Compatibility of Bacterial Antagonists of *Erwinia amylovora* with Antibiotics Used to Control Fire Blight

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ABSTRACT

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In field experiments, two bacterial antagonists that suppress fire blight, *Pseudomonas fluorescens* strain A506 and a streptomycin-resistant mutant of *Erwinia herbicola* strain C9-1 (C9-1S), were sprayed onto apple blossoms at 30% bloom. Water, streptomycin sulfate, or oxytetracycline were sprayed onto blossoms 2 and 7 days after the antagonist applications to determine the effect of these chemicals on the population dynamics of *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S during bloom. Incidences of recovery (the proportion of blossoms supporting detectable bacterial populations) and population sizes of *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S on stigmas within individual blossoms were estimated with a dilution plating assay before and after each antibiotic application. Maximum incidences of recovery of *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S from blossoms treated subsequently with water ranged from 58 to 100% and 47 to 100%, respectively; average population sizes of both strains were 10^4 to 10^6 CFU/flower. Streptomycin did not reduce the incidence of recovery or the population size of either antagonist. Oxytetracycline applications made 2 and 7 days after the antagonist applications reduced the incidence of recovery by 23 to 58% and also reduced the population size of both *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S by 10- to 100-fold. In contrast, when the first oxytetracycline treatment was delayed to 7 days after the application of the antagonists, only a slight reduction in the incidence of recovery and the population size of either antagonist was observed. The population dynamics of *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S, and presumably the degree of protection that they provide, need not be adversely affected by the concomitant usage of chemical antibiotics within the same season. Optimal integration of biological and chemical methods for suppression of fire blight, however, may require that oxytetracycline applications be delayed until after epiphytic populations of antagonists have become established on flowers.

Additional keywords: biological control.

Fire blight, caused by the bacterium *Erwinia amylovora*, is a serious disease of pome fruits (24). *E. amylovora* can establish epiphytic populations on floral surfaces that provide the inoculum for floral infection (1,4,22,23,25,27). Fire blight management programs focus on suppression of epiphytic growth of *E. amylovora* on flowers through the application of antibiotics (streptomycin or oxytetracycline) during bloom. Although streptomycin has been effective for this purpose, streptomycin-resistant isolates of *E. amylovora* are now common in many pear- and apple-growing regions in the United States (2,15,17,18).

Treatment of pear blossoms with suspensions of the bacterial antagonists *Pseudomonas fluorescens* or *E. herbicola* reduces establishment of and suppresses growth of *E. amylovora* on floral tissues (1,9,10,12,14,25,26). Over several years of testing, a mixture of *P. fluorescens* strain A506 and *E. herbicola* strain C9-1 has reduced the incidence of disease in inoculated plots in Oregon and Washington by an average of 60% (V. O. Stockwell and K. B. Johnson, *unpublished data*). Because the degree of fire blight control obtained by applications of bacterial antagonists is usually less than 100%, a strategy to integrate antagonist use with antibiotic use has been developed. This strategy requires that antibiotic-resistant antagonists become established in pear or apple blossoms at midbloom, and allows for subsequent antibiotic applications if weather-based risk advisories (23,24) indicate a high probability of infection by *E. amylovora*. In an evaluation of integrated biological and chemical control of fire blight, Lindow (12) and Lindow et al. (14) provided evidence that the disease control benefits obtained from applications of *P. fluorescens* strain A506 and antibiotics are likely to be additive; however, these studies did not measure the direct effects of antibiotic applications on the population dynamics of the bacterial antagonist on blossoms. The purpose of our study was to investigate the effect of the antibiotics streptomycin and oxytetracycline on the population dynamics of *P. fluorescens* strain A506 and of a spontaneous, streptomycin-resistant mutant of *E. herbicola* strain C9-1 (C9-1S). Our goal was to develop recommendations that provide for optimal integration of biological and chemical methods for fire blight suppression. A preliminary report has been published (19).

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. *P. fluorescens* strain A506, obtained from S. E. Lindow (Department of Environmental Science, Policy, and Management, University of California, Berkeley), is a rifampicin-resistant (100 µg/ml) derivative

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of an epiphyte recovered from pear in California (12,26). The strain is naturally resistant to streptomycin and oxytetracycline. *E. herbicola* strain C9-1 was obtained from C. Ishimaru (Department of Plant Pathology and Weed Science, Colorado State University, Fort Collins) who isolated the bacterium from 'Jonathan' apple fruit in Michigan (8). *E. herbicola* strain C9-1S is a spontaneous rifampicin- and streptomycin-resistant derivative (each antibiotic at 100 µg/ml). *E. herbicola* strain C9-1S is sensitive to 10 µg/ml of oxytetracycline in culture. *E. herbicola* strain C9-1 produces at least two antibiotics, herbicolins O and I, that inhibit growth of *E. amylovora* (8). In culture, growth rate and inhibition of *E. amylovora* by *E. herbicola* strain C9-1S are similar to that by the wild-type *E. herbicola* strain C9-1. *E. amylovora* strain 153 was isolated in 1989 from a fire blight canker on 'Gala' apple at Milton-Freewater, Oregon. *E. amylovora* strain 153 (153N) is a spontaneous nalidixic acid-resistant (100 µg/ml) derivative. Pathogenicity of *E. amylovora* strain 153N was verified in previous field experiments (9,10). All bacterial strains were stored in nutrient broth containing 15% glycerol at –80°C until use.

Bacteria used in inoculations were cultured for 4 days on nutrient agar (Difco Laboratories, Detroit) amended with 1% (wt/vol) glycerol. Rifampicin (50 µg/ml) or rifampicin and streptomycin sulfate (50 µg/ml) were added to the media for the culture of *P. fluorescens* strain A506 or *E. herbicola* strain C9-1S, respectively. Nalidixic acid (50 µg/ml) was added for the culture of *E. amylovora* strain 153N. Cultured bacteria were scraped from the agar surface, suspended in a cryoprotectant mixture of skim milk and xanthan gum, frozen, and lyophilized as described previously (9). The viable cell concentration of bacteria in the freeze-dried inocula ranged from 10^{10} to 10^{11} CFU per gram.

Experimental plot design. All experiments were conducted at the Oregon State University, Department of Botany and Plant Pathology Research Farm near Corvallis, on apple trees (1.5 to 3 m tall) spaced 6 m apart. A block of 50 34-year-old trees of cultivar Rome Beauty grafted onto 'Malling 7' rootstock was used for bacterial treatments in 1992. In 1993, the experiment was located in a block of 63 15-year-old trees of 'Rome Beauty' on 'Malling 7' rootstock. In 1994, experiments were conducted on 70 17-year-old trees of cultivar Golden Delicious grafted onto 'Malling 26' rootstock. All experiments were arranged as a split-plot design, with bacterial treatments serving as whole plots and antibiotic treatments serving as subplots. There were five, four, or three single-tree replications of each treatment combination in 1992, 1993, and 1994, respectively. All trees were sprayed routinely during bloom with fenarimol and dodine to control apple scab and powdery mildew.

Application of bacteria. Lyophilized bacteria were resuspended in water to form an aqueous inoculum with a viable cell concentration of 10^8 CFU/ml for *P. fluorescens* strain A506 or *E. herbicola* strain C9-1S and 10^7 CFU/ml for *E. amylovora* strain 153N. Table 1 summarizes the years that each bacterium was applied. Immediately after mixing, bacterial suspensions were applied until near runoff (about 3 to 4 liters per tree) to apple trees at 30% bloom with a 15-liter backpack sprayer fitted with a hand-directed spray wand. All bacterial applications were made shortly after sunrise; the dates of inoculation were 14 April 1992, 27 April 1993, and 19 April 1994.

Antibiotic spray treatments. The treatments imposed to examine the effect of streptomycin or oxytetracycline on the establishment of *P. fluorescens* strain A506, *E. herbicola* strain C9-1S, or *E. amylovora* strain 153N on apple blossoms are summarized in Table 1. In each experiment, antibiotic treatments or water were sprayed until runoff at 2 and 7 days after application of the bacteria. Streptomycin sulfate (Agristrep D 21%, Merck & Company, Inc., Rahway, NJ) was applied at a concentration of 100 µg/ml. Oxytetracycline (Mycoshield 17%, Pfizer Inc., New York) was applied at a concentration of 200 µg/ml. Water was used as a control treatment, after application of the bacteria, to provide a comparative control to measure the effect of the antibiotics on bacterial establishment on blossoms. Additional antibiotic treatments included alternated single sprays of streptomycin and oxytetracycline and repeated application of the two antibiotics as a mixture (Table 1). In 1992, streptomycin or oxytetracycline also was mixed in the spray tank with *P. fluorescens* strain A506 and applied to trees at 30% bloom. Trees at 30% bloom treated with the antagonist mixed with an antibiotic received two additional sprays of the same antibiotic 2 and 7 days later.

Recovery of bacteria from blossoms. Blossoms were sampled six times during bloom in each experiment to estimate incidences of recovery (the proportion of blossoms supporting detectable bacterial populations) and population sizes of *P. fluorescens* strain A506, *E. herbicola* strain C9-1S, or *E. amylovora* strain 153N on the pistilate surfaces. Eight (1992) or 12 blossoms (1993 and 1994) were sampled from each tree on each sampling date and placed immediately into individual wells of surface-disinfested, plastic, 12-well microtiter plates (Corning Glass Inc., Corning, NY) to avoid cross-contamination during transport to the laboratory. Blossoms were collected before each antibiotic spray, within

TABLE 1. Summary of treatments applied to apple trees[a] onto which the bacterial strains *Pseudomonas fluorescens* strain A506, *Erwinia herbicola* strain C9-1S, or *E. amylovora* strain 153N had been inoculated at 30% bloom

		Treatments					
Bacterial strain	Year	Water	Streptomycin[b]	Oxytetracycline[c]	Streptomycin-oxytetracycline[d]	Oxytetracycline-streptomycin[e]	Oxytetracycline and streptomycin[f]
P. fluorescens strain A506	1992	X	X	X			
	1993	X	X	X	X	X	X
	1994	X	X	X	X	X	
E. herbicola strain C9-1S	1993	X	X		X		X
	1994	X	X	X	X	X	
E. amylovora strain 153N	1994	X	X	X	X	X	

[a] Experimental plots were located at the Oregon State University, Department of Botany and Plant Pathology Research Farm near Corvallis. In 1992 and 1993, experiments were conducted on trees of cultivar Rome Beauty grafted onto 'Malling 7' rootstock and, in 1994, on trees of cultivar Golden Delicious grafted onto 'Malling 26' rootstock.

[b] Treatment consisted of applications of streptomycin sulfate (100 µg/ml) 2 and 7 days after application of the bacteria.

[c] Treatment consisted of applications of oxytetracycline (200 µg/ml) 2 and 7 days after application of the bacteria.

[d] Treatment consisted of an application of streptomycin sulfate (100 µg/ml) 2 days after application of the bacteria followed by oxytetracycline (200 µg/ml) 7 days after application of the bacteria.

[e] Treatment consisted of an application of oxytetracycline (200 µg/ml) 2 days after application of the bacteria followed by streptomycin sulfate (100 µg/ml) 7 days after application of the bacteria.

[f] Treatment consisted of applications of streptomycin sulfate (100 µg/ml) combined with oxytetracycline (200 µg/ml) 2 and 7 days after application of the bacteria.

24 h after each antibiotic spray, and 3 and 6 days after the second antibiotic application. The stigmas and styles of each blossom were excised and placed into a test tube containing 1 ml of sterile 10 mM potassium phosphate buffer, pH 7.1, and sonicated for 3 min. After sonication, two 100-fold serial dilutions of the wash buffer were made. A 10-µl sample of each of the three dilutions (the wash buffer and the serial dilutions) from an individual blossom was spread onto a plate of Pseudomonas agar F (Difco Laboratories), containing 50 µg/ml of cycloheximide and 100 µg/ml of rifampicin, to recover *P. fluorescens* strain A506 or *E. herbicola* strain C9-1S or onto CCT medium (7) amended with 50 µg/ml of nalidixic acid for selective recovery of *E. amylovora* strain 153N. The detection limit was 100 CFU/blossom. For each treatment combination, the total number of blossoms assayed on each sample date was 40, 48, and 36 in 1992, 1993, and 1994, respectively.

Data analysis. The SAS (Statistical Analysis Systems Inc., Cary, NC) analysis of variance procedure was used to test if the antibiotic treatments significantly affected the incidence of recovery and the mean population size of each bacterial strain on blossoms. All incidence data were arcsine square root-transformed before analysis. Mean and standard error of the log-transformed bacterial population size in individual blossoms were calculated for blossoms on which bacteria were detected. Blossoms with bacterial populations below the detection limit were not included in estimates of mean population size. Fisher's protected least significant difference at $P = 0.05$ was used to separate the means.

RESULTS

Bacterial establishment on water-treated blossoms. Spray inoculation of apple trees at 30% bloom with bacterial suspensions followed with applications of water 2 and 7 days after inoculation resulted in the establishment of epiphytic populations of *P. fluorescens* strain A506 or *E. herbicola* strain C9-1S on a high proportion of blossoms. *P. fluorescens* strain A506 was detected on 78 to 100, 81 to 96, and 58 to 92% of blossoms sampled every 2 to 3 days over a 12- to 14-day bloom period in 1992, 1993, and 1994, respectively (Fig. 1A through C). *E. herbicola* strain C9-1S was detected on 98 to 100 and 47 to 72% of blossoms sampled in 1993 and 1994, respectively (Fig. 2A and B). *P. fluorescens* strain A506 established mean population sizes in the range of 10^5 to 10^6 CFU/flower on trees inoculated with that strain and subsequently sprayed with water (Fig. 1D through F). The mean population size of *E. herbicola* strain C9-1S on blossoms ranged from 10^5 to 10^6 CFU/flower in 1993 and 10^4 to 10^5 CFU/flower in 1994 (Fig. 2C and D).

Effect of streptomycin. In all years, application of streptomycin at 2 days after the bacterial inoculations followed by a second application 5 days later did not have a detrimental effect on the incidence of recovery of *P. fluorescens* strain A506 or *E. herbicola* strain C9-1S from apple blossoms when compared with that of the water-treated controls (Figs. 1A through C and 2A and B). The mean population size of *P. fluorescens* strain A506 on blossoms treated twice with streptomycin also was similar to populations recovered from water-treated blossoms (Figs. 1D through F).

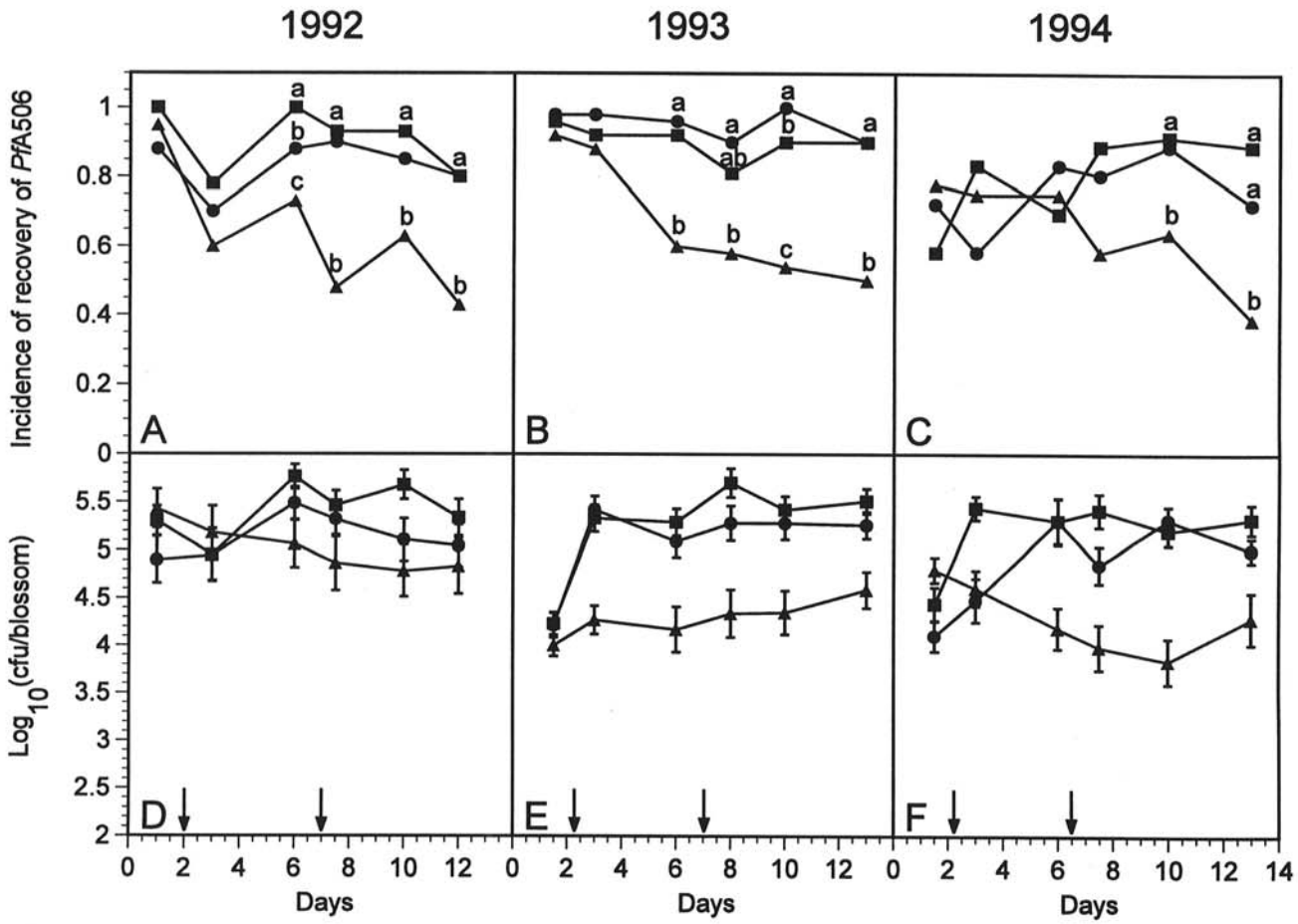

Fig. 1. The effect of repeated streptomycin or oxytetracycline sprays on **A through C,** the incidence of recovery (i.e., the proportion of blossoms with detectable populations) and **D through F,** the average population sizes of *Pseudomonas fluorescens* strain A506 on 'Rome Beauty' (1992 and 1993) or 'Golden Delicious' (1994) apple blossoms. Mean population size was calculated only from blossoms with detectable populations of the antagonist. *P. fluorescens* strain A506 was applied at day 0 (30% bloom) and sprayed with water (■), streptomycin sulfate (100 µg/ml) (●), or oxytetracycline (200 µg/ml) (▲) at 2 and 7 days (indicated by arrows on the x axis) after application of the bacterium. **A through C,** Letters positioned above a symbol indicate significant differences ($P = 0.05$) in the incidence of recovery of *P. fluorescens* strain A506 among treatment means determined on the same date according to Fisher's least significant difference procedure. **D through F,** Vertical bars represent the standard error of the average population size.

In both 1993 and 1994, the mean population size of *E. herbicola* strain C9-1S on streptomycin-treated blossoms was significantly larger than that on water-treated blossoms on two of the sampling dates after streptomycin treatments (Fig. 2C and D). In 1992, streptomycin applied as a tank mix with *P. fluorescens* strain A506 at 30% bloom and as a spray 2 and 7 days later had no significant effect on either the incidence of recovery or the population size of *P. fluorescens* strain A506 on blossoms when compared with those of the water-treated control (data not shown).

Effect of oxytetracycline. Application of oxytetracycline at 2 and 7 days after bacterial inoculation significantly reduced the incidence of recovery of *P. fluorescens* strain A506 from blossoms by 30 to 45, 23 to 40, and 25 to 58% in 1992, 1993, and 1994, respectively, when compared with those of the water-treated control (Fig. 1A through C). In 1994, mean incidence of recovery of *E. herbicola* strain C9-1S from oxytetracycline-treated blossoms sampled 7, 10, and 13 days after bacterial inoculation was reduced by 30 to 36% when compared with that of the water-treated control (Fig. 2B); although this reduction was significant only for one of the sample dates. In 1992, 1993, and 1994, the mean population sizes of *P. fluorescens* strain A506 on blossoms with detectable populations of this bacterium were approximately 10-fold lower in blossoms treated with oxytetracycline compared with those of the water-treated control (Fig. 1D through F). A similar reduction in the mean population size of *E. herbicola* strain C9-1S on blossoms with detectable populations of this bacterium was observed in late bloom in 1994 following the oxytetracycline treatments (Fig. 2D). In 1992, oxytetracycline applied as a tank mix with *P. fluorescens* strain A506 at 30% bloom and as a spray 2 and 7 days later significantly reduced the incidence of recovery of *P. fluorescens* strain A506 by 62 to 75% and reduced the mean population size of *P. fluorescens* strain A506 on blossoms by 100-fold compared with those of the water-treated control on each sampling date (data not shown).

Alternating antibiotic treatments. Streptomycin applied 2 days after the bacterial inoculations followed by oxytetracycline 5 days later had only slight effects on epiphytic populations of *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S on apple blossoms. For example, neither the incidence of recovery nor the population size of *P. fluorescens* strain A506 on blossoms receiving this treatment were reduced significantly compared with those of water-treated blossoms (Fig. 3A through D) until 3 to 7 days after the oxytetracycline treatment, when the incidence of recovery of *P. fluorescens* strain A506 from blossoms was reduced by 27 to 42% in both 1993 and 1994 (Fig. 3A and B). For *E. herbicola* strain C9-1S, treatment with streptomycin 2 days after inoculation followed by oxytetracycline 5 days later resulted in incidences of recovery and population sizes of the bacterium that were similar to or significantly greater than those detected on blossoms inoculated with *E. herbicola* strain C9-1S and then sprayed with water (Fig. 4A through D).

An application of oxytetracycline 2 days after the bacterial inoculation followed by streptomycin 5 days later significantly reduced populations of *P. fluorescens* strain A506 on apple blossoms. For example, treatment with oxytetracycline followed by streptomycin reduced the incidence of recovery of *P. fluorescens* strain A506 on blossoms by 21 and 80% in 1993 and 1994, respectively, compared with that of the water-treated control (Fig. 3A and B). The mean population size of *P. fluorescens* strain A506

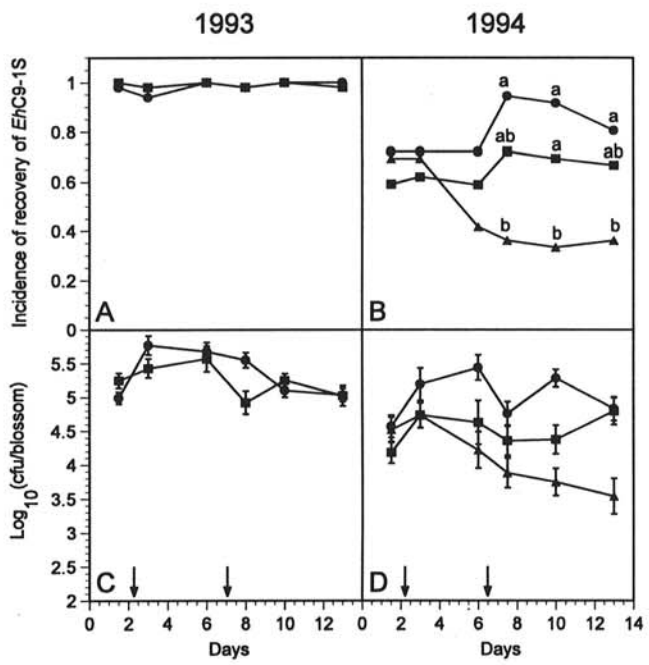

Fig. 2. The effect of repeated streptomycin or oxytetracycline sprays on **A and B,** the proportion of blossoms with detectable populations and **C and D,** the average population sizes of *Erwinia herbicola* strain C9-1S on 'Rome Beauty' (1993) or 'Golden Delicious' (1994) apple blossoms. Mean population size was calculated only from blossoms with detectable populations of the antagonist. *E. herbicola* strain C9-1S was applied at day 0 (30% bloom) and sprayed with water (■), streptomycin sulfate (100 µg/ml) (●), or oxytetracycline (200 µg/ml) (▲) at 2 and 7 days (indicated by arrows on the x axis) after application of the bacterium. **A and B,** Letters positioned above a symbol indicate significant differences ($P = 0.05$) in the incidence of recovery of *E. herbicola* strain C9-1S among treatment means determined on the same date according to Fisher's least significant difference procedure. **C and D,** Vertical bars represent the standard error of the average population size.

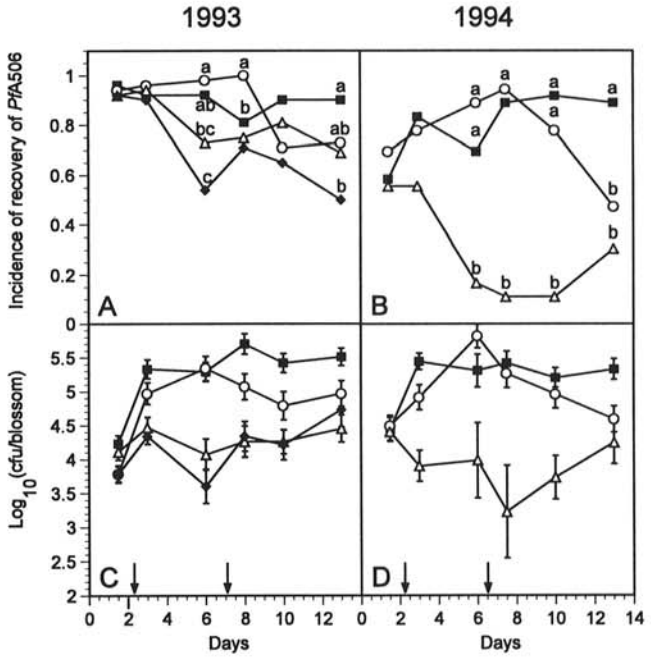

Fig. 3. The effect of alternated and combined streptomycin and oxytetracycline sprays on **A and B,** the proportion of blossoms with detectable populations and **C and D,** the average population sizes of *Pseudomonas fluorescens* strain A506 on 'Rome Beauty' (1993) or 'Golden Delicious' (1994) apple blossoms. Mean population size was calculated only from blossoms with detectable populations of the antagonist. *P. fluorescens* strain A506 was applied at day 0 (30% bloom). Antibiotic sprays or water were applied 2 and 7 days (indicated by arrows on the x axis) after initial application of *P. fluorescens* strain A506. Treatments included i) water (■); ii) streptomycin sprayed on day 2 and oxytetracycline sprayed on day 7 (○); iii) oxytetracycline sprayed on day 2 and streptomycin sprayed on day 7 (△); and iv) a mixture of streptomycin and oxytetracycline sprayed twice (◆, 1993 only). **A and B,** Letters positioned above a symbol indicate significant differences ($P = 0.05$) in the incidence of recovery of *P. fluorescens* strain A506 among treatment means determined on the same date according to Fisher's least significant difference procedure. **C and D,** Vertical bars represent the standard error of the average population size.

on blossoms from which the bacterium was detected were reduced by 10- to 100-fold (Fig. 3C and D). In contrast, an application of oxytetracycline on day 2 followed by an application of streptomycin on day 7 did not significantly reduce the incidence of recovery or the average population size of *E. herbicola* strain C9-1S on most sampling dates in 1994 compared with those observed on blossoms inoculated with *E. herbicola* strain C9-1S and then sprayed with water (Fig. 4B and D). For both *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S, mean population sizes of each bacterium on blossoms treated with oxytetracycline 2 days after inoculation followed by streptomycin 5 days later were consistently smaller than those on blossoms treated first with streptomycin (day 2) and then with oxytetracycline (day 7) (Figs. 3C and D and 4D).

Mixtures of streptomycin and oxytetracycline. In 1993, two applications of a mixture of oxytetracycline and streptomycin significantly decreased the incidence of recovery of *P. fluorescens* strain A506 from apple blossoms compared with that of the water-treated control on two of six sampling dates (Fig. 3A). On five of six sampling dates, mean population sizes of *P. fluorescens* strain A506 on blossoms treated with the antibiotic mixture also were significantly smaller than those recovered from *P. fluorescens* strain A506-treated blossoms that were sprayed with water (Fig. 3C). The incidence of recovery of *E. herbicola* strain C9-1S from blossoms treated with the antibiotic mixture averaged 90%, which was about 10% less than the incidence of recovery of *E. herbicola* strain C9-1S from water-treated blossoms (Fig. 4A). On four of six sampling dates, mean population sizes of *E. herbicola* strain C9-1S on blossoms treated with the antibiotic mixture were sigificantly smaller than those on water-treated blossoms (Fig. 4C).

Comparative effects of antibiotics on *E. amylovora*. In 1994, *E. amylovora* strain 153N became established and persisted in a high proportion (90 to 100%) of apple blossoms that were treated subsequently with water (Fig. 5A). The mean population size of *E. amylovora* strain 153N on water-treated blossoms ranged between 1×10^5 and 5×10^6 CFU/flower with the highest populations occurring on blossoms sampled 10 and 13 days after the bacterial inoculation (Fig. 5B). Spray treatments of streptomycin, oxytetracycline, and alternating applications of these antibiotics each significantly reduced the incidence of recovery of *E. amylovora* strain 153N from apple blossoms compared with that of the water-treated control (Fig. 5A through D). On four of six sampling dates, the incidences of recovery of *E. amylovora* strain 153N from blossoms treated twice with streptomycin were significantly smaller than from blossoms treated twice with oxytetracycline or from those treated with oxytetracycline (day 2) followed by streptomycin (day 7). In general, these three antibiotic treatments (streptomycin followed by oxytetracycline, oxytetracycline followed by streptomycin, and two applications of oxytetracycline) affected epiphytic populations of *E. amylovora* strain 153N similarly. Each of these treatments consistently reduced the mean population size of the pathogen detected on blossoms by 10- to 100-fold relative to populations measured on blossoms sprayed with water. On blossoms treated twice with streptomycin, populations of *E. amylovora* strain 153N were not detectable immediately after the second antibiotic application (7 days after inoculation) and averaged between 10^3 and 10^4 CFU/flower on the other sampling dates after streptomycin sprays.

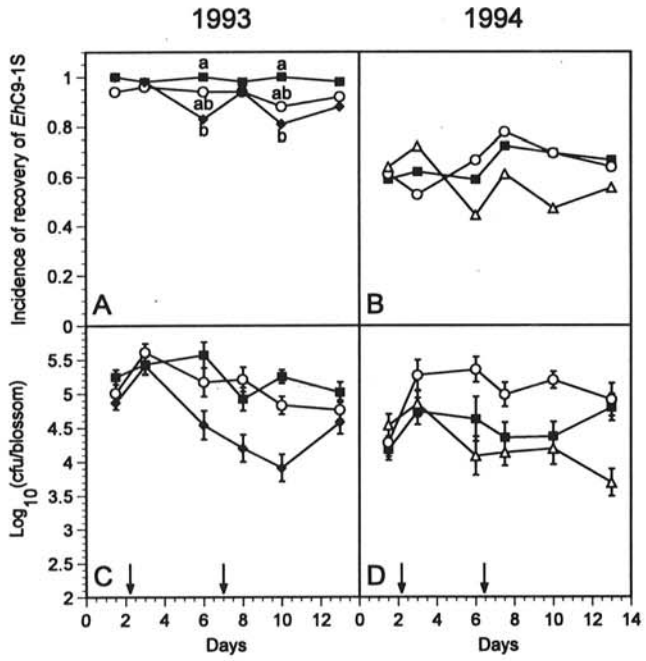

Fig. 4. The effect of alternated and combined streptomycin and oxytetracycline sprays on **A and B,** the proportion of blossoms with detectable populations and **C and D,** the average population sizes of *Erwinia herbicola* strain C9-1S on 'Rome Beauty' (1993) or 'Golden Delicious' (1994) apple blossoms. Mean population size was calculated only from blossoms with detectable populations of the antagonist. *E. herbicola* strain C9-1S was applied at day 0 (30% bloom) and sprayed 2 and 7 days later (indicated by arrows on the x axis) with one of the following treatments: i) water (■); ii) streptomycin sprayed on day 2 and oxytetracycline sprayed on day 7 (○); iii) oxytetracycline sprayed on day 2 and streptomycin sprayed on day 7 (△, 1994 only); and iv) a mixture of streptomycin and oxytetracycline sprayed twice (◆, 1993 only). **A and B,** Letters positioned above a symbol indicate significant differences ($P = 0.05$) in the incidence of recovery of *E. herbicola* strain C9-1S among treatment means determined on the same date according to Fisher's least significant difference procedure. **C and D,** Vertical bars represent the standard error of the average population size.

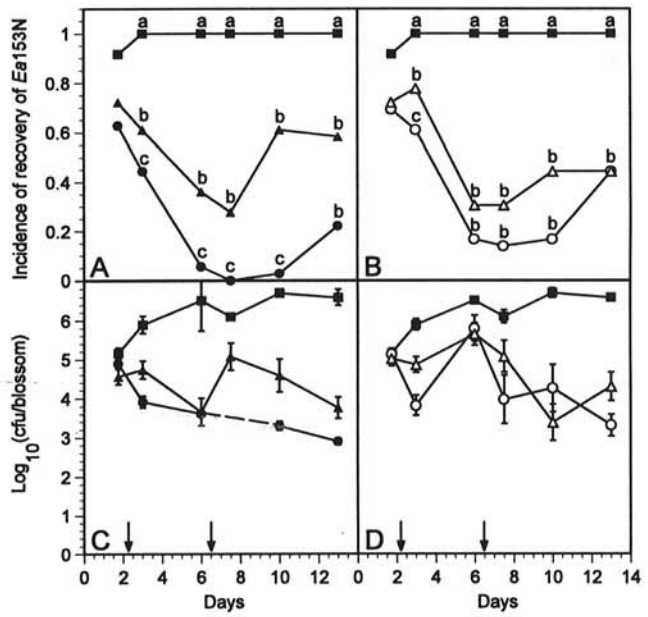

Fig. 5. The effect of streptomycin and oxytetracycline sprays on **A and B,** the proportion of blossoms with detectable populations and **C and D,** the average population sizes of *Erwinia amylovora* strain 153N on 'Golden Delicious' apple blossoms in 1994. Mean population size was calculated only from blossoms with detectable populations of the pathogen. The streptomycin- and oxytetracycline-sensitive isolate of the fire blight pathogen, *E. Amylovora* strain 153N, was applied at day 0 (30% bloom). Antibiotic sprays or water were applied at 2 and 7 days (indicated by arrows on the x axis) after application of the bacterium. Treatments included i) water (■); ii) streptomycin sulfate (100 μg/ml) sprayed twice (●, in **A and C**); iii) oxytetracycline (200 μg/ml) sprayed twice (▲, in **A and C**); iv) streptomycin sprayed on day 2 and oxytetracycline sprayed on day 7 (○, in **B and D**); and v) oxytetracycline sprayed on day 2 and streptomycin sprayed on day 7 (△, in **B and D**). **A and B,** Letters positioned above a symbol indicate significant differences ($P = 0.05$) in the incidence of recovery of *E. amylovora* strain 153N among treatment means determined on the same date according to Fisher's least significant difference procedure. **C and D,** Vertical bars represent the standard error of the average population size.

DISCUSSION

Successful integration of a biological disease control method into a conventionally managed agricultural production system requires that the biocontrol agent(s) will establish adequate populations consistently, even during periods when other control methods (e.g., chemical applications) are implemented. This study demonstrated that this requirement can be met for the antagonists *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S, which have been shown previously to reduce the incidence of the blossom blight phase of fire blight of pome fruits (10,12,14,21,26). Both bacterial strains colonized a high proportion of apple blossoms when applied once to trees at 30% bloom. In addition, both strains persisted on blossoms after the trees were treated subsequently with streptomycin or oxytetracycline. Our results indicated, however, that optimal integration of *P. fluorescens* strain A506 or *E. herbicola* strain C9-1S with oxytetracycline will require that applications of this antibiotic be delayed until after the antagonists have become established on a majority of the blossoms.

On water-treated blossoms, the degree of colonization for *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S, both in terms of proportion of blossoms colonized and their population size within individual flowers, were within the ranges (about 10^5 CFU/blossom) necessary to suppress populations of *E. amylovora* (10,20,21) and to reduce disease incidence (10,12,14). The mean population size of the antagonists generally increased after inoculation, indicating that the bacteria multiplied after application to blossoms. Moreover, because the bacteria were applied to the trees when only 30% of the flowers were open, the high incidence of recovery of *P. fluorescens* strain A506 or *E. herbicola* strain C9-1S observed later in the bloom period was likely the result of dispersal of the antagonists from colonized blossoms to blossoms that opened after inoculation. Consequently, to understand the effects of antibiotic applications on antagonist populations, we examined both the mean population size and the incidence of recovery from individual blossoms; the former parameter reflected net growth of established bacterial colonies and the latter estimated the degree to which the antagonists persisted on inoculated blossoms and dispersed successfully to flowers that were not open at the time of inoculation. These parameters are not totally independent, however, because lower population sizes on colonized blossoms will reduce the rate of secondary colonization of previously unopened blossoms.

Applications of streptomycin were not detrimental to either *P. fluorescens* strain A506 or *E. herbicola* strain C9-1S because no significant reductions in the incidence or the mean population size of the bacterial strains were attributable to treatment with this antibiotic. Furthermore, *P. fluorescens* strain A506 established well on blossoms when mixed with streptomycin in the spray tank during application. Both *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S are resistant to streptomycin (100 µg/ml) in culture and, therefore, the results obtained in the orchard experiments were not unexpected. The significant increases in the population sizes of *E. herbicola* strain C9-1S on some sampling dates after streptomycin sprays, however, were more surprising. We speculate that the streptomycin treatments suppressed the growth of indigenous bacterial epiphytes on blossoms (16), which indirectly increased the growth of *E. herbicola* strain C9-1S because of a reduced level of competition for nutrient resources (13). Cooksey (3) observed that combining a copper-resistant, nonpathogenic mutant of *Pseudomonas syringae* pv. *tomato* with a fixed copper treatment resulted in enhanced colonization of tomato leaves by the copper-resistant antagonist because of reduction of populations of competing epiphytic bacteria.

The mean population size and the incidence of recovery of *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S from blossoms treated with oxytetracycline were less than those on water-treated blossoms. Furthermore, oxytetracycline was more suppressive to populations of *P. fluorescens* strain A506 and *E. herbicola* strain C9-1 when applied 2 days after bacterial inoculation than when applied 7 days after bacterial inoculation. For example, the treatment of blossoms with oxytetracycline followed by streptomycin reduced the incidence of recovery of the antagonists more than the treatment in which streptomycin was applied first. Because the proportion of blossoms with detectable populations of bacterial antagonists after an oxytetracycline treatment was not less than the proportion of blossoms that was open when the antagonists were applied, we infer that oxytetracycline did not eradicate bacterial populations already established on blossoms. Instead, we speculate that oxytetracycline slowed the rate of bacterial multiplication on blossoms and consequently decreased both the rate of bacterial dispersal to blossoms that opened after application of the antibiotic and subsequent bacterial growth on those blossoms. Thus, application of oxytetracycline at midbloom (2 days after application of the antagonists) resulted in fewer blossoms being colonized during bloom than an application of oxytetracycline after full bloom (7 days after application of the antagonists), when most blossoms have been open long enough for dispersal and colonization to occur before the antibiotic spray. The bacteriostatic effect of oxytetracycline on the antagonists was consistent with the effect of the antibiotic in culture (5,11) and on *E. amylovora* on blossoms (17). An alternative explanation for the differential sensitivity of the antagonists to early and late sprays of oxytetracycline focuses on the physiological status of bacteria on plant surfaces at the time of application. For example, bacterial populations actively multiplying on blossoms may be more sensitive to oxytetracycline than established populations that are not growing rapidly. Huang and Burr (6) found that cells of *E. Amylovora* and *Pseudomonas syringae* pv. *papulans* in exponential growth phase were more sensitive to streptomycin than cells in stationary phase. Growth-phase related sensitivity of *P. fluorescens* and *E. herbicola* to oxytetracycline, however, has not been documented.

The detrimental effect of oxytetracycline on *P. fluorescens* strain A506 was unexpected, because the bacterium is resistant to oxytetracycline in culture and enhanced control of fire blight has been observed when *P. fluorescens* strain A506 was combined with oxytetracycline sprays (12,14). Oxytetracycline reduced the incidence of recovery and the mean population size of *P. fluorescens* strain A506, but many blossoms still contained populations of the antagonist of sufficient size to contribute to disease control. Oxytetracycline also was detrimental to *E. herbicola* strain C9-1S, but not to the degree expected based on the sensitivity of *E. herbicola* strain C9-1S to this antibiotic in culture. In both 1993 and 1994, oxytetracycline decreased the incidence of recovery of *P. fluorescens* strain A506 on more sampling dates than it reduced the incidence of *E. herbicola* strain C9-1S.

The 1994 experiments provided data for assessing the relative antibiotic-sensitivity of the antagonists and *E. amylovora*. As expected, *E. amylovora* strain 153N was affected by streptomycin applications to a much greater degree than were the streptomycin-resistant antagonist strains. The effects of two postinoculation applications of oxytetracycline on *E. amylovora* strain 153N, however, were generally similar to the effects of this treatment on *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S (i.e., oxytetracycline caused moderate reductions in both incidence of recovery and mean population size of all three bacteria). Previous reports (17) have concluded that oxytetracycline is bacteriostatic when applied to sensitive bacterial populations on blossoms, whereas streptomycin can be eradicative. Our results with *E. amylovora* strain 153N also showed that oxytetracycline did not have the same ability as streptomycin to reduce the incidence of recovery or the mean population size of epiphytic bacterial colonies on floral surfaces. Furthermore, the order of application was not critical for suppression of populations of this streptomycin-sensitive strain of *E. amylovora*. A single applica-

tion of streptomycin followed by oxytetracycline resulted in a decrease in the population size of *E. amylovora* strain 153N similar to that caused by a single application of oxytetracycline followed by streptomycin. If the pathogen was resistant to streptomycin, we would expect that the order of antibiotic application would influence the magnitude of the population size decrease, as was observed with *P. fluorescens* strain A506 or *E. herbicola* strain C9-1S (i.e., an early application of oxytetracycline followed by streptomycin decreased the populations of these antagonists to a greater degree than an early application of streptomycin followed by oxytetracycline).

The population data collected in this study demonstrated that integration of biological and chemical control for fire blight can be achieved, but that specific strategies for using these control measures in combination are needed. For example, *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S should be applied to trees in midbloom (about 30 to 60% bloom), regardless of the risk of fire blight development, because they require time to become established on blossoms and to grow to population sizes sufficient to suppress *E. amylovora*. Populations of indigenous *E. amylovora* generally are detected in orchards only after full bloom (23). This delay in the appearance and population development of the pathogen provides time for the establishment of the antagonists before full bloom. If weather-based risk advisories (23,24) indicate that conditions are conducive for fire blight, our data indicate that antibiotics can be applied without compromising the activity of the bacterial antagonists. Both *P. fluorescens* strain A506 and *E. herbicola* strain C9-1S appeared to be fully compatible with streptomycin, and both were affected less by oxytetracycline if its application was delayed until the antagonists were established on most of the blossoms. Maintaining this delay would be relatively easy in orchards in which streptomycin could be applied first. This option, however, may be limited to production areas in which the efficacy of streptomycin has not been compromised by a high level of streptomycin-resistance in the *E. amylovora* population (2,15,17,18). In areas where the presence of streptomycin-resistant isolates of *E. amylovora* necessitates the exclusive use of oxytetracycline, an additional antagonist application, nearer to full bloom, may hasten the establishment of antagonist populations on a large proportion of the blossoms, thereby limiting the detrimental effects of an early oxytetracycline application to the antagonists.

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