Dissemination of Bacteria Antagonistic to *Erwinia amylovora* by Honey Bees

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ABSTRACT

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Foraging honey bees (*Apis mellifera*) were tested for ability to disseminate bacteria antagonistic to *Erwinia amylovora* to apple and pear flowers in commercial orchards. The bacteria, *Pseudomonas fluorescens* and *E. herbicola*, previously known to provide biological control of fire blight, were placed on apple or cattail pollen at populations of 10^9 and 10^8 bacteria per gram, respectively. There was no significant decline in viable bacteria on either pollen over 3 wk at 4 C. These bacteria-treated pollens were placed in pollen inserts in the entrances of beehives. Honey bees emerging from these hives through bacteria-pollen mixtures acquired an average of 10^5 and 10^4 cfu per bee of *P. fluorescens* and *E. herbicola*, respectively. *E. herbicola* was detected on 92% of the apple flowers in a 2.6-ha orchard 2 days after the start of one study. In a pear orchard, 72% of the flowers within 7.6 m of the hive were colonized with *P. fluorescens* (average population of 10^2 cfu per flower) 8 days after the start of the study. Our study showed that bees can be efficient vectors of antagonistic bacteria for biological control of fire blight; disease control could not be evaluated because of frost and absence of disease in the test orchards.

Biological control of fire blight has been experimentally demonstrated by spraying apple or pear orchards with high populations of antagonistic bacteria (1,5,9). However, this technique delivers the antagonistic bacteria indiscriminately to the orchard, where only a very small percentage of the bacteria is actually deposited on the flower. Multiple applications may be necessary because flowers do not open simultaneously. The protection of flowers with streptomycin or copper sprays is successful because most infections occur through the flower (13,17). This finite infection court also limits the number of sites that must be protected in order to achieve control. Therefore, protecting only the flower with either bactericides or antagonistic bacteria should provide effective control of blossom blight.

Epiphytic populations of *Erwinia amylovora* (Burrill) Winslow et al develop almost exclusively on the stigmata of healthy flowers of fire blight hosts (3,9,13,19). Bacteria isolated from apparently healthy flowers include *E. amylovora* and other saprophytic bacteria, often in numbers exceeding 10^5 to 10^6 cfu per flower. It appears that colonization of the stigma with either antagonistic bacteria or *E. amylovora* may be a critical juncture determining whether the flower remains healthy or becomes infected. Flower-visiting insects seeking nectar and pollen are known to be inadvertent vectors of *E. amylovora* as well as beneficial vectors of pollen and antagonistic bacteria (12,14). There is ample evidence that honey bees (*Apis mellifera* L.) function effectively in disseminating *E. amylovora* between flowers during their foraging activities (11,18). Honey bees could also be used to deliver antagonistic bacteria to the stigma, the precise site where pathogen and antagonistic bacteria interact (3,9). Because of the foraging habits of bees, the bacteria would be deposited on the flower soon after it opens. Thus, the repeat applications and critical timing necessary with broadcast spraying would be avoided.

Some commercial growers provide supplementary pollen to honey bees to increase its availability in fruit orchards. Hive or pollen "inserts" are placed in the entrance of hives, and fresh, viable pollen is added during periods of peak bee activity. As they exit the hive, foraging bees are forced to pass through the insert, thus coating their bodies with pollen. This practice has been shown to increase pollination efficiency (7). We investigated this method of delivery by placing pollen laden with antagonistic bacteria in the inserts, thus providing a mechanism to enable bees to acquire the beneficial bacteria and disseminate them to flowers in commercial apple and pear orchards. Details of a preliminary study were previously reported (16).

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MATERIALS AND METHODS

Survival of antagonists on pollen. The antagonistic bacteria used in these studies were previously shown to provide biological control of fire blight (1,5). *Pseudomonas fluorescens* (Trevisan) Migula strain A506 from pear (isolated by S. E. Lindow, University of California, Berkeley) is naturally resistant to streptomycin and copper. *E. herbicola* (Löhnis) Dye subsp. *herbicola* strain Eh318 from apple (isolated by S. V. Beer, Cornell University, Ithaca, NY) was selected for resistance to rifampicin and nalidixic acid.

The bacterial inoculum was prepared by either of two methods. In one method, the antagonistic bacteria were grown on plates of King's medium B (KB) (4) for 24 hr. Each 100-mm-diameter petri plate was flooded with 5 ml of sterile distilled water (SDW), and the bacteria were dislodged from the surface of the medium with a bent glass rod. The bacterial suspension was pipetted to a sterile glass tube, vortexed, and then thoroughly mixed with apple pollen (pollen and anther sacs, Antles Pollen Supplies, Inc., Wenatchee, WA) in a flat dish. The resulting mixture was spread on a piece of aluminum foil and allowed to air-dry for 0.5–1 hr in a laminar flow hood. The bacterial suspension from two heavily streaked plates was used to inoculate 1 g of pollen.

A second method was developed to reduce the time involved in preparing bacterial inoculum. The antagonistic bacteria were grown overnight at 29 C in 50 ml of enriched liquid medium (0.2% K_2HPO_4, 0.05% KH_2PO_4, 1.0% yeast extract, 0.5% glycerol, and, after autoclaving, 0.5 ml/100 ml of 5.0% $MgSO_4 \cdot 7H_2O$). The bacteria were pelleted by centrifugation (4,000 *g* for 5 min), resuspended in 25 ml of SDW, and mixed with cattail pollen and dried as described above. The amount of an overnight culture used to inoculate 5 g of pollen was 50 ml.

Pollen of cattail (*Typha latifolia* L.) was collected from flowering spikes by shaking the spikes into a paper bag. Approximately 3 kg of pollen was collected in 2 hr. The antagonistic bacteria were grown and mixed with the cattail pollen as described above for apple pollen.

Survival of bacteria on apple and cattail pollen was determined by mixing 1.0 g of bacteria-treated pollen in 10 ml of SDW and spreading serial dilutions in 0.1-ml aliquots on three replicate plates of KB. All bacteria-pollen mixtures were stored at 4 C and sampled at the time of preparation and again at 1, 2, 4, and 21 days.

The survival of A506 on apple pollen was tested in a pollen insert for comparison with the survival at 4 C. The bacteria-pollen mixture was prepared as above, and 10 g was placed in an insert in a hive. Samples of 0.5 g were taken 1 and 24 hr later and mixed in a ratio of 1 g of pollen to 10 ml of SDW. This suspension was vortexed for 30 sec, and serial dilutions were plated on KB supplemented with 100 μg/ml of streptomycin.

Acquisition of antagonistic bacteria by honey bees. Active honey bee hives with two standard brood boxes were used to determine the acquisition by bees of the bacteria on cattail pollen. Hive inserts (Antles Pollen Inc.) were placed into the hive entrances at least 24 hr before the mixtures were added to allow the bees time to adjust to the device. The bacteria-pollen mixtures (7.5 g) were added between 10 a.m. and noon on days when the temperatures were between 15 and 21 C. Ten bees exiting through the inserts within either 1 or 24 hr after addition of the bacteria-pollen mixtures were collected individually in sterile disposable plastic tubes (17 × 100 mm), transported back to the laboratory, and processed within 2 hr. Each bee was vortexed in 1.0 ml of SDW for 30 sec. A 0.1-ml aliquot of the wash was spread on KB supplemented with streptomycin or with rifampicin plus nalidixic acid at 100 μg/ml each to select for A506 or Eh318, respectively. Plates were incubated at 29 C for 24–48 hr and examined for the presence of colonies typical of Eh318 or A506.

Dissemination of antagonistic bacteria in orchards. Active hives with two standard brood boxes were moved to orchards, and inserts were placed in hive entrances at least 24 hr before the addition of 2.5–5.0 g of the bacteria-pollen mixture. Depending on the orchard and level of bee activity, the inserts were replenished with mixture at 2- to 3-hr intervals for up to three applications. These field studies were conducted in pear and apple orchards in Box Elder and Cache counties, Utah. Details for pollen addition in each orchard are recorded below with A506 used in both a pear and an apple orchard in 1990 and Eh318 used in a pear orchard in 1990 and an apple orchard in 1991.

The deposition of bacteria on flowers was quantified by sampling pear or apple flowers either in bulk in resealable bags or individually in sterile disposable tubes (17 × 100 mm). Bulk samples of 25 or 50 flowers were collected at each of four distances (distances varied depending on the orchard) radiating from the hives. No more than one flower was collected per cluster. These flowers were washed in SDW (0.5 ml per flower) for 30 sec, and serial dilutions of the wash water were plated on the appropriate selective medium. Individual flowers were carefully removed from the tree by slipping the sterile tube opening over the flower and cutting the pedicel off by capping the tube. Each flower was vortexed for 30 sec in 1.0 ml of SDW, and a 0.1-ml aliquot was spread on the appropriate medium.

In Box Elder County (1990), two hives were placed at the center of a 20-ha orchard of 12-yr-old Bartlett pear trees, 3 days after full bloom. The Eh318-apple pollen mixture was estimated to contain 10^8 cfu/g and was added at the rate of 2.5 g per insert at 10 a.m. and noon for a total of 10 g in the orchard. Flower samples were taken at distances of <7.6, 15.2, 30.5, and 61 m.

Failure to detect Eh318 bacteria in the Box Elder County pear orchard suggested a more intensive method was required in the Cache County pear orchard (1990) to establish a bacterial population in flowers. Therefore, this experiment was performed in a 1.5-ha Bartlett pear orchard at the early bloom stages and the bacteria-pollen mixture was added to the inserts in two hives over a 2-day period. The A506-apple pollen mixture contained 1.6×10^8 and 2.7×10^7 cfu/g on the 2 days, respectively. Approximately 5 g of the mixture was added to each insert at 10 a.m., 11 a.m., and 2 p.m. for a total of 30 g in the orchard. A bulk sample of 25 flowers was taken immediately before the mixture was added to the inserts. Bulk samples of 25 flowers each were taken from <7.6, 7.6, 15.4, and 30.5 m at 1, 2, 4, and 6 days after the first addition of the bacteria-pollen mixture.

Five hives with inserts were moved into a 2.6-ha apple orchard in Cache County (1990) 2 days before the transmission studies began. Hives were placed 1 m apart in the center of the orchard. Approximately 4 g of A506-apple pollen mixture was added during early bloom at 9 a.m. and 2 p.m. on three consecutive days, with populations of A506 at 2.3, 1.9, and 4.4 × 10^8 cfu/g on each day,

Table 1. Survival of *Pseudomonas fluorescens* strain A506 and *Erwinia herbicola* strain Eh318 on apple or cattail pollen stored at 4 C

Bacterium / Pollen	Colony-forming units per gram of mixture after storage (days)[a]					
	0	1	2	4	21	Mean
A506						
Apple	3.4×10^9	7.0×10^8	2.1×10^9	1.3×10^9	2.0×10^9	1.6×10^9
Cattail	1.7×10^9	3.7×10^9	1.2×10^9	1.2×10^9	NT	1.7×10^9
Eh318						
Apple	6.1×10^8	1.5×10^8	3.7×10^8	1.5×10^8	6.0×10^8	3.1×10^8
Cattail	7.0×10^7	3.7×10^8	5.0×10^8	3.0×10^8	NT	2.5×10^8

[a] Values are means of three replications. NT = not tested.

respectively. This resulted in a total of 40 g of bacteria-pollen mixture per day. The distances for blossom collection were marked at <7.6, 15.2, 30.5, and 61 m from the hives. The orchard was in full bloom by the second day of pollen addition. Bulk samples of 25 flowers were taken from each distance at 0, 1, 2, and 3 days after the addition of the bacteria-pollen mixture. Twenty-five individual blossoms were also sampled at each distance from the hive 3 days after the first addition.

During the second year in the Cache County apple orchard (1991), we studied the dissemination of Eh318 grown in liquid cultures and placed on cattail pollen. Five hives with two brood boxes were spaced throughout the orchard approximately 45–50 m apart. Over three consecutive days, 5 g of bacteria-pollen mixture was placed in the hive inserts at about 10 a.m. for a total of 75 g of bacteria-pollen mixture. Bulk samples of 25 blossoms were collected from throughout the orchard and checked for the presence of A506 as well as Eh318. Individual blossom samples were checked only for Eh318.

Table 2. Survival of *Pseudomonas fluorescens* strain A506 on apple pollen placed in hive inserts in Cache County, Utah, orchards, 1990

Orchard Pollen inserted	Recovery of A506 on pollen in inserts (cfu/g)	
	After 1 hr	After 24 hr
Pear		
20 April	1.6×10^8	7.8×10^7
21 April	2.7×10^7	3.5×10^7
Apple		
24 April	2.3×10^8	3.0×10^7
25 April	1.9×10^8	1.0×10^8
26 April	4.4×10^8	4.3×10^8

RESULTS

Survival of antagonists on pollen substrates. The recovery of A506 from apple and cattail pollen substrates averaged about one order of magnitude higher than that of Eh318 throughout the 3 wk of storage at 4 C, with populations averaging 10^9 and 10^8 cfu/g of pollen, respectively (Table 1).

The population of A506 on apple pollen in inserts averaged 2.1×10^8 cfu/g on pollen sampled within 1 hr and 1.3×10^8 cfu/g after 24 hr in the insert under orchard conditions (Table 2).

Acquisition of antagonistic bacteria by honey bees. Every bee exiting through the insert within 1 hr after the addition of bacteria-pollen mixtures carried populations of the inoculated bacteria. There were no significant differences between cattail and apple pollen with either bacterial strain. The bacteria recovered from bees exiting through cattail pollen and apple pollen had average populations of 8.0×10^3 and 5.0×10^3 of A506 and 2.5×10^3 and 2.5×10^3 of Eh318, respectively. This study was repeated two times, with similar results. The recovery of marked bacteria from bees 24 hr after exposing them to the bacteria-pollen mixtures was considerably less with both strains and pollen mixtures. The recovery was consistently better with A506, where 80% of the bees carried an average population of 7.3×10^2 cfu per bee, whereas Eh318 was recovered from only 50% of the bees, with 5.6×10^2 cfu per bee. Results of two repetitions of this study were similar.

Dissemination of antagonistic bacteria in orchards. In the Box Elder County pear orchard (1990), Eh318 was not detected from bulk samples of 100 pear flowers collected at the four distances before the bacteria-apple pollen mixture was added to the hives. Individual flowers (25 from each distance) sampled 1 day and again 1 wk after the bacteria-pollen mixture was added also showed no detectable levels of Eh318. Bees exiting the hive through the insert before the addition of the bacteria-pollen mixture were devoid of Eh318. However, 3 hr after the mixture was added, 100% of 20 bees were carrying Eh318, with a mean population of 1×10^3 cfu per bee (SD = 1.5×10^2).

A bulk sample of 100 flowers taken from throughout the Cache County pear orchard (1990) before release of the bacteria was found to be free of any bacteria that resembled A506 on KB-streptomycin selective media. Very low populations of A506 were detected in the bulk flower samples up to 4 days after the initial release and only in samples taken from within 7.6 m of the hives (Table 3). After 6 days, A506 was detected in bulk samples at all four distances from the hives, with the highest population in flowers taken from trees immediately around the hives. The presence of A506 in the bulk samples was used as the signal to sample individual flowers. Twenty-five individual flowers were sampled within a 7.6-m radius of the hives 8 days after the first addition of bacteria-pollen mixture. Strain A506 was detected on 72% of the flowers. The populations were highly variable and ranged from 10 to 1,000 cfu per flower, with a mean population per flower of 287.8 cfu (SD = 296.4 cfu). Additional data were not obtained from this orchard because a −6 C frost damaged the flowers.

No A506 bacteria were detected in a bulk sample of 100 flowers taken from throughout the Cache County apple orchard (1990) before the release of the antagonistic bacteria. Strain A506 bacteria were detected in bulk flower samples at all distances sampled 1 day after the first addition of the A506-apple pollen mixture (Table 4). The mean population per flower over all distances at 3 days after the start of the study was 3.2×10^2 cfu ($n = 100$). Populations of A506 per individual flower 3 days after the first addition of pollen were quite variable but still readily detected in a sizable number of flowers at all distances. Strain A506 was present in 52, 24, 40, and 56% of the flowers at <7.6, 15.2, 30.5, and 61 m from the source hives, respectively, but because of high variability, there was no significant difference among distances. The mean populations at each distance, from near to far, were 122, 153, 37, and 35 cfu per flower (SD

Table 3. Dissemination of *Pseudomonas fluorescens* strain A506 by honey bees in a Cache County, Utah, pear orchard, 1990

Days after first addition of mixture[a]	Colony-forming units per flower according to distance from hives[b]			
	<7.6 m	7.6 m	15.4 m	30.5 m
0	0	0	0	0
1	10	0	0	0
2	2.5	0	0	0
4	0	13.5	0	0
6	1,160	195	35	490

[a]The bacteria-apple pollen mixture was placed in inserts in two hives on days 0 and 1 at 1.6×10^8 and 2.7×10^7 cfu/g, respectively.
[b]A bulk sample of 25 flowers was taken at each distance.

Table 4. Dissemination of *Pseudomonas fluorescens* strain A506 by honey bees in a Cache County, Utah, apple orchard, 1990

Days after first addition of pollen[a]	Colony-forming units per flower according to distance from hives[b]			
	<7.6 m	15.2 m	30.5 m	61 m
0	0	0	0	0
1	750	500	75	25
2	380	330	345	220
3	565	380	300	35

[a]The bacteria-apple pollen mixture was placed in inserts in five hives on days 0, 1, and 2 at 2.3, 1.9, and 4.4×10^8 cfu/g, respectively.
[b]A bulk sample of 25 flowers was taken at each distance.

= 189, 216, 65, and 60 cfu, respectively). A frost of −6 C on 30 April froze 100% of the open flowers as well as most of the unopened flower buds. Bulk samples of 25 apparently healthy flowers taken after the frost (8 days after the first pollen addition) had low populations of A506, i.e., 35, 65, 2.5, and 2.5 cfu per flower at each of the four distances, respectively. A sample taken 10 days after pollen addition had populations of 25, 0, 0, and 0 cfu per flower at the four distances. Pollen collected from the inserts on 20 April, 6 days after the first addition and 3 days after last addition, had a A506 population of 4.4×10^8 cfu/g.

Endemic populations of Eh318 and A506 were not detected in a bulk sample of 25 flowers taken from the Cache County apple orchard (1991) before the bacteria-pollen mixture was added to the hive inserts. One day after the bacteria-pollen mixture was added to the inserts, Eh318 was detected in a bulk sample of 25 flowers, with a population of 1.65×10^3 cfu per flower. On the second day of the study (24 hr after the second addition of pollen), the bulk sample of 25 flowers had an average of 5.4×10^3 cfu per flower. At the same time, 92% of the 25 individual flowers contained detectable populations of Eh318 ranging from 10 to 5,700 cfu per flower, with a mean of 3.5×10^2 cfu ($\pm 1.5 \times 10^2$ cfu). On the third day of the study, but still 24 hr after the most recent addition of pollen to the inserts, 96% of the individual flowers sampled contained Eh318, with populations ranging from 10 to 1,500 cfu per flower and a mean population of 2.2×10^2 cfu ($\pm 3.8 \times 10^2$). Four days after the first addition of pollen (2 days after the most recent addition of pollen), only 72% of the 25 individual flowers contained detectable populations of Eh318. The average number of bacteria per flower was 1.7×10^2 ($\pm 2.3 \times 10^2$). Precipitation on the second and third days after the first addition of pollen totaled 13 mm, with a particularly heavy downpour on the third day. This rain was apparently responsible for the decline in the bacterial population in the flowers.

DISCUSSION

In this study, honey bees were shown to disperse antagonistic bacteria from pollen inserts to flowers in commercial pear and apple orchards. The antagonistic bacteria were detected in apple flowers over 60 m from the hives within 24 hr after bee exposure to the bacteria-apple pollen mixture. In some cases, individual flowers had bacterial populations exceeding 10^3 cfu. However, the deposition of high numbers on a flower may not be necessary because the antagonists will multiply on the flower. The actual population of antagonists necessary to provide satisfactory biological control of fire blight is not known. However, populations ranging from 10^5 to 10^8 cfu per flower may be necessary (1). It is more important that a high percentage of flowers become colonized with the antagonistic bacteria for biological control of fire blight to succeed. We demonstrated that up to 72% of the flowers in a Bartlett pear orchard and 96% of those in an apple orchard were colonized by marked bacteria. In the 1991 study in a Cache County apple orchard, antagonistic bacteria were present in nearly 100% of the flowers within 24 hr after the addition of the bacteria-pollen mixtures when weather conditions were favorable for bee activity. During the 5-day study, when the bacteria-pollen mixture was placed in the hives, the weather was warm (daily maximum temperatures were 15–26 C) and bee activity was therefore high.

Honey bees collecting either pollen or nectar actively scramble over the anthers and contact the stigma in over 90% of the flower visits (6,8). Pollen has been observed with scanning electron microscopy on over 90% of the stigmata of McIntosh apple flowers (2). Therefore, the bacteria on the pollen originating from inserts are likely to be deposited on the stigma, the appropriate site for subsequent growth of the antagonists (13,19).

The stigmata may be the only sites that require colonization by the antagonists in order to prevent flower infections. Under normal conditions, the stigma is the location where *E. amylovora* is present and where it increases in numbers (3,9,13). Epiphytic populations develop on the stigma and are moved to the floral cup with rain or heavy dews. Therefore, colonization and protection of the stigma by antagonistic bacteria should result in a reduced incidence of fire blight.

Conditions were unfavorable for the development of epiphytic populations of *E. amylovora* and subsequent fire blight in the orchards studied in 1990 (15). The hard frost during full bloom damaged most of the existing flowers and the developing buds, which precluded the opportunity to test for biological control of fire blight. In 1991, conditions were favorable for a single high-risk day in the Cache County apple orchard, according to the MARYBLYT model (10), but the cooperating grower applied streptomycin. However, the absence of fire blight was due to the lack of an epiphytic population rather than to the streptomycin application or biological control. Further studies will include efforts to demonstrate fire blight control using this technique.

Increasing the titer of bacteria on pollen should improve the effectiveness of this technique. Also, the bacteria must survive long enough on the pollen to be transported by bees to flowers. Once there, bacteria can multiply on the stigmata to effective numbers. At this stage of our studies, we did not consider long-term survival of the bacteria on the pollen to be necessary. At some future date, survival may need to be improved in order to package and deliver a product to the grower. We attained populations of *P. fluorescens* as high as 3.7×10^9 per gram of cattail pollen by simply adding a liquid suspension of bacteria to the pollen. Population levels and survival of the antagonists were similar on the apple pollen and cattail pollen. However, cattail pollen was easier to prepare and use, and the bees were not adversely affected by the presence of cattail pollen in the inserts.

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