in the lowest proportion of blossoms with detectable populations of *Ea*153nal^R (Fig. 1H and I). In contrast to 1991, nalidixic acid reduced recovery of the pathogen from blossoms in 1992 (Fig. 1H and I).

Development of fire blight. At Medford in 1991, two sprays of the combination of *Pf*A506 and *Eh*C9-1rif^R resulted in a cumulative total of 3.5 (1%) blighted blossom clusters per tree compared to 25–35 (8%) diseased clusters in control treatments (Fig. 4A). In 1992, two or four bacterial antagonist applications reduced the incidence of diseased blossom clusters by about 50% compared to the water and nalidixic acid controls, which had 111 (44%) and 133 (37%) diseased clusters per tree, respectively (Fig. 4B). *Ea*153nal^R was isolated from all diseased blossom clusters sampled from the plots at Medford in both years.

At Wenatchee in 1992; incidence of fire blight in trees that received two or three applications of *Pf*A506 and *Eh*C9-1rif^R averaged 8 (2.9%) and 11 (5.0%) diseased blossom clusters/tree, respectively, compared to an average of 17 (9.0%) diseased clusters in trees treated with water.

Streptomycin was the most effective treatment for control of fire blight (Fig. 4A–C) in each experiment. At Medford in 1991 and at Wenatchee in 1992, however, the reduction of disease incidence in trees that received two applications of streptomycin was not statistically superior to the reduction of disease obtained with two applications of the bacterial antagonists.

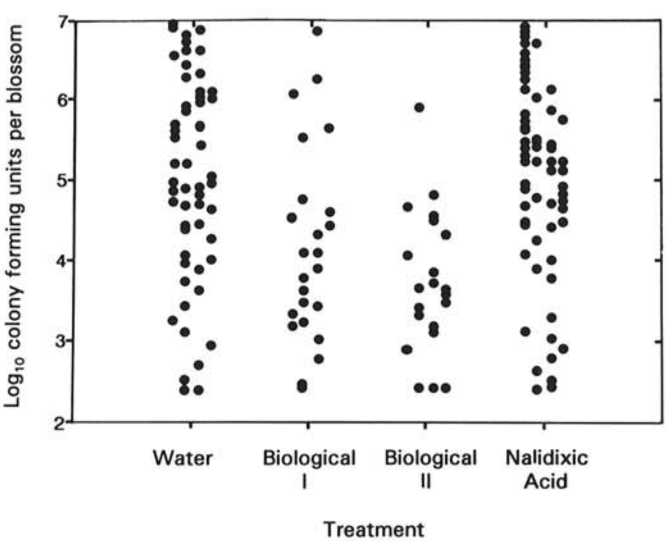

Fig. 3. Population size of honey bee-dispersed *Erwinia amylovora* strain 153nal^R in individual blossoms of pear cv. Bartlett sampled on 24 April 1991 in Medford, OR. Columns within a treatment represent blossoms sampled from the same tree. The treatments 'biological I' and 'biological II' were sprayed with combined suspension of *Pseudomonas fluorescens* strain A506 (10^8 cfu/ml) and *Erwinia herbicola* strain C9-1rif^R (10^8 cfu/ml) twice or four times during bloom, respectively.

DISCUSSION

Honey bees infested with freeze-dried *E. amylovora* introduced the pathogen into 27–49% of water-treated pear blossoms, re-

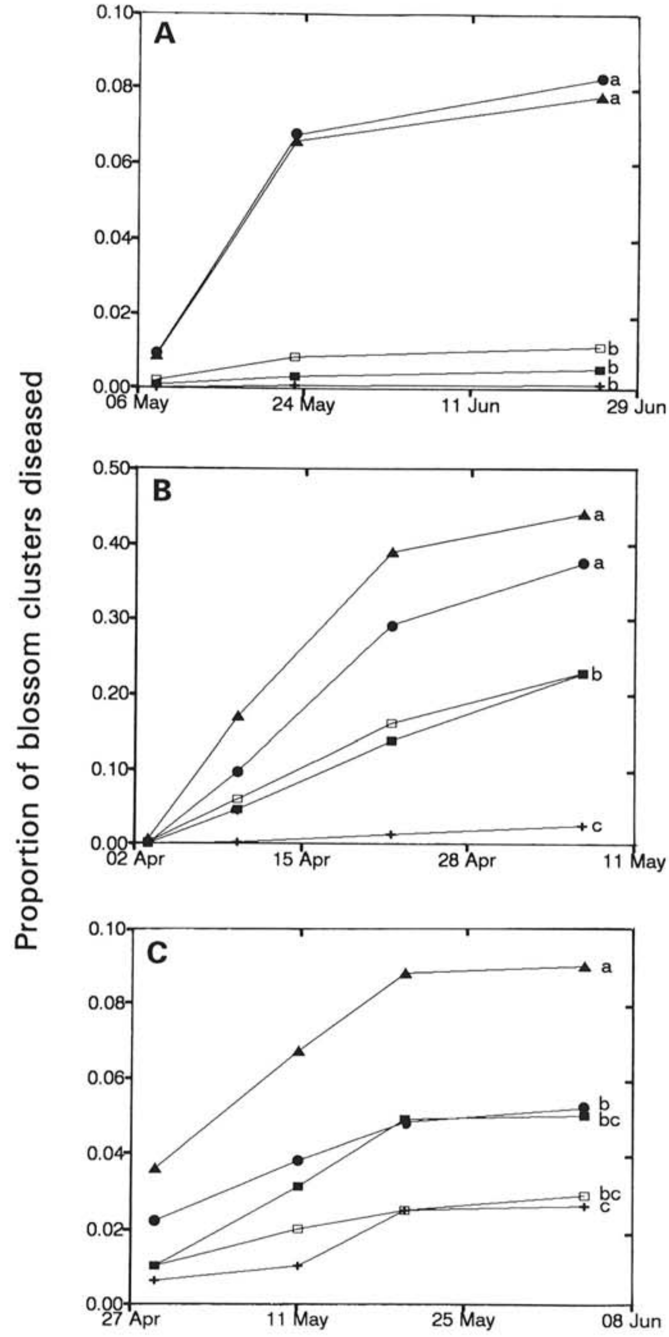

Fig. 4. Proportion of pear blossom clusters diseased with fire blight in Medford, OR, in, **A,** 1991 and, **B,** 1992, and Wenatchee, WA, in, **C,** 1992. Symbols: water control (▲), biological I (□), biological II (■), streptomycin +, nalidixic acid ●. The treatment 'biological I' was sprayed with a combined suspension of *Pseudomonas fluorescens* strain A506 (10^8 cfu/ml) and *Erwinia herbicola* strain C9-1rif^R (10^8 cfu/ml) twice during bloom; 'biological II' was the same combination of bacteria applied three (Wenatchee) or four (Medford) times during bloom. Within a panel, letters positioned near data points indicate significant differences ($P = 0.05$) among the means according to Fisher's protected least significant difference test.

sulting in a significant fire blight epidemic in each of the three experiments. In the two experiments conducted at Medford, the amount of time that bees foraged on blossoms was not affected by the chemical or biological treatments applied to individual trees. Consequently, we assume the proportion of blossoms to which the fire blight pathogen was introduced was uniform among trees, and conclude that differences in detection of *Ea*153nal^R among treatments was the result of direct effects of the treatments on epiphytic growth of pathogen populations.

The antagonistic bacteria *Pf*A506 and *Eh*C9-1rif^R applied in combination became established and persisted in a high proportion of the blossoms at Medford in 1991 and Wenatchee in 1992 (Fig. 1A, C, D, F) but not at Medford in 1992 (Fig. 1B and E). In addition, the mean populations of *Pf*A506 and *Eh*C9-1rif^R in blossoms were 1 to 2 log units smaller at Medford in 1992 (Table 3) compared to the other experiments (Tables 1 and 2). Growth and persistence of the antagonists in blossoms may have been influenced by environmental conditions during each experiment, although the nature of such stresses was not clearly apparent. The mean daily temperature at Medford in 1992 averaged 18 C and rainfall totaled 7 mm during the period when bacterial antagonists were applied. In contrast, at Medford in 1991, the average daily temperature during early bloom was 16 C and rainfall totaled 40 mm; Wenatchee in 1992 averaged 9 C during early bloom and no measurable rainfall was recorded. Alternatively, we considered that large populations of indigenous epiphytic bacteria on pear blossoms may have influenced establishment and growth of the antagonists in the 1992 Medford experiment. This hypothesis was tested by placing subsamples of blossom washings obtained on the last sampling date (2 April) on nonantibiotic-amended Pseudomonas Agar F in addition to plating on PFR and CCT-nal. More than 95% of blossoms from the water and two antagonist treatments did not have detectable populations of bacteria other than *Pf*A506, *Eh*C9-1rif^R, and *Ea*153nal^R.

*Pf*A506 and *Eh*C9-1rif^R provided significant disease control in each season, despite differences in the levels of establishment and persistence of these bacteria in blossoms among the experiments. Based on these differences, we propose that two effects of bacterial antagonists on populations *E. amylovora* contributed to control of fire blight in the field. The first effect was observed only at Medford in 1991, where the applied antagonists reduced the proportion of blossoms that had population sizes of *Ea*153nal^R greater than 10^5 cfu per blossom (Fig. 3). For each treatment in the 1991 Medford experiment in which epiphytic populations of *E. amylovora* were detected, a ratio of the number of blossoms with *Ea*153nal^R populations greater than 10^5 cfu per blossom to the number of diseased blossom cluster per tree can be calculated. The resulting values are 1.1 and 1.3 for the water and nalidixic acid treatments, respectively, and 1.3 and 1.0 for the two and four applications of bacterial antagonists, respectively. Calculation of the same ratio, but with detectable *Ea*153nal^R populations of any size as the numerator, gives values of 2.4 and 2.3 for the water and nalidixic acid treatments, respectively, and 6 and 21 for two and four applications of the bacterial antagonists, respectively. The more consistent ratios obtained by limiting the number of blossoms in the numerator to those with *Ea*153nal^R populations greater than 10^5 cfu per blossom suggests that the probability of a blossom cluster becoming diseased is dependent on the epiphytic population size of the pathogen within individual blossoms. Thomson et al (14) also hypothesized that the probability of a pear blossom being infected by *E. amylovora* is dependent on the epiphytic population size of the pathogen on the blossom. Assuming this hypothesis is correct, at least a portion of the disease control obtained by applications of bacterial antagonists to pear blossoms in 1991 was likely the result of *Pf*A506 and/or *Eh*C9-1rif^R competing and limiting epiphytic populations of *E. amylovora* on floral surfaces. This postestablishment competition resulted in a smaller population size of the pathogen (Fig. 3) and lower probability of infection.

Secondly, bacterial antagonists reduced the proportion of blossoms on which honey bee-dispersed *E. amylovora* became established (i.e., those blossoms with detectable pathogen populations). This effect was observed in each experiment (Fig. 1G–I) but was probably most important at Medford in 1992, where the proportion of blossoms with detectable antagonist populations and corresponding population sizes of the antagonists were relatively small. In this experiment, the treatment that received two applications of the antagonists had a 49% reduction in disease even though the mean proportion of blossoms with detectable populations of either *Pf*A506 or *Eh*C9-1rif^R never exceeded 38%. As noted above, the bloom period at Medford in 1992 may not have been conducive to epiphytic bacterial growth on stigmatic surfaces of pear, and the observed reduction in disease control could not be attributed to effects of the antagonists on established pathogen populations. Instead, bacterial antagonists may have occupied the few preferred sites on the pear blossoms that could support bacterial growth, and *E. amylovora* was excluded preemptively from sites where it could survive environmental stress (10).

Use of *Pf*A506 and *Eh*C9-1rif^R in combination revealed several characteristics of these bacterial strains that were likely important in attaining fire blight control. At Medford in 1991 and Wenatchee in 1992, both bacterial strains spread to blossoms on trees that were not treated directly with the antagonist suspension. This ability to spread to nontreated blossoms may have been the reason that in each experiment, two applications of the combination of *Pf*A506 and *Eh*C9-1rif^R were as effective as three or four. *Pf*A506, because of its natural resistance to streptomycin, also spread to a high proportion of blossoms treated with this antibiotic. This indicates potential for mixed applications of *Pf*A506 and streptomycin in an integrated biological and chemical control program (9). Factors that likely enhanced disease control were the size of the populations of *Pf*A506 and *Eh*C9-1rif^R that developed in blossoms, the length of time that large populations were sustained in blossoms, and the relative compatibility of these antagonists as indicated by the positive correlations of their population sizes within individual blossoms. In laboratory studies, Wilson and Lindow (17) reported that the carrying capacity (i.e., upper limit) for *Pf*A506 in pear blossoms was in the range of 10^5 to 10^6 cfu per blossom. At both Medford in 1991 and Wenatchee in 1992, mean populations of both *Pf*A506 and *Eh*C9-1rif^R in treated blossoms were consistently in this range during the latter half of the bloom period. The positive correlations of the population sizes of *Pf*A506 and *Eh*C9-1rif^R within individual blossoms may indicate that the carrying capacity for the total population size of bacterial epiphytes on a floral surface is variable among individual blossoms, perhaps influenced by differences in stage of blossom development, age, nutritional status, or microenvironment.

Streptomycin and nalidixic acid treatments were included in the experimental design for different purposes. The streptomycin treatment represented the standard for disease control on which to base the relative efficacy of antagonist treatments. This antibiotic was the most effective treatment in each experiment; however, it should be noted that *Ea*153nal^R is sensitive to streptomycin and that two applications of *Pf*A506 and *Eh*C9-1rif^R were not statistically inferior to streptomycin at Medford in 1991 and Wenatchee in 1992. In pear-growing regions where streptomycin-resistant strains are abundant, such as Washington State (8), the antagonists could be more effective than streptomycin for fire blight control. Nalidixic acid was included as an experimental treatment in an attempt to limit the spread of bacterial antagonists from trees on which they were applied to trees on which epiphytic populations of *Ea*153nal^R could develop without biological competition. Use of nalidixic acid for this purpose, however, was not effective; it did not limit the population size or proportion of blossoms with detectable populations of the antagonists compared to water-treated controls. Furthermore, in studies conducted in 1992, this antibiotic reduced the proportion of blossoms on which *Ea*153nal^R was detected, and in Wenatchee, decreased disease severity.

In conclusion, spray applications of the antagonistic bacteria *Pf*A506 and *Eh*C9-1rif^R, in combination, controlled fire blight in two pear production areas in the western United States. Disease control was correlated to reduced establishment of *E. amylovora* in blossoms treated with the bacterial antagonists, and at one field site, by suppressed growth of the fire blight pathogen on floral surfaces treated with the antagonist suspension. *Pf*A506 and *Eh*C9-1rif^R, applied in combination, were most effective as disease control agents when high populations (mean size $> 10^5$ cfu per blossom) of each antagonist became established and persisted in a high proportion of pear blossoms over most of the bloom period. In one experiment, reduced efficacy of bacterial antagonists was attributed to poor establishment and small population size in pear blossoms. The conditions that favor establishment of *Pf*A506 and *Eh*C9-1rif^R in blossoms, and the degree to which these antagonist strains interact to control fire blight warrant further investigation.

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