Temperature and Pomaceous Flower Age Related to Colonization by *Erwinia amylovora* and Antagonists

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ABSTRACT

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Fire blight of apple and pear is initiated by epiphytic populations of *Erwinia amylovora* on flower stigmas. Predicting this disease and managing it with microbial antagonists depends on an understanding of bacterial colonization on stigmas. Detached 'Manchurian' crab apple flowers were inoculated with *E. amylovora* and subjected to a range of constant temperatures or various fluctuating temperature regimes. Results may have application to disease risk assessment systems such as the Cougarblight model, which now are based on in vitro growth of the pathogen. In other experiments, detached crab apple flowers and attached 'Gala' apple flow-

ers were maintained at different temperatures for various periods before inoculation with *E. amylovora* or antagonists (*Pseudomonas fluorescens* strain A506 and *Pantoea agglomerans* strains C9-1 and E325). Maximum stigma age supporting bacterial multiplication decreased as temperature increased, and was reduced by pollination. Stigmas were receptive to bacteria at ages older than previously reported, probably due to less interference from indigenous organisms. The study revealed antagonist limitations that possibly affect field performance (e.g., the inability of strain A506 to grow on relatively old stigmas conducive to the pathogen). Such deficiencies could be overcome by selecting other antagonists or using antagonist mixtures in the orchard.

Additional keywords: biological control, *Malus × domestica*.

Fire blight, a major disease of apple and pear trees, is most commonly initiated by epiphytic populations of *Erwinia amylovora* that develop on blossoms (1,39). Under dry climatic conditions, bacteria colonize flower stigmas, and subsequent rain or heavy dew facilitates movement to the floral cup (hypanthium) where infection generally occurs (37). Management programs have emphasized risk assessment (5) and suppression of *E. amylovora* on floral parts with antibiotics (31,39). The reliance on streptomycin, in particular, led to the development of pathogen resistance in many production areas (24,25).

Suppression of *E. amylovora* on flowers with microbial antagonists is a relatively new alternative (8,14) or complementary measure to antibiotics (22,35). *Pseudomonas fluorescens* strain A506, the active ingredient in BlightBan A506 (NuFarm Inc., Sugar Land, TX), initially was selected based on its inhibition of an ice-nucleating strain of *P. syringae* on corn leaves, and only later was found to reduce fire blight incidence by preemptively excluding the pathogen (22). Another bacterial antagonist of commercial interest is *Pantoea agglomerans* (syn. *E. herbicola*) strain C9-1 (14), which was selected based on in vitro production of antibiotics inhibitory to *E. amylovora* (12). Other microbial antagonists similarly have been identified in screening assays involving artificial media (3) or immature pear fruit (2,10,41). Laboratory screening with detached crab apple flowers led to the recent discovery of *P. agglomerans* strain E325, which was exceptionally effective in suppressing populations of *E. amylovora* on flower stigmas (26,27,30). This antagonist is being developed commercially under the product name "Bloomtime Biological" (Northwest Agricultural Products, Pasco, WA).

The effects of bacterial antagonists on the blossom blight phase of fire blight in field trials have often been inconsistent (8,40). This may be due to instability of antagonist populations, environmental conditions, or host physiological changes associated with flower development and senescence. Improvements in efficacy will depend partly on an increased understanding of these factors.

The flower stigma is of considerable importance as the site of pathogen establishment and the site where antagonist adaptation is critical for effective biological control. Growth of *E. amylovora* on stigmas is dependent on temperature, as reflected by disease-forecasting systems (5). A major component of these systems is the accumulation of degree hours or degree days prior to a wetting event. In some cases, degree values have been adjusted based on in vitro bacterial growth, presumed to simulate the epiphytic growth of bacteria in relation to temperature (4,33).

Flower stigmas are dynamic, progressing to full maturity and undergoing senescence in a matter of days. Studies related to this focused mainly on the interaction between stigmatic surfaces and pollen grains as influenced by environmental factors (20,34). It is known, for example, that temperature affects the duration of stigma receptivity to pollen, and that stigma senescence is accelerated after pollination (34). A relationship between stigma age and stigma capacity to support bacterial growth only recently was indicated in field studies with apple (38) and pear (23). Information generally is lacking regarding interrelationships of temperature, stigma age, pollination, and bacterial colonization on stigmatic surfaces. Such knowledge is relevant to disease risk assessment and to the effective use of biological agents for disease management. The present study examines these relationships using crab apple flowers in the laboratory (26) and apple blossoms in the field.

MATERIALS AND METHODS

Microorganisms. The pathogen used was *E. amylovora* strain Ea153 and antagonists were *Pseudomonas fluorescens* strain A506 and *Pantoea agglomerans* strains C9-1 and E325. All

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strains, except A506, which was first isolated with natural antibiotic resistance, are antibiotic-resistant derivatives generated spontaneously at the source laboratories. Thus, the pathogen strain is resistant to nalidixic acid and all antagonist strains are resistant to rifampicin. Original strain codes (i.e., without added letters indicating specific derivative types) are used in this article. Strain Ea153, obtained from K. Johnson (Oregon State University, Corvallis), was isolated from cankers on 'Gala' apple in Oregon and later used in studies with apple and pear (16,17). Strain A506, originally from S. Lindow (University of California, Berkeley), was isolated from pear in California (22). Strain C9-1 originally came from C. Ishimaru (Colorado State University, Fort Collins), who isolated it from 'Jonathan' apple fruit in Michigan (12), but the rifampicin-resistant derivative used in field studies in Oregon (17) was obtained from K. Johnson. Strain E325 was isolated from Gala apple blossoms near Wenatchee, WA, in 1994 (26).

Bacteria were cultured on nutrient yeast dextrose agar (NYDA; nutrient broth, 8 g; yeast, 5 g; dextrose, 5 g; agar, 15 g; and deionized water, 1 liter) for 24 h at 24°C. Inoculum suspensions were prepared in 10 mM potassium phosphate buffer (pH 7.0) and 0.03% Tween 20, and adjusted to 10^6 , 10^7 , or 10^8 CFU/ml.

Plant material. Laboratory experiments were performed with detached blossoms of crab apple (*Malus* sp. 'Manchurian') trees, 1.6-cm minimum stem diameter, received from a local nursery (Van Well Nursery, Wenatchee, WA). Trees were induced to bloom in a greenhouse as previously described (26). Newly opened flowers with nondehiscent anthers were collected and maintained by submerging the cut end of the pedicle in 10% sucrose contained in a 2-ml vial. Vials with flowers were supported by plastic tube racks placed in a 4-liter plastic container. Unless otherwise indicated, relative humidity (RH) was established at 90% by flooding the bottom of the container with 1 liter of a glycerol solution (392 ml of glycerol, 808 ml of deionized water) and using a lid to create a closed chamber (13).

Field experiments were performed with apple (*Malus × domestica* Borkh. Gala) in 2000 and 2001. Trees were 6 years old and grown on M7 rootstock in an experimental block at Columbia View near Wenatchee, WA.

Inoculation and bacterial population size. As described previously (26,29), flowers were inoculated with a micropipette. Approximately 0.1 to 0.2 μ l of inoculum suspension was applied per flower by touching a droplet to each stigma (normally five per flower) to form a thin film of moisture.

Population size of bacterial strains on the stigmas of individual flowers was determined by placing stigmas, along with stylar tissue, in sterile microcentrifuge tubes containing 1 ml of sterile buffer (10 mM potassium phosphate, pH 7.0). Tubes were vortexed briefly and placed in a sonication bath for 60 s. Samples were again vortexed, and serial dilutions were spread on CCT medium (11), amended with nalidixic acid (100 μ g/ml) for detection of strain Ea153 or on NYDA amended with rifampicin at 25 ppm and cycloheximide at 50 ppm for detection of strains A506, C9-1, and E325. Plates were incubated at 24°C.

Constant versus fluctuating diurnal temperatures. A test was performed to determine whether fluctuating diurnal temperatures typical of the orchard environment have a different effect on microbial growth on stigmas than that of constant temperatures, which are more feasible in the laboratory. Detached crab apple flowers were inoculated with Ea153 by applying a suspension of 10^6 CFU/ml to the stigmatic surfaces of each flower. Flowers were supported in open containers placed in four different controlled environment chambers, each programmed for a different diurnal temperature range (14°C constant, 10 to 18°C, 7 to 21°C, or 4 to 24°C) as indicated by plotted temperature readings recorded every 15 min. In each chamber, the daily average temperature was 14°C. All chambers were set at a constant 90% RH. Ten flowers were sampled after 0, 24, 48, and 72 h to determine bacterial population size.

Temperature range and optimum for bacterial growth. To determine the temperature range and optimum for growth of bacterial strains on flower stigmas, stigmas of detached crab apple flowers were inoculated with strain Ea153, A506, C9-1, or E325 using a suspension of 10^8 CFU/ml. The estimated starting population size on each flower was $\approx 10^4$ CFU. Flowers were incubated for 24 h at temperatures ranging from 4 to 40°C in increments of 4°C, and then evaluated for bacterial population size. Five flowers were used per strain per temperature.

Temperature, stigma age, and pollination. The effect of temperature, pollination, and stigma age on bacterial colonization of stigmas was studied in the laboratory as follows. Crab apple flowers were collected, anthers were removed with microscissors, and then half of the flowers were pollinated with a mixture of 'Rome' and 'Red Delicious' apple pollen and the other half were left unpollinated. Pollen was applied with an artist paint brush by lightly touching the stigmas of each flower. Flowers were held for 0, 3, 6, 9, or 12 days at 4, 9, 14, 19, or 24°C prior to bacterial inoculation using a suspension of 10^8 CFU/ml. Flowers were collected on different dates, but all flowers were inoculated on the same date. After inoculation, flowers were incubated for 24 h at 24°C. Five pollinated and five nonpollinated flowers were used per stigma age and temperature. The experiment was performed separately with strains Ea153, A506, and E325.

A similar experiment was done to determine the influence of these same factors on the interaction between antagonist and pathogen. Flowers were collected and pollinated or not pollinated as described. Flowers were held for 0, 1, 2, 3, 4, 5, 6, 8, or 10 days at 14 or 24°C prior to inoculation. All flowers were inoculated on the same date with antagonist strain E325, C9-1, or A506 using a suspension of 10^8 CFU/ml. After 24 h of incubation at 24°C, flowers were inoculated with pathogen strain Ea153 using a suspension of 10^7 CFU. After another 24 h incubation at 24°C, population size of both antagonist and pathogen on each flower was determined. Five flowers were used per stigma age and temperature. The experiment was done separately with pollinated and nonpollinated flowers.

Field experiment. The interrelationships of temperature, pollination, stigma age, and bacterial colonization also were studied in the field with Gala apple. To manipulate temperature and prevent bees from naturally pollinating flowers, individual trees were enclosed in an aluminum frame covered with translucent polyethylene 0.15 mm thick. Each enclosure was 3.0 m wide, 2.4 m long, and 3.7 m high. In 2000 and 2001, a total of eight and six trees, respectively, were enclosed. An electric heating and cooling unit was placed in each enclosure. For half of the enclosed trees, the thermostat was set to 10°C and, for the other half, it was set to 24°C. The electric units did not have the capacity to maintain these temperatures in the noninsulated enclosures; however, they did establish two different temperature ranges during bloom. Temperature, RH, and vegetative wetness were monitored inside each enclosure and at one nonenclosed site in the experimental block.

The trees initially were selected based on their abundance of flower buds, and compatibility of size and shape to enclosure dimensions. After trees were selected and enclosed at scattered sites within the orchard block, it was randomly determined which would be subjected to the low-temperature range and which to the high-temperature range.

When blossoms at the "popcorn" stage appeared in peak numbers, branches were flagged on two sides of the tree. One side was designated for nonpollinated flowers and the other for pollinated flowers. On one day, all open flowers were removed; the next day, all unopened flowers were removed. Remaining flowers that opened during the 24-h period were used in the experiment. On the first day that flowers were open, half of the flowers on each tree were pollinated with a mixture of Rome and Red Delicious apple pollen using an artist paint brush, and the other half were left unpollinated. There were a minimum of 200 pollinated

and 200 nonpollinated flowers on each tree. Blossoms were sampled every 1 or 2 days, and cut flowers were maintained and stigmas inoculated as described for crab apple flowers. On the day that flowers were collected, inoculations were made with strain Ea153, E325, or A506 using a suspension of 10^8 CFU/ml. Flowers were supported in open containers and incubated in a controlled environment chamber set at 24°C and 90% RH. Sampling from high- and low-temperature enclosures was started on 20 and 21 April 2000, respectively, and on 27 and 28 April 2001, respectively. Five pollinated and five nonpollinated flowers were collected per strain per tree on each sampling date. Population size expressed as log CFU was averaged for each set of five blossoms prior to statistical analyses.

Additional flowers, five pollinated and five nonpollinated, were collected per tree per date for later examination with a scanning electron microscope (SEM). Stigmas excised from the flowers were placed in the fixative Histochoice (Amesco, Solon, OH), dehydrated in an ethanol series, and critical point dried in a Samdri prt-3 Critical Point Drying Apparatus (Tousimis Res. Corp., Rockville, OH). Dried material was coated with platinum using a Desk II sputter coater (Denton Vacuum, Inc., Morrestown, NJ) and viewed with a Hitachi Model S-530 (Hitachi Instruments Inc., San Jose, CA) equipped with a Quartz PCI digital interface (Quartz imaging Corp., Vancouver, BC) at an accelerating voltage of 10 to 15 kV.

Statistical analyses. The test for establishing the temperature range and optimum for growth of specific bacterial strains on

flower stigmas was performed three times, and all other laboratory experiments were performed twice. Population data from both laboratory and field experiments were log-transformed prior to analysis. Data from multiple trials of each laboratory experiment were tested for homogeneity according to Hartley's F-max test before being pooled and analyzed. Analysis of variance (ANOVA) was performed using SAS (version 8.1; SAS Institute, Cary, NC) and means were separated according to the least significant difference (LSD) test ($P \leq 0.05$). Laboratory data establishing temperature range and optimum for bacterial growth and all field data also were subjected to nonlinear regression analysis using SigmaPlot 8.0 software (Jandel Scientific, San Rafael, CA).

RESULTS

Constant versus fluctuating constant diurnal temperatures.

When flower stigmas were inoculated with strain Ea153 and flowers were incubated at constant or fluctuating temperatures averaging 14°C, means for bacterial population size increased from $\approx \log 1.6$ to $> \log 5.0$ CFU in a 72-h period. At 24, 48, or 72 h, population size on flowers subjected to different diurnal temperature cycles were not different according to ANOVA and the LSD test ($P \leq 0.05$) (Fig. 1).

Temperature range and optimum for bacterial growth. Following the 24-h incubation of inoculated flowers at various temperatures, none of the bacterial strains increased on stigmas at 4 or 8°C beyond the starting level; however, at 12°C, all strains grew to approximately the same population size (Fig. 2). Conversely, the upper temperature limits of the bacterial strains were quite different. Regression curves indicated that the highest temperatures allowing growth of strains Ea153 and A506 were approximately 39 and 35°C, respectively, and the upper limits for strains C9-1 and E325 were above 40°C. Approximate optimal

Fig. 1. Effect of constant and fluctuating temperatures on growth of *Erwinia amylovora* strain Ea153 on stigmas of detached crab apple flowers. **A**, Hourly temperatures plotted for chambers programmed to a constant 14°C or diurnal fluctuations averaging 14°C and **B**, daily population size per flower for the different temperature schemes. Each point is the mean of 20 flowers, and bars represent standard error.

Fig. 2. Temperature range and optimum for growth of *Erwinia amylovora* strain Ea153, *Pantoea agglomerans* strains C9-1 and E325, and *Pseudomonas fluorescens* strain A506 on stigmas of detached crab apple flowers. Each point is the mean population size for 15 flowers after a 24-h incubation, and vertical bars represent standard error. All regressions had a significance of $P < 0.003$. The Weibull equation (43) was used for Ea153 and A506, resulting in R^2 of 0.89 and 0.97, respectively. The Quadratic equation ($y = y_0 + ax + bx^2$) was used for C9-1 and E325, resulting in R^2 of 0.88 and 0.77, respectively. Horizontal hatched line represents estimated population size at time of inoculation.

temperatures for strains Ea153 and A506 were 28 and 24°C, respectively; optima for strains C9-1 and E325 could not be confidently estimated because their temperature ranges are broad, extending outside the range of the experiment. Maximum population size of all antagonist strains was similar (i.e., near log 6 CFU per flower), but the maximum for Ea153 exceeded this level by more than a half log unit. Populations of the pathogen generally were larger than those of the antagonist strains at temperatures from 20 to 32°C.

Temperature, stigma age, and pollination. The time period that crab apple stigmas had the capacity to support bacterial populations decreased in length as temperature increased; generally, it was shorter when flowers were pollinated rather than nonpollinated (Fig. 3). For each bacterial strain used, the effect of tempera-

ture, stigma age, pollination, and the interactions of these parameters (i.e., temperature–age, temperature–pollination, and age–pollination) all were highly significant ($P < 0.0001$) according to ANOVA. The data indicate a diminishing capacity of stigmas to support bacterial growth or sustain populations present at the time of inoculation (Fig. 3). This decline was noticeably more rapid with strain A506. On pollinated flowers held for just 3 days at temperatures from 14 to 24°C prior to inoculation and incubation, mean populations for A506 were below the starting level. After longer holding periods at these temperatures, A506 was almost undetectable. In contrast, populations of Ea153 and E325 on pollinated flowers held 3 days before inoculation were always near or above the initial size regardless of the holding temperature. When pollinated flowers were held for 6 days at 4 or 9°C, Ea153

Fig. 3. Effect of temperature, stigma age, and pollination on growth and survival of **A and B**, *Erwinia amylovora* strain Ea153, **C and D**, *Pantoea agglomerans* strain E325, and **E and F**, *Pseudomonas fluorescens* strain A506 on detached crab apple flowers. Flowers were nonpollinated (**A**, **C**, and **E**) or pollinated (**B**, **D**, and **F**) and held at various temperatures for various periods before being inoculated and incubated at 24°C for 24 h. Each point is the mean population size for 10 flowers, and vertical bars represent standard error. Horizontal hatched lines represent estimated population size at time of inoculation.

and E325 populations grew to near-maximum levels. Even after 9 or 12 days, pollinated flowers held at 4°C continued to support growth of these bacteria, and those held at higher temperatures sustained populations of between log 1 and log 4 CFU per flower.

Experiments were complicated further when flowers were inoculated with two bacterial strains instead of one to determine the influence of the above factors on antagonist–pathogen interactions (Figs. 4 and 5). The effects of temperature, stigma age, and pollination on individual strain populations in the dual inoculations were similar to those shown in the previous tests. A look at antagonist populations revealed again that, as stigma age increased, colonization and survival of strain A506 was negatively affected earlier and to a greater degree than the other antagonists. On nonpollinated flowers held at 14°C (Fig. 4A), population size of C9-1 and E325 was near log 6 CFU on stigmas of all ages inoculated (up to 10 days), but A506 populations were significantly smaller than this on stigmas inoculated at 8 and 10 days. On nonpollinated flowers held at 24°C for more than 5 days prior to inoculation (Fig. 4B), all antagonist populations were near or below the starting size and A506 populations were again smaller than those of the other antagonists. Pollinated flowers held at 14°C for more than 5 days (Fig. 5A) or at 24°C for more than 4 days (Fig. 5B) supported little or no growth of any of the antagonists. On pollinated flowers held at either temperature for more than 1 or 2 days, A506 populations were always numerically smaller, and in several cases significantly different ($P \leq 0.05$) from those of the other antagonists.

During the period when nonpollinated flowers at 14 or 24°C supported growth of the pathogen, as indicated by control flowers with *E. amylovora* alone (Fig. 4C and D), the level of pathogen suppression due to individual antagonist strains was relatively constant. Results with pollinated flowers were similar (Fig. 5C and D), though pathogen populations varied more over time, particularly at 24°C. The performance of antagonists relative to each other was consistent, with strain E325 most effective, followed by strains C9-1 and A506.

Field experiment. Average hourly temperature and RH around enclosed Gala apple trees during bloom in 2000 and 2001 are presented in Figure 6. During the experimental period in 2000, the overall average temperature for the low range was 12.8°C and the average for the high range was 21.1°C. In 2001, overall averages for the low and high temperature ranges were 13.8 and 22.0°C, respectively. Overall averages for RH in low- and high-temperature enclosures was 76 and 68%, respectively, in 2000, and 71 and 69%, respectively, in 2001. This compared with an average outside RH of 49 and 54% in 2000 and 2001, respectively. Detection of free moisture on plant surfaces was negligible.

In 2000, flowers were sampled daily and inoculated in the laboratory over a period of 8 days (Fig. 7). Flowers from low-temperature enclosures always supported bacterial populations that grew 1 to 3 log units beyond the starting level (Fig. 7A and B). Flowers from high-temperature enclosures sampled late during the 8-day period failed to support growth, but maintained populations at or below the starting level (Fig. 7C and D). In

Fig. 4. Effect of stigma age on the interaction between bacterial antagonists and *Erwinia amylovora* strain Ea153 on nonpollinated detached crab apple flowers at **A and C**, 14°C and **B and D**, 24°C. Population size of **A and B**, antagonists *Pantoea agglomerans* strains C9-1 and E325 and *Pseudomonas fluorescens* strain A506 and **C and D**, Ea153 in response to pre-inoculation with antagonists. Flowers were held for various periods at 14 or 24°C, inoculated with antagonist, incubated at 24°C for 24 h, then inoculated with Ea325 and incubated at 24°C for 24 h. Each point is the mean for 10 flowers, and vertical bars represent standard error. Asterisk above day number indicates that means are different according to least significant difference test ($P \leq 0.05$). Horizontal hatched lines represent estimated population size at time of inoculation.

2001, sampling from low-temperature enclosures was done every 2 days for 14 days (Fig. 8A and B). During the expanded period of sampling, it was shown that even 12-day-old stigmas could support bacterial growth. As shown in the previous year, flowers from high-temperature enclosures in 2001 failed to support growth late in an 8-day sampling period (Fig. 8C and D). The effect of pollination on the capacity of stigmas to support growth is not as obvious as that of temperature in Figures 7 and 8, but nonetheless is real. Pollination had a negative effect ($P \leq 0.001$) on the capacity of stigmas to support each bacterial strain. Regressions tended to be flatter for pollinated than for non-pollinated flowers. Regressions also indicated that the change in stigmas from a state of being able to support growth to one that merely allowed bacteria to survive occurred earlier on pollinated than nonpollinated flowers.

A general comparison of bacterial strains on apple flowers as affected by temperature, stigma age, and pollination can be made based on regression curves (Figs. 7 and 8). Populations of pathogen strain Ea153 usually grew to a larger size on stigmas, often by 0.5 to 1 log unit, than the antagonist strains. Populations of antagonists E325 and A506 were similar in size on relatively young stigmas. As stigmas became older, population means for E325 were closer to those of the pathogen, and means for A506 decreased to values more widely separated from the other means. This was most apparent with pollinated flowers in 2001 (Fig. 8B and D). On pollinated flowers at the low temperature, populations of A506 were near the starting size on stigmas inoculated at 8 days, and barely detectable on stigmas inoculated at 14 days. In con-

trast, populations of Ea153 and E325 under these conditions were greater than the starting size at 12 days and near or just below this level at 14 days. For pollinated flowers at high temperature, A506 did not grow on 5-day-old stigmas, and was nearly undetectable on stigmas inoculated at 8 days. Both Ea153 and E325 grew when stigmas were 5 days old and survived at the starting level when stigmas were inoculated at 8 days.

SEM examination of stigmas of different ages revealed that changes in surface morphology generally corresponded to the capacity of stigmas to support bacterial growth, particularly that of strains Ea153 and E325. When flowers were open for less than 24 h, papillae appeared bulbous and turgid (Fig. 9A and D). As stigmas aged, increasing portions of the papillae collapsed, interstices widened, and the underlying cuticular layers became more visible (Figs. 9 and 10). Rate of papillae degeneration was greater in high-temperature enclosures (Fig. 9) than in low-temperature enclosures (Fig. 10). Under high temperatures, most papillae were collapsed and melded to the cuticular matrix 8 days after flower opening (Fig. 9C and F); under low temperatures, papillae were not totally collapsed until 12 or 14 days (Fig. 10C and F). At the stage when all papillae were collapsed, strains Ea153 and E325 grew minimally or only survived at the starting population level (Fig. 8B and D).

DISCUSSION

The temperature range and optimum for bacterial growth on flower stigmas varied among strains tested (Fig. 2). *E. amylovora*

Fig. 5. Effect of stigma age on the interaction between bacterial antagonists and *Erwinia amylovora* strain Ea153 on pollinated detached crab apple flowers at **A** and **C**, 14°C and **B** and **D**, 24°C. Population size of **A** and **B**, antagonists *Pantoea agglomerans* strains C9-1 and E325 and *Pseudomonas fluorescens* strain A506 and **C** and **D**, Ea153 in response to pre-inoculation with antagonists. Flowers were held for various periods at 14 or 24°C, inoculated with antagonist, incubated at 24°C for 24 h, then inoculated with Ea325 and incubated at 24°C for 24 h. Each point is the mean for 10 flowers, and vertical bars represent standard error. Asterisk above day number indicates that means are different according to least significant difference test ($P \leq 0.05$). Horizontal hatched lines represent estimated population size at time of inoculation.

generally grew to higher population levels than did antagonists at temperatures from 20 to 32°C, which is consistent with a recent study by Johnson et al. (18) indicating higher growth rates of the pathogen compared with antagonist strains C9-1 and A506. Nevertheless, populations of *Pantoea agglomerans* strains C9-1 and E325 were relatively large at these temperatures (i.e., near log 6 CFU per flower) and were close to or larger than populations of *E. amylovora* at all other temperatures within the pathogen range. This was not the case with *Pseudomonas fluorescens* strain A506, which grew poorly or not at all at the upper temperature range of *E. amylovora*. However, temperatures seldom reach such high extremes during typical apple and pear bloom periods in the northwestern United States. The difference in temperature optima of strains A506 and C9-1 is thought to be one way the two antagonists complement each other when applied as a mixture, based on observations in multiple field trials over a number of years (14,15; K. B. Johnson, *personal communication*). The dominant strain in these studies tended to be A506 during relatively low-temperature years and C9-1 in high-temperature years. Results of the laboratory experiment presented in Figure 2 did not indicate an advantage of A506 on stigmas based on temperature, because population sizes were always similar to, or less than, those of C9-1. It is conceivable, though, that strains might respond differently in the field to interactions involving temperature and other variables (e.g., bloom progression, bee dispersal, and rain).

The incubation of flowers under constant or fluctuating temperatures averaging 14°C (Fig. 1) demonstrated that, within the range from 4 to 24°C, the diurnal pattern of temperature variation does not, in itself, affect growth of *E. amylovora* on stigmas; growth always was dependent on mean temperature. Given this result, it was deemed unnecessary to simulate field temperature fluctuations in other laboratory experiments. However, the constant temperature of 24°C in several of the experiments (Figs. 3 to

5) is not meant to represent a daily mean temperature, because this rarely occurs during bloom in most pear and apple production areas. It was included for comparison to 14°C, and to gain insight into flower–bacteria interactions during daily periods when temperatures exceed 24°C. The temperature average of 21 or 22°C in field enclosures also was extreme, but occasionally occurs on successive days (7,9). The average of 13 or 14°C in other enclosures is more typical of daily temperature means during bloom in Washington State.

Given the importance of temperature in fire blight risk assessment (5), it is appropriate to consider the relevance of our data to existing disease models. In the Cougarblight risk assessment model developed by Smith (33) for the northwestern United States, potential growth of *E. amylovora* on stigmas is reflected by a 4-day accumulation of degree hours above 15.5°C. The degree-hour values were adjusted according to a curve, based on bacterial doubling rates at various temperatures in liquid medium (32). The same in vitro data was similarly used in a European disease risk assessment system (4). Preliminary application of our in vivo data for *E. amylovora* (Fig. 2) to the Cougarblight model by its developer was encouraging, resulting in greater amplitude of plotted degree totals during periods of high risk and actual infection, but not during low-risk periods (T. J. Smith, *personal communication*). The modification included lowering the temperature threshold below 15.5°C, because significant growth occurred on flowers at temperatures below this limit. Such a change also is consistent with data in Figure 1. If we were to use the 15.5°C threshold to predict results of this experiment, we would anticipate no growth at the constant of 14°C, and increasing growth with higher peak temperatures in the diurnal cycles. We found, however, that population sizes for the different temperature regimes were the same. Adjusting threshold temperature downward brings predicted and actual growth closer together, and

Fig. 6. Temperature and relative humidity in enclosures surrounding ‘Gala’ apple trees in **A and C**, 2000 and **B and D**, 2001. Presented are average hourly temperatures (**A and B**) and relative humidity (**C and D**) in high- and low-temperature enclosures and at one nonenclosed site within orchard block.

may improve the model; of course, any modification to predictive disease models must be validated in the field. Although the relation of temperature to pathogen growth is critical to fire blight risk assessment, other temperature-dependent processes, such as rate of flower opening and pathogen spread by pollinating insects, also are involved.

Laboratory and field experiments indicated that flower stigmas potentially can support bacterial growth at older ages than reported recently by Thomson and Gouk (38). These researchers found that *E. amylovora* did not multiply on Gala apple flowers when stigmas were older than 3 or 4 days at time of inoculation, and indicated similar results for strain A506 and *Pantoea agglomerans* strain Eh318. Procedures used were quite different from those of the present study with Gala apple. In the previous investigation, flowers were marked at time of opening on successive days, all flowers were inoculated on day 6 or 9, and then flowers were monitored daily for population. In contrast, we enclosed trees, started experiments with all flowers newly opened, sampled and inoculated flowers daily, and incubated the detached flowers at near-optimal temperature for growth. Although different approaches were employed, both studies are instructive. The former results may be more representative of what happens under typical field conditions when stigmas of various ages are inoculated artificially. Our results may be more indicative of maximum stigma ages allowing colonization and growth by introduced bacteria, when conditions are optimal and indigenous organisms are ab-

sent. Both studies also have shortcomings. The previous report (38), consisting of one year's data from locations in the United States and New Zealand, lacks critical weather information, such as temperatures prior to inoculation and RH throughout the experimental period. In the present study, an inherent weakness lies in artificial methods (i.e., enclosing trees and inoculating and incubating detached flowers) that could produce artifactual or misleading results.

In the study by Thomson and Gouk (38), flower stigmas inoculated at day 3 or 4 supported bacterial growth up to day 6 or 8, but stigmas inoculated after day 4 were not at all conducive to bacterial growth. A similar phenomenon occurred with strain A506 on pear blossoms (23). These observations indicate the involvement of factors other than, or in addition to, stigma aging. Possibly, naturally occurring epiphytic organisms increased to a level that preempted or limited colonization by the introduced bacteria. Thomson and Gouk (38) discounted this, even though natural populations at days 4 and 5 approached or exceeded log 6 CFU, which is considered to be near-maximum capacity on flowers (14, 23). Stockwell et al. (36) suggested that indigenous organisms on pear blossoms could be sufficiently high to interfere with antagonist establishment, but subsequent indirect analysis (19) did not support this. In a recent report by Lindow and Suslow (23) focused on intratree movement of strain A506, stigmatic populations of this antagonist increased during 12 or 13 days to approximately log 6 CFU on flowers inoculated within 5 days of open-

Fig. 7. Size of bacterial populations on stigmas of 'Gala' apple flowers sampled from trees in 2000 after various periods and inoculated in the laboratory. Single trees were enclosed and maintained at **A and C**, a low-temperature range (average of 12.8°C) or **B and D**, a high-temperature range (average of 21.1°C). All flowers used opened within the same 24-h period. Half of the flowers were pollinated (**B and D**) and half were left unpollinated (**A and C**). Immediately afterward, the first samples were collected (day "0"). On each sampling day, stigmas of detached flowers were inoculated with *Erwinia amylovora* strain Ea153, *Pantoea agglomerans* strain E325, or *Pseudomonas fluorescens* strain A506, and flowers were incubated at 24°C for 24 h before determining population size. Standard error, represented by vertical bars, was calculated based on the average values for five flowers from each of four replicate trees. Regressions had a significance of $P \leq 0.05$ (*), $P \leq 0.01$ (**), or $P \leq 0.001$ (***). Horizontal hatched lines represent estimated population size at time of inoculation.

ing; eventual populations decreased, however, with further increases in flower age at inoculation. The authors suggested possible competition with indigenous organisms, because population sizes of strain A506 and indigenous bacteria had an inverse curvilinear relationship. In our investigation, the use of tree enclosures reduced interflower spread of microorganisms by flying insects, as supported by previous research (30); thus, natural microbial colonization of flowers was less likely to interfere with our inoculations.

It also could be argued that high RH in tree enclosures and laboratory chambers promoted atypical bacterial growth on flower stigmas. Higher moisture possibly extended the period of wet exudates on stigma surfaces, or prolonged the turgid condition of stigma papillae. The time period when all or some stigma papillae appeared bulbous and turgid, according to SEM, generally corresponded to the period when stigmas supported bacterial growth. This is comparable to observations by Thomson and Gouk (38), even though stigma receptivity to bacteria in the two studies was of dissimilar duration. Micrographs are presented for this reason. They indicate that the life of stigma papillae may extend for much longer periods than previously reported (38). Part of this may be explained by a difference in temperature. Overall average RH in the tree enclosures was $\approx 71\%$, which was only 19% higher than the outside level. These considerations point to another explanation for the extended life of papillae in tree enclosures. Flowers sampled for SEM were not inoculated with bacteria and were as-

sumed to have low contamination from indigenous or introduced organisms (30). Thus, it is conceivable that epiphytic microorganisms themselves cause an increase in the rate of stigma senescence. An understanding of moisture and microbial colonization in relation to stigma senescence will require further investigation.

Our study was an attempt to gain insight into flower-microbe interrelationships by controlling variables such as bacterial spread, temperature, and pollination. Unfortunately, flower age in relation to inoculation and colonization was not evaluated on nonenclosed trees, but only on enclosed trees; thus, we cannot be conclusive regarding the effect of indigenous microorganisms. It is plausible, however, that flowers were receptive to bacteria at older stigma ages than in other studies because indigenous organisms were not a significant factor. In our controlled experiments with detached flowers and enclosed trees, duration of stigma conduciveness to bacteria was inversely related to temperature and negatively affected, though slightly, by pollination. Although these relationships were firmly established, the information may have little predictive value in the orchard, because bacterial colonization normally is complicated by indigenous organisms that vary in type and number, and depend on location and conditions (36). The effect of pollen on bacterial colonization of flower stigmas deserved consideration because of its relation to flower physiology (20,34) and previous research indicating a role of pollen in the microbial ecology of the phyllosphere (6,21,42). Possibly, pollen negatively affected bacteria because they competed with bacteria

Fig. 8. Size of bacterial populations on stigmas of 'Gala' apple flowers sampled from trees in 2001 after various periods and inoculated in the laboratory. Single trees were enclosed and maintained at **A and C**, a low-temperature range (average of 13.8°C) or **B and D**, a high-temperature range (average of 22.0°C). All flowers used opened within the same 24-h period. Half of the flowers were pollinated (**B and D**) and half were left unpollinated (**A and C**). Immediately afterward, the first flower samples were collected (day "0"). On each sampling day, stigmas of detached flowers were inoculated with *Erwinia amylovora* strain Ea153, *Pantoea agglomerans* strain E325, or *Pseudomonas fluorescens* strain A506, and flowers were incubated at 24°C for 24 h before determining population size. Standard error, represented by vertical bars, was calculated based on the average values for five flowers from each of three replicate trees. Regressions had a significance of $P \leq 0.01$ (*), $P \leq 0.001$ (**), or $P \leq 0.0001$ (***). Horizontal hatched lines represent estimated population size at time of inoculation.

for space and nutrients on stigma surfaces, or pollen germination and pollen tube growth indirectly affected bacteria by accelerating stigma senescence (34).

A comparison of bacterial strains on stigmas of various ages revealed a failure of strain A506 to grow on stigmas in late senescence stages that did support growth of *P. agglomerans* and *E. amylovora*. This difference, particularly evident on pollinated and dual-inoculated stigmas, may have been due to depletion of nutrients or build-up of toxins differentially affecting the bacterial strains. It also may relate to the low osmotolerance of strain A506, a characteristic previously associated with poor growth and survival of this organism in the sugar-rich environment of the flower hypanthium (28,30). The comparatively low capacity of A506 to grow on old stigmas should be noted; however, it is unknown whether this characteristic relates to biocontrol efficacy in the orchard. Even if the phenomenon proves inconsequential for individual old flowers, it still may relate to total populations in the orchard and overall efficiency of interflower spread of bacteria and their colonization on younger flowers. The idea of using

mixtures of antagonists has been advanced in studies with strains A506 and C9-1 by Johnson and collaborators (14,15,17). The case can be made that deficiencies of one antagonist in a mixture (e.g., inability to grow on old stigmas) are compensated for by another.

Except for the unexpected results with strain A506, experiments indicated a general constancy of antagonist behavior on aging stigmas relative to the pathogen. The data contribute to an understanding of the flower-microbe system and increase confidence in the utilization of antagonists for suppressing populations of *E. amylovora* on flowers. This was reinforced by dual inoculations in which we evaluated the ability of antagonists to preemptively exclude *E. amylovora* on flower stigmas of various ages (Figs. 4 and 5). Although antagonists differed from each other in efficacy, the effect of individual antagonists on the pathogen was consistent as flowers aged. It may be concluded that stigma aging itself, and associated plant physiological changes, do not significantly alter antagonist-pathogen interactions during the period when stigmas support bacterial growth.

Fig. 9. Scanning electron micrographs of 'Gala' apple stigmas sampled from trees in high-temperature enclosures in 2001 (22°C average). Shown are stigmas sampled at **A and D**, 0 days, **B and E**, 4 days, and **C and F**, 8 days from time of flower opening.

Fig. 10. Scanning electron micrographs of 'Gala' apple stigmas sampled from trees in low-temperature enclosures in 2001 (13.8°C average). Shown are stigmas sampled at **A and D**, 4 days, **B and E**, 8 days, and **C and F**, 14 days from time of flower opening.

In summary, aspects of temperature–flower–bacteria interrelationships relevant to fire blight were examined in detail, either for the first time or by using a different and more controlled approach. Data relating temperature to pathogen growth on stigmas could be used to improve disease risk assessment models presently based on *in vitro* growth. Duration of stigma receptivity to bacteria was dependent on temperature and pollination. It was generally longer than previously reported, probably due to less interference from indigenous organisms. If this is true, early application of biocontrol agents during bloom may be more important than we have realized for their establishment on individual flowers and subsequent spread within orchards. The controlled, but artificial, experimental methods also revealed antagonist limitations that possibly affect field performance. Such deficiencies could be overcome by selecting other antagonists or using antagonist mixtures in the orchard.

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