

## Dispersal of *Erwinia amylovora* and *Pseudomonas fluorescens* by Honey Bees from Hives to Apple and Pear Blossoms

K. B. Johnson, V. O. Stockwell, D. M. Burgett, D. Sugar, and J. E. Loper

First author: Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331-2902; second and fifth authors: USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR 97331; third author: Department of Entomology, Oregon State University, Corvallis, 97331; fourth author: Department of Botany and Plant Pathology, Oregon State University, Southern Oregon Experiment Station, Medford, 97502. Research supported in part by the Winter Pear Control Committee and by the Oregon Agricultural Experiment Station. This is Oregon Agricultural Experiment Station Technical Paper 10,008. Technical assistance of T. Sawyer, K. Theiling, and J. Jenkins is gratefully acknowledged. Accepted for publication 27 January 1993.

### ABSTRACT

Johnson, K. B., Stockwell, V. O., Burgett, D. M., Sugar, D. and Loper, J. E. 1993. Dispersal of *Erwinia amylovora* and *Pseudomonas fluorescens* by honey bees from hives to apple and pear blossoms. *Phytopathology* 83:478-484.

Ability of honey bees (*Apis mellifera*) to disperse the fire blight pathogen, *Erwinia amylovora* strain 153nal<sup>R</sup>, and *Pseudomonas fluorescens* strain PFA506, a biocontrol agent for fire blight, from their hives to pistillate surfaces of pome fruit blossoms was investigated. Two hives, one to disperse Ea153nal<sup>R</sup> and the other to disperse PFA506, were placed in an apple orchard of cultivar Rome (1990 and 1991) or Golden Delicious (1992) during bloom. Pollen inserts were attached to each hive, which forced the bees to walk through a freeze-dried preparation of either bacterium as they exited. PFA506 inoculum contained 10<sup>10</sup> to 10<sup>11</sup> colony-forming units per gram (cfu/g) and was dispersed at 10<sup>5</sup> to 10<sup>6</sup> cfu/bee. Inoculum of Ea153nal<sup>R</sup> contained 10<sup>9</sup> to 10<sup>11</sup> cfu/g and was dispersed at 10<sup>4</sup> to 10<sup>6</sup> cfu per bee. In several experiments, a lognormal distribution described the variation in density of bacterial cfu/bee. Each year, apple blossoms were sampled at various distances from the hives. Maximum

frequency of recovery of PFA506 in sampled blossoms ranged from 23 (1990) to 81% (1991). Detection of Ea153nal<sup>R</sup> in blossoms was more variable, ranging from <1 (1990) to 72% (1992). In experiments on pear, a single beehive was placed in a planting of 40 16-yr-old pear trees of cultivar Bartlett enclosed within a cage constructed of 30% shade cloth. Bees within the enclosed area were inoculated with freeze-dried Ea153nal<sup>R</sup> as they exited the hive through a pollen insert. Maximum detection of Ea153nal<sup>R</sup> in blossoms was 41% in 1991 and 27% in 1992. Incidence of fire blight was 9 and 41% of blossom clusters in 1991 and 1992, respectively. Among all experiments, the estimated efficiency of bees as bacterial vectors averaged 20 blossoms per hour of foraging activity. Use of honey bees to disperse bacteria should prove valuable as a method to inoculate flowers of pome fruits with *E. amylovora*, and to study ecology of bacterial epiphytes within conventionally managed orchards.

*Additional keyword:* biological control.

In the western United States, fire blight, caused by the bacterium *Erwinia amylovora*, is a sporadic but serious disease of pear (*Pyrus communis* L.) and is of increasing importance on apple (*Malus × domestica* Borkh.). Under the semiarid conditions typical of this region, control measures focus on limiting the blossom blight phase of the disease. Chemical control and sanitation are the principal practices employed; however, resistance of *E. amylovora* to the antibiotic streptomycin in Washington (8) and California (11) has made chemical control of fire blight less reliable.

Biological control of fire blight is a promising approach (1,7,16) that if integrated with conventional practices could add stability to disease management programs. Most successful experiments on biological control of fire blight involve spraying aqueous suspensions of bacterial antagonists on blossoms (1,7,16). The nonpathogenic antagonists colonize stigmatic and hypanthial surfaces within blossoms (3,16) and are thought to exclude *E. amylovora* preemptively from these nutrient-rich surfaces where disease is initiated (3,14,16,17).

The blossom blight phase of fire blight is influenced by insect activity within an orchard. Van der Zwet and Kiel (18) considered insects to be the most important means of dissemination for *E. amylovora* and listed 77 genera associated with spread of the disease. Honey bees (*Apis mellifera* L.), because of their importance as pollinators, have been intensively studied with regard to disease transmission (5,12,19). Most studies conclude that honey bees are important in transmitting *E. amylovora* from flower to flower but not from overwintering cankers to flowers (12,18).

Beehives are routinely placed in Oregon and Washington pear and apple orchards during the bloom period to aid in pollination (10). Dispensers known as pollen inserts are sometimes attached to the entrance of hives in order to inoculate bees with supplemental pollen as they exit to begin foraging. Inserts are designed so that bees leaving a hive must walk through the pollen within the dispenser, whereas most returning bees avoid reinoculation (10). With this method, honey bees have been shown to disseminate pollen contaminated with bacterial antagonists of *E. amylovora* to pear flowers (15), but the efficiency of honey bees as bacterial vectors is not known.

The purpose of this study was to investigate the efficiency with which honey bees introduce either *E. amylovora* or a nonpathogenic, epiphytic strain of *Pseudomonas fluorescens* to blossoms of apple or pear. A second objective was to evaluate bee dissemination of *E. amylovora* as an inoculation method for further studies on fire blight epidemiology and control.

### MATERIALS AND METHODS

**Inoculum preparation.** A spontaneous, nalidixic-acid resistant mutant of *E. amylovora* strain 153 (*Ea153nal<sup>R</sup>*) and *Pseudomonas fluorescens* strain A506 (*PFA506*) were used as inoculum. *Ea153* was originally isolated in 1989 from fire blight cankers on Gala apple in Milton-Freewater, OR. *PFA506*, obtained from S. Lindow (Dept. of Plant Pathology, University of California, Berkeley) is a rifampicin-resistant (200 mg/L) derivative of a strain found on pear in California that has controlled fire blight when applied in an aqueous suspension during bloom (7).

Each bacterial strain was cultured separately at 20–25 C in petri dishes or on sterilized cafeteria trays that contained nutrient agar (Difco Laboratories, Detroit, MI) amended with 1% glycerol. Rifampicin (100 mg/L) or nalidixic acid (25 mg/L) was added

for culture of *PfA506* or *Ea153nal*<sup>R</sup>, respectively. Cultures were harvested after 4 days with a spatula and mixed one to one (w/w) with a cryoprotective suspension of skim milk (18%) and xanthan gum (2%) in distilled water (6). In 1990 only, *Ea153nal*<sup>R</sup> was mixed 1:10 with the milk/gum suspension. After mixing, culture suspensions were frozen at -80 C and lyophilized for 24-48 h. Dried cultures were ground through a series of metal screens, the smallest of which had a mesh size of 0.30 mm. In 1990 and for the *Ea153nal*<sup>R</sup> preparation used in the pear experiment in 1991, the dried cultures were mixed one to one (w/w) with powdered sucrose during the grinding process. Dried bacterial preparations were stored at -15 C. The number of viable colony-forming units (cfu) per gram of each bacterial preparation was assayed periodically by resuspending the preparation in sterile potassium phosphate buffer (0.05 M, pH 6.5; SPB) and plating aliquots of serial dilutions (1:9) on Difco *Pseudomonas* agar F amended with rifampicin (100 mg/L)(PFR) for *PfA506* or on CCT medium (4) amended with nalidixic acid (50 mg/L) for *Ea153nal*<sup>R</sup>. Colonies of *PfA506* on PFR were enumerated after 2-3 days incubation at 20-25 C, whereas colonies of *Ea153nal*<sup>R</sup> counted after 3-4 days.

**Bacterial dispersal.** Experiments to measure bacterial dispersal by bees were initiated by placing a beehive(s) in plantings of apple or pear trees at 5-10% bloom. At this time, a pollen insert (Antles Pollen Supplies, Inc., Wenatchee, WA) was attached to each hive and filled with freeze-dried inoculum of a bacterial strain on days when the bees were expected to forage. Inoculum (5-10 g) was placed into a pollen insert every 30 min to 2 h, depending on the size of a hive and amount of bee activity.

Bacterial concentrations on bees were determined by capturing individual bees as they exited the pollen insert in autoclaved, 250-ml bottles that contained 10 ml of SPB. Captured bees were refrigerated (4 C) overnight, during which time they drowned. Neither bacterial strain grew significantly when refrigerated for this length of time. Bottles were shaken on a vortex mixer for 30 sec and 0.01-ml aliquots of the buffer and two 100-fold serial dilutions were spread on PFR for *PfA506* or on CCT-nal for *Ea153nal*<sup>R</sup>. Twelve to 24 bees were captured on each of two or three dates in each experiment. Collections were made throughout the day with most bees captured 15-30 min after the inoculum supply within the pollen insert was replenished.

Bee activity within each experimental planting was quantified during periods when the pollen inserts contained bacterial inoculum by recording the number of hours the bee colonies actively foraged, and by making hourly assessments of the rate of bee inoculation and the number of bees foraging in trees assigned an experimental treatment. Rate of bee inoculation was defined as the number of bees that exited through a pollen insert per minute. The number of bees foraging in a tree were counted by closely examining the entire tree canopy for periods of 30-60 sec (10). Numbers of blossoms per tree were estimated by counting the number of blossom clusters per tree and multiplying by the average number of blossoms per cluster.

**Detection of bacteria in blossoms.** Apple and pear blossoms were sampled three to five times during bloom to determine if bacteria inoculated onto bees were detectable on the pistillate surfaces of flowers. On each sampling date, 22-33 blossoms with mature (dark-colored) anthers were removed in a haphazard pattern from each tree and placed individually into wells of ethanol-disinfested egg cartons to avoid cross-contamination during transport. In the lab, either the styles and stigmas (apple) or whole pistils including ovaries and nectaries (pear) were excised from blossoms with flamed tweezers or a scalpel. For each blossom, excised floral tissues were placed into a test tube that contained 2.24 ml of SPB. Racks of test tubes that contained pistils were agitated in a bath-type sonicator for 60 sec. After sonication, a 0.01-ml aliquot of the blossom wash and of a 1:224 dilution were spread on separate halves of an agar surface in a petri dish. In the apple experiments, population sizes of total aerobic bacteria were estimated on *Pseudomonas* agar F, strain *PfA506* on PFR, and *Ea153nal*<sup>R</sup> on CCT-nal. In the pear experiments, only populations of *Ea153nal*<sup>R</sup> were assessed.

Detection limits for the wash solution and the 1:224 dilution were  $2.24 \times 10^2$  and  $5.0 \times 10^4$  cfu, respectively. After every 11 blossoms, a set of test tubes that contained only SPB were similarly processed as controls. Bacterial colonies were counted on each plate after 2-3 days incubation at 20-24 C. Throughout the apple experiments, only bacterial colonies that produced a fluorescent pigment and had a size, morphology, and color similar to that of *PfA506* were recovered on PFR. On CCT-nal, most colonies were of the same size and morphology as the original strain of *Ea153nal*<sup>R</sup>, although a bacterium with more translucent colony was isolated occasionally. Pathogenicity of a subset of *Ea153nal*<sup>R</sup> isolates recovered on CCT-nal was verified each year by inserting each isolate into an immature pear fruit with a dissecting needle and incubating the inoculated fruit in a high humidity chamber at 20-24 C. Presence or absence of fire blight symptoms on the fruit was noted after 6 days.

**Apple experiments.** In the springs of 1990, 1991, and 1992, two beehives were placed near the edge of an apple planting at the Oregon State University, Dept. of Botany and Plant Pathology Research Farm near Corvallis, OR, when the trees were at 5-10% bloom. One hive was designated to disperse *PfA506* and the other to disperse *Ea153nal*<sup>R</sup>. In 1990, the planting consisted of 50 32-yr-old trees of cultivar Rome on Malling 7 rootstock arranged five rows wide by 10 rows deep with individual trees spaced 6 m apart. The 1991 planting contained 63 12-yr-old trees of cultivar Rome on Malling 7 rootstock (7 rows wide  $\times$  9 rows deep; 6 m between trees). In 1992, the experiment was established in a 70-tree planting (7 rows wide  $\times$  10 rows deep; 6 m between trees) of 13-yr-old Golden Delicious on Malling 26 rootstock. In all years, routine applications of fenarimol and dodine were applied to the plantings during the experimental period to control apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*).

During 1990, trees designated for sampling were located 12, 24, and 56 m from the hives. Bacterial inoculum was placed in pollen inserts on 13, 14, and 18-21 April. Blossoms were sampled on 13, 18, 23, and 30 April. On 23 and 30 April, an additional sample of 44 blossoms was taken from the planting of 12-yr-old Rome apple, which was located 100 m northeast of the 32-yr-old Rome planting. The intent of this sample was to provide a comparative control as to the source of the antibiotic-resistant bacteria within the experimental planting. In late May, the experimental trees were examined for fire blight symptoms.

Trees designated for sampling in 1991 were located 12, 24, and 42 m from the edge of the orchard where two beehives were positioned. Bacterial inoculum was placed in pollen inserts on 30 April and 1-4 May; blossoms were sampled on 29 April and 2, 6, and 12 May. As a comparative control, an additional sample of 44 blossoms was taken on 2, 6, and 12 May from the block of 32-yr-old Rome apple, which was used for the experiment in the previous year. Fire blight was assessed on 6 and 17 June. Isolation of *Ea153nal*<sup>R</sup> was attempted from all blighted blossom clusters observed in the trees by macerating diseased tissue in SPB and then streaking the suspension onto CCT-nal.

During 1992, blossoms were sampled from trees located in rows 6, 24, and 36 m from the edge of the planting where the beehives were positioned. Bacterial inoculum was placed in pollen inserts on 1-3, 6-8, and 10 April. Blossoms were sampled on 31 March, 5, 12, and 16 April. An additional 44 blossoms were sampled on 5, 12, and 16 May from a planting of 38-yr-old apple trees (cultivar Jonathan) located 200 m to the west of the experimental planting. On 11 May, fire blight was assessed and 25 diseased blossom clusters were sampled for *Ea153nal*<sup>R</sup> as described above.

**Pear experiments.** In March of 1991 and 1992, four adjacent rows of 10 16-yr-old Bartlett pear trees located at the Southern Oregon Experiment Station in Medford were enclosed with polypropylene shade cloth (30% shade, 2.2 mm mesh, Nicolon Corp., Norcross, GA) to confine bee flight activity within the plot during bloom. Trees were spaced 2.5 m apart with 4 m between rows. Dimensions of the shade-cloth enclosure were 25  $\times$  18  $\times$  4.5 m in height, supported by a frame constructed of 2.5-cm-diameter galvanized steel conduit and 1-cm-diameter braided steel cable.

As part of a larger study on fire blight biology, 20 trees within the cage were assigned experimental treatments (four replications of five treatments) and remaining trees served as buffers between treated trees. Only results from the water-treated control are presented in this paper. The shade cloth enclosure was dismantled in early May of each year.

A single beehive was placed inside the enclosure at 5% bloom (10 April 1991 and 20 March 1992). Inoculum of *Ea153nal*<sup>R</sup> was placed within a pollen insert attached to the hive on 14–17 April 1991 and 21–24 March 1992. Water (3 L) was applied to each

tree with a hand-directed backpack sprayer on 10, 13, 15 and 17 April 1991 (5, 35, 75, and 95% bloom, respectively) and on 18, 20, 22 and 24 March 1992 (3, 25, 65, and 90% bloom, respectively). In 1991, blossoms were sampled on 10, 14, 18, 21, and 24 April; in 1992, blossom sampling dates were 19, 23, 26, and 29 March and 2 April. Blighted blossom clusters per tree were counted on 8 and 23 May and 26 June in 1991 and on 3, 10, and 22 April and 7 May in 1992. Blighted clusters were pruned from the trees as they were counted, and an attempt was made to isolate *Ea153nal*<sup>R</sup> from 25 of these blighted clusters as

TABLE 1. Experimental conditions under which honey bees, placed in apple or pear orchards, were inoculated with freeze-dried preparations of *Erwinia amylovora* strain 153nal<sup>R</sup> or *Pseudomonas fluorescens* strain A506

Experimental condition	Dispersal experiment				
	Apple/Corvallis <sup>a</sup>			Pear/Medford <sup>b</sup>	
	1990	1991	1992	1991	1992
Bloom period	13 Apr–30 Apr	29 Apr–12 May	31 Mar–13 Apr	10 Apr–24 Apr	17 Mar–1 Apr
Average daily minimum temperature (C)	6.8 (2.7) <sup>f</sup>	5.3 (3.3)	5.6 (3.0)	2.7 (3.1)	2.4 (3.4)
Average daily maximum temperature (C)	17.4 (4.1)	16.7 (3.5)	16.1 (4.1)	16.2 (3.4)	18.0 (3.0)
No. of days with rain	11	6	10	9	6
Average amount of rain (mm/day) <sup>d</sup>	5.4 (5.7)	6.2 (6.7)	6.2 (8.6)	3.3 (3.7)	2.3 (1.4)
Inoculum concentration (log <sub>10</sub> cfu/g):					
<i>Ea153nal</i> <sup>R</sup>	9.0 (0.6)	11.0 (0.5)	11.1 (0.3)	11.1 (0.2)	11.1 (0.3)
<i>PfA506</i>	10.1 (0.5)	10.7 (0.2)	10.6 (0.2)	... <sup>c</sup>	...
No. of days bees were inoculated	6	5	7	3	4
Hours per day bees were inoculated	3.8 (1.1)	4.4 (0.7)	3.5 (1.3)	3.7 (0.8)	3.9 (0.9)
Amount of inoculum dispensed per day (g):					
<i>Ea153nal</i> <sup>R</sup>	35 (29)	84 (13)	41 (19)	17 (3)	9 (5)
<i>PfA506</i>	46 (37)	91 (21)	39 (15)	...	...
Bee inoculation rate <sup>f</sup> (bees/min):					
<i>Ea153nal</i> <sup>R</sup>	48 (13)	91 (21)	62 (25)	32 (14)	15 (10)
<i>PfA506</i>	49 (12)	85 (20)	42 (19)	...	...
Average no. of bees per tree <sup>g</sup>	9.0 (5.5)	6.5 (3.2)	5.0 (5.1)	6.2 (5.0)	5.8 (3.8)
Bee hours per tree <sup>h</sup>	205	143	122	69	90
Blossoms per tree	3,770 (602)	2,582 (1,364)	4,543 (2,486)	2,444 (553)	1,644 (633)
Blossoms inoculated per bee hour: <sup>i</sup>					
<i>Ea153nal</i> <sup>R</sup>	<1	15	44	15	5
<i>PfA506</i>	9	31	28	...	...

<sup>a</sup> Apple cultivar was Rome in 1990 and 1991, and Golden Delicious in 1992; trees were located near Corvallis, OR.

<sup>b</sup> Pear cultivar was Bartlett; trees were located near Medford, OR.

<sup>c</sup> Values followed by standard deviation represent means averaged over the experimental period.

<sup>d</sup> Mean presented was averaged over days with rain.

<sup>e</sup> Bees were not used to disperse *PfA506* on pear trees.

<sup>f</sup> Average number of bees per minute that exited a pollen insert containing bacterial inoculum.

<sup>g</sup> Mean of individual counts of number of bees foraging in each tree; counts were taken at hourly intervals on days when pollen inserts contained bacterial inoculum.

<sup>h</sup> Value presented was obtained by summing the hourly counts of bees per tree on days that pollen inserts contained bacterial inoculum; sums were adjusted for partial hours that occurred at the end of inoculation periods.

<sup>i</sup> Value presented is average number of blossoms with a detectable population of the given bacterial strain divided by bee hours per tree (see text for further explanation of the numerator).



described above.

**Data analysis.** For each experiment, data on viable colony-forming units per gram of dried bacterial preparation, weather conditions, and bee activity (inoculation rate, foraging hours per day, and number per tree) were summarized by computing the mean and standard deviation. Distributions of bacterial colony-forming units per bee were compared among experiments by plotting the logarithm of the value obtained for each bee against tabulated normal-order statistics (i.e., rankits [2,9]); the Shapiro-Wilk statistic also was computed to test normality of log-transformed bacterial colony-forming units per bee with the univariate procedure of SAS (Statistical Analysis Systems, Cary, NC). Ratios of the mean logarithm of bacterial colony-forming units per bee to the logarithm of inoculum concentration (cfu/g) were calculated for each experiment to estimate the amount of inoculum carried by a bee (gram per bee) as it exited a pollen insert.

For the apple experiments, the mean proportion and standard error of blossoms that had detectable populations of total bacteria, *PfA506*, and *Ea153nal<sup>R</sup>* were calculated for each sampling date by averaging the values obtained for individual trees. Mean and standard deviation of population size of bacteria and bacterial strains on apple stigmas were calculated by averaging the logarithm of values obtained for blossoms on which bacteria or a bacterial strain were detected; i.e., blossoms with bacterial populations below the detection limit were not included in the mean. The effect of distance of a tree from the hives on the number of bees per tree and the proportion of blossoms with detectable populations of dispersed bacteria was evaluated by dividing the values obtained for the two most distal trees on each sampling date by the value obtained on the same date for the tree closest to the hive. These relative values of number of bees per tree and proportion of blossoms containing dispersed bacteria were regressed on distance from a hive.

In the pear experiments, the proportion and standard error of blossoms with detectable populations of *Ea153nal<sup>R</sup>* were calculated for each sampling date by averaging the values obtained for individual trees. Mean population size of *Ea153nal<sup>R</sup>* was computed as described above for apples.

Efficiency of bees as vectors of bacteria to blossoms was calculated for each experiment by dividing number of blossoms per tree with a detectable population of a dispersed bacterial strain by an estimate of number of hours individual, inoculum-infested bees foraged on a tree (units of the ratio are blossoms per bee hour). Number of blossoms inoculated per tree was calculated as the product of average number of blossoms per tree by the average proportion of blossoms with a detectable population of a bacterial strain. Data from the sampling date with the highest average proportion of blossoms with a detectable population of a bacterial strain was chosen for this calculation. Bee hours on a given tree was obtained by summing the hourly counts of bees per tree over all days the inserts contained inoculum. These sums were adjusted for partial hours that occurred at the end of inoculation periods. In apple experiments, in which two hives, one with inoculum of *PfA506* and the other with *Ea153nal<sup>R</sup>*, were present in the orchard, bee hours per tree was partitioned into bee hours per tree for each bacterial strain by multiplying total bee hours by the average rate at which bees were inoculated with a given strain at the hive divided by the average combined rate of bee inoculation for both hives.

## RESULTS

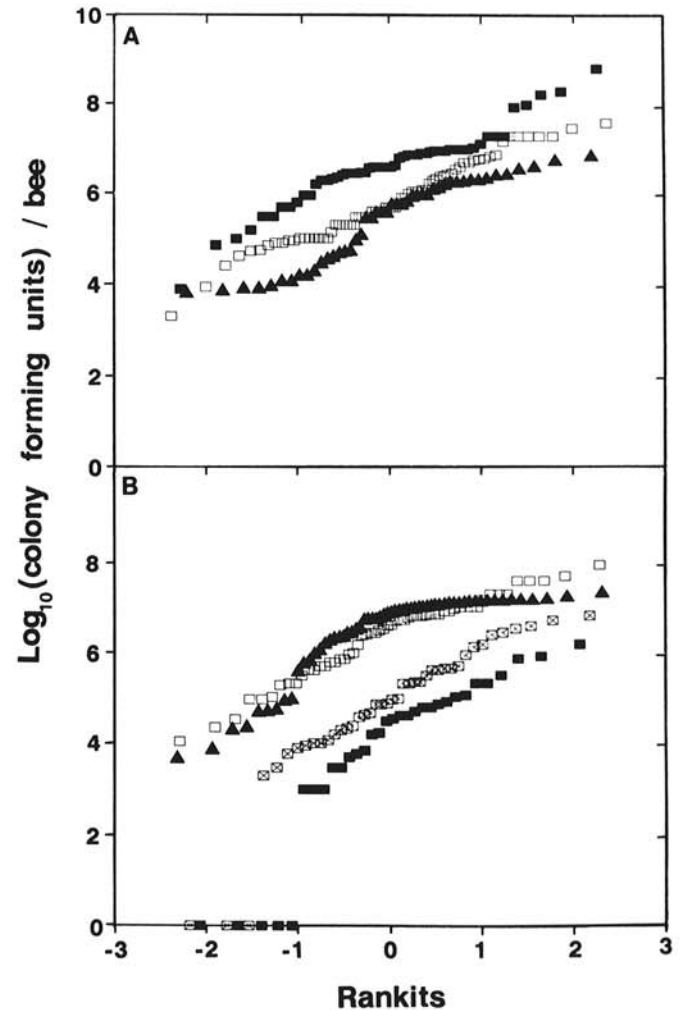
**Experimental conditions.** In all five experiments, weather conditions were favorable for honey bee foraging on about half the days during apple or pear bloom. Most often, unfavorable conditions for bee activity occurred during cloudy periods that were accompanied by rain or during periods of strong winds. Number of days with rain and average daily maximum temperature were similar in Corvallis and Medford, but Corvallis had warmer nights and averaged more than twice the amount of rain per day than Medford (Table 1).

Bees were inoculated with bacteria for about 4 h per day on

5–7 days in the apple experiments and for 3 or 4 days in pear experiments (Table 1). Rate of inoculation of honey bees and amount of bacterial inoculum dispensed in pollen inserts per day were correlated ( $r = 0.96$ ) but variable among experiments (Table 1). Both the size of a bee colony and environmental conditions affected the rate with which bees exited the hive. In the 1990 and 1991 apple experiments, the rates at which bees were inoculated with *PfA506* and *Ea153nal<sup>R</sup>* were similar, but in 1992, 48% more bees passed through the pollen insert containing *Ea153nal<sup>R</sup>* than that containing *PfA506* (Table 1).

On average, counts of number of bees per tree were highest in the 32-yr-old planting of apple cultivar Rome used in the 1990 experiment and lowest in the 13-yr-old planting of apple cultivar Golden Delicious used in 1992 (Table 1). The average ratio of number of bees per tree to bee inoculation rate, however, was higher in pear experiments conducted within the shade-cloth enclosure (0.25) than in apple experiments conducted in the open (0.12). During periods the pollen inserts contained bacterial inoculum, cumulative bee hours per tree ranged from 122 to 205 in the apple experiments and from 69 to 90 in the pear experiments (Table 1). Estimates of blossoms per apple tree were approximately twice those obtained in pear (Table 1).

**Inoculum concentration and bacteria per bee.** Concentration of *PfA506* in freeze-dried preparations was greater than  $10^{10}$  cfu/g in all experiments (Table 1). Concentration of *Ea153nal<sup>R</sup>* was about  $1 \times 10^{11}$  cfu/g in all experiments except in 1990 when



**Fig. 1.** Rankit diagrams of number of colony-forming units of **A**, *Pseudomonas fluorescens* strain A506 and **B**, *Erwinia amylovora* strain 153nal<sup>R</sup> on individual honey bees captured as they exited a pollen insert in 1990 (solid square), 1991 (open square = Corvallis and crossed square = Medford), and 1992 (solid triangle). Within an experiment, the degree of linearity of a data set indicates how closely the sample followed the lognormal distribution (2,9).

the concentration was deliberately decreased to  $1 \times 10^9$  cfu/g (Table 1).

Bees carried concentrations of freeze-dried bacteria that ranged from less than the detection limit ( $10^3$  cfu/bee) to more than  $10^8$  cfu/bee; the mean bacterial concentration per bee among experiments ranged from 4.5 (*Ea153nal<sup>R</sup>*, 1990) to 6.6  $\log_{10}$  cfu/bee (*PfA506*, 1990). Plots of  $\log_{10}$  bacterial colony-forming units per bee against normal-order statistics (rankits) approximated a straight line in most experiments (Fig. 1A and B), indicating that the variation in number of bacteria per bee was described by a lognormal distribution (2,9). Computed values of the Shapiro-Wilk statistic (W) were greater than 0.95 ( $P > 0.03$ ) for all log-transformed distributions measured in 1990 and 1991 but less than 0.90 ( $P < 0.001$ ) for *PfA506* and *Ea153nal<sup>R</sup>* in 1992. Ratios of bacterial colony-forming units per bee to inoculum concentration (cfu/g) indicated that bees carried from 10  $\mu$ g per bee (*Ea153nal<sup>R</sup>*, pear, 1991) to 310  $\mu$ g per bee (*PfA506*, 1990); for the other five experiments, bees carried between 70 and 300  $\mu$ g per bee.

**Apple experiments.** Bacteria, including *PfA506* and *Ea153nal<sup>R</sup>*, were readily recovered from apple stigmas. In general, incidence of total aerobic bacteria on stigmas of individual blossoms increased over the bloom period with the maximum detection occurring in samples taken in late petal fall (Fig. 2). Within an experiment, the proportion of blossoms with detectable populations of total aerobic bacteria was always greater than that of *PfA506* or *Ea153nal<sup>R</sup>* (Fig. 2A–C). Other aerobic bacteria were recovered from blossoms that contained *PfA506* or *Ea153nal<sup>R</sup>* in 47 and 58% of the samples, respectively.

Detection of *PfA506* on blossoms increased from 0% on the first sample date to 26, 82, and 30% of blossoms sampled in late bloom of 1990, 1991, and 1992, respectively (Fig. 2A–C). Similarly, detection of *Ea153nal<sup>R</sup>* increased from 0% to 43 and 70% of blossoms in 1991 and 1992, respectively (Fig. 2B and C), but not in 1990 (Fig. 2A), when a lower inoculum concentration was used (Table 1). Average population size of both *PfA506* and *Ea153nal<sup>R</sup>* ranged from 3.1 to 4.7  $\log_{10}$  cfu per blossom (Table 2); measured population sizes of *PfA506* in 1992 were smaller than in the other 2 yr. Distance to a tree from a hive did not significantly ( $P < 0.05$ ) affect detection of either dispersed bacterial strain in blossoms in any year. Counts of bees per tree significantly declined ( $P < 0.05$ ) by 0.5% per meter from the hive in 1991 and by 0.9% per meter in 1992; distance did not affect the counts of bees per tree in 1990.

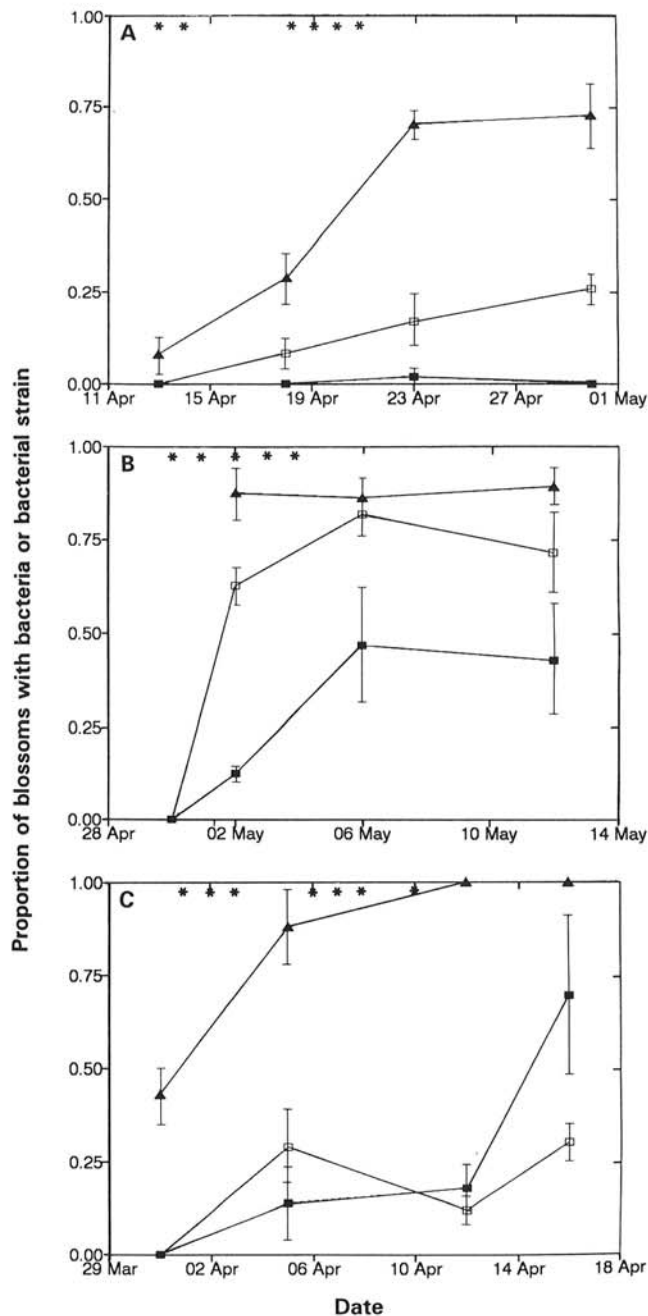
Both *PfA506* and *Ea153nal<sup>R</sup>* were recovered from blossoms sampled in adjacent plantings of apple located 150–200 m from the planting in which the hives were positioned. In 1990, *PfA506* was detected in two blossoms of 96 blossoms sampled from the adjacent planting and two different blossoms also had detectable populations of *Ea153nal<sup>R</sup>*. In 1991, over all sampling dates, detection of *PfA506* and *Ea153nal<sup>R</sup>* in blossoms sampled from the adjacent planting averaged 55 and 94%, respectively, of the proportions obtained in blossoms sampled in the planting where the hives were located; in 1992, these averages were 30 and 37% for *PfA506* and *Ea153nal<sup>R</sup>*, respectively.

In 1990, fire blight was not observed in the 32-yr-old planting of cultivar Rome used for the bee dispersal experiment. In 1991, a total of seven blighted blossom clusters were observed within the 12-yr-old planting of cultivar Rome. Twelve-year-old trees of cultivar Golden Delicious trees averaged  $27 \pm 7$  (standard error of the mean) blighted clusters per tree in 1992. Nalidixic acid-resistant *E. amylovora* was recovered from each sampled diseased blossom cluster in both years.

**Pear experiments.** Incidence of *Ea153nal<sup>R</sup>* in pear blossoms increased from 0% on the first sampling date to 41 and 27% of blossoms sampled on the last date in 1991 and 1992, respectively (Fig. 3A and B). Average population size of *Ea153nal<sup>R</sup>* in blossoms also increased from  $3.4 \pm 0.8$  (standard deviation) (1991) and  $3.2 \pm 0.6$  (1992)  $\log_{10}$  cfu per blossom while bees were present in the enclosure to  $4.9 \pm 1.3$  (1991) and  $5.4 \pm 1.6$  (1992)  $\log_{10}$  cfu per blossom on the last sampling date. Each season, symptoms of fire blight began to develop about 10 days after the last blossom

sample. Over a period of several weeks, incidence of disease increased to an average of 24 (8%) and 111 (41%) blighted clusters per tree in 1991 and 1992, respectively (Fig. 3C and D). Pear trees that bordered the outside of the enclosed area did not develop fire blight symptoms in either year. All isolations from blighted blossom clusters in both years resulted in recovery of nalidixic acid-resistant *E. amylovora*.

**Bee vector efficiency.** Estimated efficiencies of honey bees as vectors of freeze-dried bacteria from hive to blossoms were variable among experiments, ranging from  $<1$  (*Ea153nal<sup>R</sup>*, 1990) to 44 blossoms per bee hour (*Ea153nal<sup>R</sup>*, apple, 1992) (Table 1). Excluding the low concentration inoculum of *Ea153nal<sup>R</sup>* that was used in 1990, the efficiency of honey bees as bacterial vectors



**Fig. 2.** Proportion of apple blossoms with detectable populations of total aerobic bacteria (solid triangle), *Pseudomonas fluorescens* strain A506 (open square) and *Erwinia amylovora* strain 153nal<sup>R</sup> (solid square) in A, 1990, B, 1991, and C, 1992. Honey bees from hives positioned at the edge of 50–70 tree apple plantings were inoculated with *Pseudomonas fluorescens* strain A506 and *Erwinia amylovora* strain 153nal<sup>R</sup> on dates indicated by \*'s near the top of each panel. Length of the vertical line through a data point is twice the standard error of the mean.

TABLE 2. Mean populations of total aerobic bacteria and of bacterial strains inoculated onto honey bees recovered from stigmatic surfaces of apple blossoms<sup>a</sup>

Bacterial population	1990				1991			1992		
	13 Apr	18 Apr	23 Apr	30 Apr	2 May	6 May	12 May	5 Apr	12 Apr	16 Apr
Total aerobic bacteria	2.8 (0.2) 6	4.4 (1.3) 20	3.7 (1.0) 46	4.3 (1.2) 48	3.8 (1.0) 58	4.8 (1.0) 58	4.5 (1.1) 59	4.1 (1.2) 58	5.7 (0.9) 66	6.6 (0.6) 66
<i>Pseudomonas fluorescens</i> strain A506	ND <sup>b</sup>	4.7 (1.4) 5	4.1 (1.3) 11	3.7 (1.2) 17	3.5 (0.6) 41	4.2 (1.0) 53	4.2 (1.0) 47	3.3 (0.9) 19	3.1 (0.8) 4	3.1 (0.8) 20
<i>Erwinia amylovora</i> strain I53nal <sup>R</sup>	ND	ND	5.6 1	ND	3.7 (1.1) 8	4.3 (1.1) 31	3.7 (1.1) 26	3.9 (0.8) 9	3.3 (1.0) 14	3.8 (1.2) 46

<sup>a</sup> Populations are expressed as log<sub>10</sub> (cfu) per blossom followed by the standard deviation and number of blossoms averaged; only blossoms from which bacteria or the given bacterial strain were detected were averaged in the mean.

<sup>b</sup> Bacterial strain was not detected.

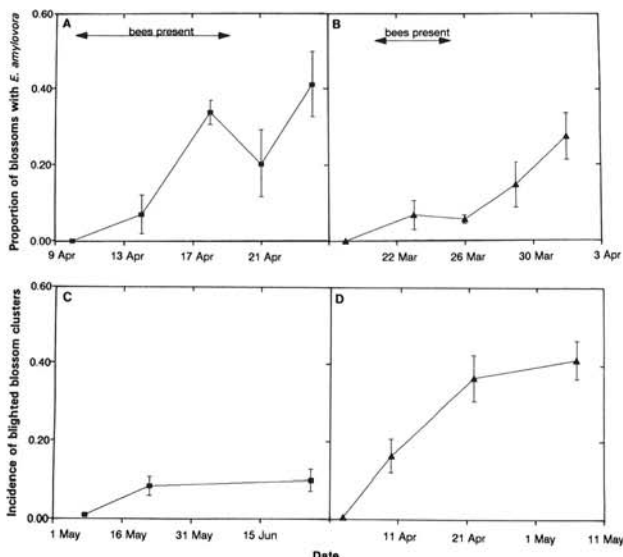


Fig. 3. Incidence of A,B, *Erwinia amylovora* strain I53nal<sup>R</sup> in pear blossoms and, C,D, fire blight strikes in pear trees in the springs of 1991 and 1992, respectively. The pear trees were enclosed within a cage of 30% shade cloth. Honey bees from a hive positioned within the enclosure were inoculated with *Erwinia amylovora* strain I53nal<sup>R</sup> on three (1991) or four (1992) dates during the period indicated near the top of each panel. Length of the vertical line through a data point is twice the standard error of the mean.

averaged among experiments was 19.8 blossoms per bee hour for *Ea153nal<sup>R</sup>* and 22.7 blossoms per bee hour for *PfA506*.

## DISCUSSION

Honey bees were successfully used to disperse dried preparations of viable bacteria from hives to blossoms, and bacteria vectored in this manner can become established on stigmatic surfaces within these blossoms. Assuming all isolates of *PfA506* and *Ea153nal<sup>R</sup>* detected on blossoms originated from bees, the estimated efficiency of this process averaged 20 blossoms per bee hour for bees that exited a pollen insert filled with bacterial inoculum that contained 10<sup>10</sup> to 10<sup>11</sup> cfu/g (Table 1). We observed that foraging bees visited about seven blossoms per minute (or 400 blossoms per hour); thus, with an efficiency of 20 blossoms per bee hour, the average bee successfully inoculated a subsequently detectable population of bacteria to one flower every 20 blossom visits. A second assumption important to this estimate is that the bees observed in trees originated at a hive placed in the experimental planting. This was certainly the case for the pear experiments conducted inside the enclosure. For the apple experiments, we knew of no other managed hives in the immediate area (0.5 km radius), and wild honey bee populations in the area were small relative to the size of the hives placed in the plantings.

An unknown factor in these calculations is the importance of primary spread from hive to blossom relative to secondary spread from flower to flower either by bees or by rain. It is likely that primary spread was important in our experiments because the average number of bacterial colony-forming units on a bee exiting a pollen insert was 1 to 2 log units greater than the average population within individual blossoms.

The conclusion that honey bees dispersed bacteria to flowers is tempered somewhat, however, by the high frequencies of *PfA506* and *Ea153nal<sup>R</sup>* found in apple blossoms sampled in 1991 and 1992 from adjacent plantings located 100–200 m from the experimental sites. The adjacent sites were sampled to serve as controls on the recovery of antibiotic-marked bacteria from blossoms sampled in the planting located next to the hives. The presence of *PfA506* and *Ea153nal<sup>R</sup>* in the adjacent plantings opens the possibility that the bacteria had become part of the indigenous microflora. It is more likely, however, that the presence of *PfA506* and *Ea153nal<sup>R</sup>* in the adjacent plantings also was the result of honey bee inoculation. *PfA506* and *Ea153nal<sup>R</sup>* were not detected in blossom samples made prior to infestation of bees with bacterial inoculum. Moreover, the bees colonies used in the apple experiments were relatively large (40,000–80,000 adult bees per hive) and the distance of the adjacent plantings was within the potential range of foraging workers (10). In 1991 and 1992, the adjacent plantings were along a linear path between the hives and large groves of big leaf maple (*Acer macrophyllum*) that were in full bloom at the time each experiment was initiated. The relative density of bees foraging adjacent plantings and big leaf maple appeared to be similar to that observed in the apple trees located next to the hives, and many of the bees foraging on flowers in the adjacent plantings had visible deposits of dried bacterial inoculum on their bodies. We also found that *PfA506* and *Ea153nal<sup>R</sup>* did not survive well as epiphytes on plant surfaces between seasons (V. O. Stockwell, unpublished).

Pollen inserts proved to be an effective means of inoculating honey bees with bacteria. About 80% of bees beginning foraging activity exited a hive through the inserts, although many bees tried to avoid contact with the dried bacterial preparations (the sucrose added to some inoculum preparations did not alter this avoidance behavior). At times, the pollen insert was slid to the right or left on the hive entrance platform to increase the proportion of bees that exited the hive through the pollen insert. For the same reason, tape was used to narrow the size of the openings at the sides of the pollen insert where returning bees reenter a hive. At periods of peak foraging activity in large hives, outgoing honey bees emptied a pollen insert containing 10 g of inoculum in 15 min. During slower periods, the dried inoculum in the pollen insert often absorbed some moisture, and if not occasionally stirred, formed a crust. When the inoculum crusted, outgoing bees formed channels through the inoculum to avoid contaminating themselves. The pollen inserts were cleaned and dried daily to reduce crusting of the inoculum preparations.

The relatively small bee colonies (15,000–20,000 adult bees per



hive) placed within the shade-cloth enclosure required 1 or 2 days of adjustment before they actively foraged on the pear trees within the cage. Pears are not a preferred food for honey bees (10), and on the first day after placement in the enclosure, many bees hung on the shade cloth, mostly in the upper southeast and southwest corners. Mortality of these bees was high, but after 2 days, this behavior occurred less frequently and other bees began to increase their foraging on pear flowers. Bee foraging activity per tree within the enclosure was uniform with no apparent directional gradients occurring in 1992; in 1991 foraging activity decreased slightly from the southern to northern portion of the enclosure. Also in 1991, when the bee hive was left inside the enclosure for 9 days, it was necessary to provide the hive with an additional source of sugar to supplement the low sugar concentration in pear nectar.

Employing honey bees to disperse bacteria to pome fruit blossoms is probably most useful as a research tool to mimic natural inoculation of flowers with the fire blight pathogen and/or to study how conventional disease management practices may influence establishment and proliferation of bacterial epiphytes on floral surfaces. All experiments in which bees have been used to inoculate pear with *E. amylovora* (Fig. 2 and another conducted in Wentachee, WA [R. McLaughlin, *personal communication*]) and one of three experiments with apple in this study have resulted in significant fire blight epidemics. Also, in contrast to spray inoculations of *E. amylovora* that we have conducted, fire blight epidemics initiated by honey bee dispersal of the pathogen have been uniform among trees and have not been so severe that proven chemicals for control of fire blight appear ineffective (K. B. Johnson and V. O. Stockwell, *unpublished*).

As a means to implement fire blight biocontrol, economic considerations and relatively poor biological efficiency may limit the usefulness of honey bees as dispersal agents of bacterial antagonists. The pollen inserts used in the study required nearly constant monitoring during periods of bee foraging activity to ensure that a large proportion of outgoing bees were inoculated with bacteria as they exited the hive. Amount of inoculum dispersed by bees in each apple experiment was similar to that needed to thoroughly spray a 50–100 tree planting with an aqueous suspension of bacteria ( $10^7$  cfu/ml), but the frequency of recovery and population size of bee-dispersed bacteria in blossoms was generally smaller than what we have obtained in comparable spray applications (13; K. B. Johnson and V. O. Stockwell, *unpublished*). Among seasons, the temporal rate of establishment of the bee-dispersed bacterial antagonist Pfa506 in blossoms was probably too variable to consistently protect trees from disease (Fig. 2A–C). Related to this, three additional attempts were made to conduct the Pfa506 and Ea153nal<sup>R</sup> dispersal experiments in open plantings of apple and pear located in Corvallis, OR (K. B. Johnson, *unpublished*). These experiments were unsuccessful because bees did not forage in blossoms within the experimental plantings owing to cold, wet weather in one case, and coincidental bloom in peach and sour cherry orchards in the other two.

## LITERATURE CITED

1. Beer, S. V., Rundle, J. R., and Wodzinski, R. S. 1984. Recent progress in the development of biological control for fire blight—A review. *Acta Hort.* 151:195-203.
2. Harter, H. L. 1961. Expected values of normal order statistics. *Biometrika* 48:151-165.
3. Hattigh, M. J., Beer, S. V., and Lawson, E. W. 1986. Scanning electron microscopy of apple blossoms colonized by *Erwinia amylovora* and *E. herbicola*. *Phytopathology* 76:900-904.
4. Ishimaru, C. A., and Klos, E. J. 1984. New medium for detecting *Erwinia amylovora* and its use in epidemiological studies. *Phytopathology* 74:1342-1345.
5. Kiett, G. W., and Ivanoff, S. S. 1941. Transmission of fire blight by bees and its relation to nectar concentration of apple and pear blossoms. *J. Agric. Res.* 62:745-753.
6. Kloepper, J. W., and Schroth, M. N. 1981. Development of a powder formulation of rhizobacteria for inoculation of potato seed pieces. *Phytopathology* 71:590-592.
7. Lindow, S. E. 1984. Integrated control and role of antibiosis in biological control of fire blight and frost injury. Pages 83-115 in: *Biological Control on the Phylloplane*. C. Windels and S. E. Lindow, eds. American Phytopathological Society, St. Paul, MN.
8. Loper, J. E., Henkels, M. D., Roberts, R. G., Grove, G. G., Willet, M. J., and Smith, T. J. 1991. Evaluation of streptomycin, oxytetracycline, and copper resistance of *Erwinia amylovora* isolated from pear orchards in Washington state. *Plant Dis.* 75:287-290.
9. Loper, J. E., Suslow, T. V., and Schroth, M. N. 1984. Lognormal distribution of bacterial populations in the rhizosphere. *Phytopathology* 74:1454-1460.
10. Mayer, D. F., Johansen, C. A., and Burgett, D. M. 1986. Bee Pollination of Tree Fruits. Pacific Northwest Extension Publication 282, Washington State University Cooperative Extension Service, Pullman. 10 pp.
11. Moller, W. J., Schroth, M. N., and Thomson, S. V. 1981. The scenario of fire blight and streptomycin resistance. *Plant Dis.* 65:563-568.
12. Pierstorff, A. L., and Lamb, H. 1934. The honey bee in relation to the overwintering and primary spread of the fire blight organism. *Phytopathology* 24:1347-1357.
13. Stockwell, V. O., Loper, J. E., and Johnson, K. B. 1991. Colonization of pear stigmas by beneficial bacteria for control of fire blight. (Abstr.) *Phytopathology* 81:1198.
14. Thomson, S. V. 1986. The role of the stigma in fire blight infections. *Phytopathology* 76:476-482.
15. Thomson, S. V., Hansen, D. R., Flint, K. M., and Vandenberg, J. D. 1992. Dissemination of bacteria antagonistic to *Erwinia amylovora* by honey bees. *Plant Dis.* 76:1052-1056.
16. Wilson, M., Epton, H. A. S., and Sigeo, D. C. 1990. Biological control of fire blight of hawthorn with *Erwinia herbicola* under protected conditions. *Plant Pathol.* 39:301-308.
17. Wilson, M., Sigeo, D. C., and Epton, H. A. S. 1989. *Erwinia amylovora* infection of hawthorn blossom: II. The stigma. *J. Phytopathol.* 127:15-28.
18. van der Zwet, T., and Keil, H. L. 1979. Fire Blight: A Bacterial Disease of Rosaceous Plants. *Agric. Handb.* 510, United States Dep. Agric., Science and Education Administration, Washington, DC. 200 pp.
19. van Laere, O., De Greef, M., and De Wael, L. 1981. Influence of the honey bee on fire blight transmission. *Acta Hort.* 117:131-141.