Temporal Dynamics of the Biocontrol Agent *Pseudomonas fluorescens* Strain A506 in Flowers in Inoculated Pear Trees

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ABSTRACT

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The colonization of individual flowers in mature pear orchards by *Pseudomonas fluorescens* strain A506 applied at different times during bloom was measured to determine the receptivity of flowers to colonization and the extent of intra-tree movement over time. Strain A506 populations in flowers open at inoculation were initially about 10^4 cells per flower and increased to approximately 10^6 cells per flower in flowers that were inoculated within about 5 days of opening. However, eventual populations decreased with further increases in flower age at inoculation

to as few as about 10^3 cells per flower when inoculated flowers were more than 10 days old. Populations of strain A506 on flowers that opened after inoculation was initially very low at the time of petal expansion (<100 cells per flower) but increased rapidly with time after flower opening. The maximum population of strain A506 that developed on such flowers decreased with increasing time between inoculation and petal expansion; 10^4 to 10^5 cells of strain A506 eventually colonized flowers that opened within 7 days of inoculation, whereas fewer than 100 cells colonized flowers that opened 24 days or more after inoculation. Large total bacterial populations on A506-treated trees were associated with significant reductions in populations of *Erwinia amylovora* and reduced incidence of fire blight and severity of fruit russet.

Fire blight, caused by the bacterium *Erwinia amylovora*, is one of the most serious diseases of pear and apple. Although the disease is widely distributed in most pome fruit growing regions of the world (1,3,33), it is particularly prominent in the western United States. The disease generally results from a build-up of inoculum of the pathogen under relatively warm temperatures ($>15^\circ\text{C}$) on the pistillate surface of flowers (7,31). This epiphytic inoculum can initiate infection if moved to the hypanthium by rain, which also may dilute nectar sufficiently to allow growth and survival of the pathogen at this site (26,31,33). The disease is currently managed by repeated applications of bactericides, primarily streptomycin, onto flowers to prevent epiphytic populations from developing (33). The frequent application of streptomycin has resulted in widespread resistance in most growing areas, limiting its effectiveness (7,11,20,33).

Biological control of fire blight can also be achieved by applying bacterial antagonists onto flowers before the development of epiphytic populations of *E. amylovora*. Such antagonists have been used by themselves (7–10,15,28,29,32,33) or in conjunction with antibiotics (19,29) for the management of fire blight. Several antibiotic-producing strains of *Pantoea agglomerans* have been found to reduce disease if applied to flowers before *E. amylovora* (7,28). Likewise, *Pseudomonas fluorescens* strain A506, which apparently inhibits *E. amylovora* by competing with the pathogen for growth-limiting resources (18,35), is registered and commercialized for control of fire blight (Blightban A506, NuFarm, Sugar Land, TX).

An understanding of the microbial ecology of flowers and of the infection process of *E. amylovora* reveals several features of this disease that should make its control conducive to the use of antagonistic microorganisms (7). Flowers of most pome fruits

apparently emerge in the spring without substantial populations of indigenous bacteria; such populations usually develop only after several days after emergence of flowers (5,16,19,30). Although such noncolonized plant tissue probably facilitates the growth of the pathogen if it is among the initial immigrants to a flower, it is also a suitable habitat for the rapid growth of introduced antagonists (7–10,19). Flowers are apparently susceptible to infection by *E. amylovora* only for a short period of time (7 to 10 days) after flower emergence (1,33). Thus, there is only a narrow window of time during which pathogen populations need to be suppressed by antagonists to achieve disease control. Perhaps for these reasons, the biological control of fire blight using *Pantoea agglomerans* and *Pseudomonas fluorescens* often has been successful, resulting in reductions in disease incidence averaging 50 to 80% (7), although reports of lesser control have also been made (34).

Several features of the process of fire blight infection make control of this disease difficult. In most cultivars of pear and in some apple cultivars, flowering can be asynchronous, occurring over a period of up to 6 weeks. If weather conditions are suitable, infection can occur at any time during this period. The prolonged flowering period requires multiple applications of bactericides to ensure that newly emerged flowers contain a protective bactericide. Often, those flowers emerging later in the spring, after the senescence of the main cohort of flowers, are most at risk of infection due to seasonal increases in daytime temperature. Thus, the cost of disease management is directly proportional to the duration of bloom in most seasons. If repeated application of antagonistic bacteria were similarly required to achieve biological control of fire blight, the cost of this strategy might be prohibitive.

The prolonged bloom of pear is a challenge for successful biological control of fire blight and may account for variation in disease suppression in different years. Unlike chemical bactericides that must be directly applied to flowers to act as a deterrent to pathogen growth, antagonistic bacteria can undergo secondary

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movement to uninoculated flowers (8,10,23,28,32). Insects, particularly honeybees, are effective vectors for movement of bacteria into flowers (8,32). In principle, only a small number of such dispersed cells might be needed to allow colonization of flowers that are newly-opened since antagonists were spray-inoculated onto a tree. Although *Pseudomonas fluorescens* strain A506 and *Pantoea agglomerans* C9-1 and E325 were both found to move from treated trees to untreated trees nearby (23,28), we lack detailed information on the process of such secondary colonization of flowers within a treated tree. Because not all flowers emerge simultaneously, suppression of growth of *E. amylovora* must occur in a succession of flowers over a considerable period of time, and the movement of inoculum of antagonists from flower to flower within a treated tree would be important in the process of biological control.

Because there are practical and economic constraints on how frequently a biocontrol agent can be applied to an orchard, we studied the process of colonization of pear flowers that were inoculated with *Pseudomonas fluorescens* strain A506 as well as cohorts of flowers that emerged after inoculation in an orchard environment on mature trees. In this study, we monitored the process of colonization of individual flowers in each of many different cohorts with known emergence times relative to that of spray inoculation. In this way, we achieved considerable insight into the process by which secondary colonization of flowers occurs in an orchard setting. This information will address issues related to the timing and frequency with which biocontrol agents need be applied to trees to achieve sufficient flower colonization with antagonists to yield biological control of fire blight.

MATERIALS AND METHODS

Bacterial strains and culture media. The source and characteristics of *Pseudomonas fluorescens* strain A506 has been described previously (35). This streptomycin-resistant strain is a spontaneous mutant resistant to rifampicin (100 µg/ml) and was selected as described in a previous study (33). Estimates of total bacteria were made on King's medium B (12) containing cycloheximide (100 µg/ml) (KB) to inhibit fungal growth. Recovery of strain A506 was on KB containing rifampicin (100 µg/ml) (KBR). *E. amylovora* was quantified on modified Miller-Schroth medium (with sorbitol) (MS) (22). Inoculum of strain A506 was from a lyophilized preparation of cells of strain A506 (containing approximately 10^{11} cells per g) recovered from broth culture. This preparation, supplied by Advanced Genetic Sciences, Oakland, CA, was equivalent to the registered biological pesticide now available as Blightban A506 (NuFarm).

Field plot design. All field studies were conducted in two commercial orchards containing mature cv. Bartlett pear trees. One orchard was located about 15 km west of Vacaville, CA, and the other about 8 km east of Sacramento, CA; both orchards were located in major pear growing districts and thus represented a majority of the pears grown in California. The trees in both orchards were approximately 4 m apart within a row, and 7 m separated adjacent rows of trees. Most trees were about 4 to 5 m in height. All of the trees in the tests were sprayed with solutions of streptomycin sulfate (100 µg/ml) with commercial air-blast sprayers by cooperating growers. This bactericide was sprayed at intervals that the cooperators determined, generally every 3 to 7 days, starting about 10 days after first bloom depending on prevailing weather conditions. The fungicide benomyl was occasionally applied to control pear scab (*Venturia pyrina* Aderhold). Field tests were conducted on trees organized in a randomized complete block design with five replications. Each replication consisted of five trees in a given row in two (Vacaville) or four (Sacramento) adjacent rows of trees (10 to 20 trees per replicate, respectively); at least 12 trees separated groups of treated trees within a row, and different replicate blocks were separated by at least 10 rows of trees to minimize movement of inoculum from one block to another. At 3- to 4-day intervals, starting when each tree had at least 100 open flowers (designated first bloom), between 500 and 1,000 pear flowers on trees assigned to a given treatment that had opened within the last few hours or that would open during the day (petals partially but not completely open) were randomly tagged with a small colored plastic tie (placed at the base of the pedicel) to designate a flower age group. First bloom in both trial sites occurred on the same day in a given year. First bloom occurred on 13 March 1992 and 16 March 1993. Trees at both sites were spray-inoculated with a suspension of *Pseudomonas fluorescens* strain A506 at 20% bloom, 90% bloom, or both at 20 and 90% bloom. Additionally, at the Vacaville site, trees were also sprayed with strain A506 at either 5% bloom or 50% bloom. In 1992, strain A506 was applied to trees at the Vacaville site at 3, 5, 7, and 12 days after first bloom, whereas strain A506 was applied to trees at the Sacramento site at 6 and 12 days after first bloom. In 1993, strain A506 was sprayed onto trees at the Vacaville site at 3, 5, 8, and 11 days after first bloom, and at the Sacramento site at 5 and 11 days after first bloom. Suspensions of strain A506 were prepared by diluting lyophilized bacterial preparations with tap water to a concentration of approximately 10^8 cells per ml. Bacteria were applied to near wetness (about 3 liters per tree) with a piston-pressurized handgun sprayer within 1 h of preparation.

Enumeration of bacterial populations on plants. For each treatment schedule of strain A506 application, epiphytic bacterial populations from up to 20 individual flowers were estimated for each age group of flowers that had been defined at each sampling time. Individual flowers were placed in small, sterile, sealable plastic bags immediately after collection in the field and trans-

Fig. 1. Daily maximum (□) and minimum (○) temperatures at a site near the field trial at Vacaville, CA in 1992 (top panel) and 1993 (bottom panel). Time is indicated as the number of days after first bloom (13 March 1992 and 16 March 1993). Daily rainfall amounts at this site are also indicated. Daily temperatures and the days of occurrence and amounts of rainfall at a second field site near Sacramento were very similar for a given year.

ported on ice to the laboratory within about 4 h, and 5 ml of sterile washing buffer (24) was added to the bags. The flowers were submerged in the buffer and the bags were sonicated for 7 min in an ultrasonic cleaning bath (Bransonic 52) to dislodge bacteria from the flowers as in other studies (2). The bags were then manually agitated for 10 s to disperse the bacterial cells prior to enumeration. Appropriate dilutions of the flower washings were then plated onto KB, KBR, and MS media and incubated at 24°C. Total bacteria were counted after 4 days growth on KB, whereas pinkish orange dome-shaped mucoid colonies characteristic of *E. amylovora* on MS medium were enumerated after 6 days growth, and strain A506 was enumerated on KBR after 3 days. Only colonies

that resembled *Pseudomonas fluorescens* strain A506 were recovered on KBR, indicating that this medium was highly selective for the antagonistic bacterium. Representative presumptive colonies of *E. amylovora* were isolated with a toothpick from dilution plates and stab-inoculated into immature pear fruits. The presence of a spreading blackened water-soaked area with beads of ooze around the inoculated site 3 days after incubation at 30°C confirmed the identity of isolates as *E. amylovora*.

Measurement of fire blight disease and fruit russet. Fire blight infection was visually rated by viewing trees from ground level and counting the number of infected fruiting or vegetative shoots on each tree that exhibited wilting or discoloration typical

Fig. 2. Population size of *Pseudomonas fluorescens* strain A506 on flowers of pear trees treated with this strain at **A**, 5% bloom, **B**, 50% bloom, or **C**, 90% bloom (indicated by the arrows) or on **D**, untreated trees that emerged 1 day (■), 4 days (○), 7 days (▲), 13 days (◇), 17 days (△), 24 days (□), 31 days (●), and 38 days (◆) after the date of first bloom at a plot near Vacaville, CA, in 1992. The vertical bars represent the standard error of the mean of log-transformed population sizes at a given sampling time after first bloom.

of fire blight disease. Most fire blight strikes were enumerated about 2 weeks after infections had become apparent so disease was obvious and not easily missed during observation. A total of about 100 mature fruits were collected from each replicate of each treatment. The severity of russet of each fruit was estimated visually using assessment keys as described previously (18).

Statistical methods. The goodness-of-fit of the normal distribution of log-transformed bacterial population sizes of individual tissue samples was tested by the Shapiro-Wilk (*W*) statistic and calculated using SAS (release 6.04, SAS Institute, Cary, NC). Because most distributions were adequately described by a log-

normal distribution, all estimates of bacterial population sizes were log-transformed prior to analysis. Measurements of fruit russet, expressed as the percentage of the fruit surface damaged, were subjected to inverse sine transformation before analysis. Samples in which population sizes of *E. amylovora* were not detected were assigned the log-transformed value of 0. Analysis of variance of log-transformed epiphytic bacterial population sizes was computed using the general linear models procedure of SAS. Separation of treatment means was determined by Fisher's unprotected least significant difference test; this method controls the comparison-wise error rate.

RESULTS

By tagging large numbers of individual flowers as they opened at frequent intervals throughout the spring we were able to ascertain when inoculum of *Pseudomonas fluorescens* strain A506 had been applied to a tree relative to the time a flower had opened, as well as the temporal population dynamics on many different cohorts of flowers after they opened. Because sampling of flowers for epiphytic bacterial populations is inherently destructive, this strategy allowed us to make repeated estimates of population sizes of different cohorts of flowers that had emerged at 3- to 4-day intervals throughout the spring. Because the date when each flower opened was known, we could unambiguously determine its age when spray inoculation occurred, or how long after inoculation the flower opened. By varying the time when spray inoculations were made, we thus were able to sample cohorts of flowers that opened at various times both before and after spray inoculation of strain A506.

The weather conditions during the bloom period in March and April of 1992 and 1993 were typical for this area, with slowly increasing temperatures and progressive decreases in rainfall (Fig. 1). Mean temperatures at a given time after bloom initiation were similar in the 2 years. Rainfall occurred on seven occasions in 1992 and on 10 occasions in 1993 (Fig. 1). Typical of most pome fruit growing regions in the arid western United States, the frequency of rainfall was highest early in the bloom period (Fig. 1).

The population dynamics of strain A506 on flowers that had opened by the time of spray inoculation of trees differed greatly from that of flowers that opened after inoculation. The population sizes and temporal patterns of colonization of flowers from trees inoculated on a given date were remarkably similar in both field trials in a given year as well as between years. For clarity, therefore, primary colonization patterns are illustrated only for flowers sampled at the Vacaville trial in 1992. Strain A506 achieved high population sizes on all flowers that were open at the time of spray inoculation (Fig. 2). Although an average of approximately 10^4 cells per flower were recovered shortly after inoculation, populations usually increased to about 10^6 cells per flower within 5 days after inoculation (Fig. 2). Generally, the highest population sizes achieved by strain A506 in flowers that were directly inoculated occurred in flowers that had opened shortly before inoculation. For example, the maximum population size of strain A506 on flowers that opened on the day of first bloom was $10^{5.5}$ cells per flower, whereas the maximum population size of strain A506 on flowers that opened on flowers emerging 4 and 7 days later was over 10^6 cells per flower (Fig. 2B). In contrast to flowers that were open at the time of inoculation, flowers that were closed usually harbored very low populations of strain A506 shortly after opening (Fig. 2). The population size of strain A506 in newly-opened flowers, however, generally increased rapidly after flowers opened (Fig. 2). The maximum population size achieved on such flowers, however, tended to decrease with increasing time that had elapsed between the time of inoculation of the trees and the opening of a particular cohort of flowers (Fig. 2). Thus, flowers that opened shortly after inoculation usually achieved population sizes of approximately 10^6 cells per flower within 5 to 8 days in

Fig. 3. Population size of *Pseudomonas fluorescens* strain A506 on flowers of pear trees treated at 5% bloom (4 days after first bloom) **A**, that were open for 1 day at the time of spray inoculation or **B**, that emerged 4 days after spray inoculation of trees in a field trial located near Vacaville, CA, in 1992. The open triangles represent estimates of population sizes of strain A506 on 20 individual flowers at each sampling time, whereas the vertical bars represent the standard error of the mean of log-transformed population sizes. From 1 to 14 flowers at a given time for which no strain A506 was detected were assigned the log value of 0.

most cases, whereas flowers that opened 2 weeks or more after inoculation with strain A506 never developed large populations of the strain (Fig. 2). Interestingly, the maximum population size of strain A506 on flowers that opened shortly after inoculation with this strain was larger than that on flowers that had opened up to 10 days earlier and were directly inoculated (Fig. 2C). The population size of strain A506 remained close to the maximum size achieved on a given cohort of flowers throughout the sampling period, although in some cases population sizes declined at later sampling times as daytime temperatures increased and rainfall became less frequent (Fig. 2). For example, the population size of strain A506 on freshly opened flowers that were directly inoculated remained above 10^6 cells per flower for over 20 days (Fig. 2A and B), whereas the population size of strain A506 on flowers that opened after inoculation rapidly reached populations of only about 10^5 cells per flower and were maintained for many days (Fig. 2B). Although untreated control trees were located a minimum of 50 m from the closest treated trees, many flowers acquired measurable populations of strain A506 (Fig. 2D). Mean population sizes of strain A506 in most cohorts of flowers were, however, less than about 10^3 cells per flower (Fig. 2).

The heterogeneity of population sizes of strain A506 in different cohorts of flowers differed greatly. Although population sizes of strain A506 in newly-opened flowers that were open at the time of inoculation increased from about 10^4 to nearly 10^6 cells per flower, the range of observed population sizes at a given sampling time was always low (Fig. 3A). In contrast, upon opening, flowers that were closed at the time of inoculation harbored a wide range of strain A506 population sizes (Fig. 3B). While mean population sizes of strain A506 increased from at or below the detection limit (500 cells) on such flowers to over 10^5 cells per flower within the first 3 to 5 days after opening, population sizes became more homogeneous as flowers aged (Fig. 3B). Frequency distributions of population sizes of strain A506 in flowers that were closed at the time of inoculation further reveal the dramatic change in both size and heterogeneity of strain A506 with time (Fig. 4). Although strain A506 was undetectable on most flowers 1 day after their opening, a small proportion had population sizes as high as 10^5 cells per flower (Fig. 4). By 7 days after opening, most flowers harbored over 10^4 cells per flower, and by 14 days all flowers had at least 10^5 cells per flower (Fig. 4). Clearly, strain A506 efficiently colonizes even flowers that were closed at the time of inoculation, rapidly developing high populations in most flowers.

The maximum population size of strain A506 that developed in flowers that were open at the time of spray inoculation was strongly influenced by how long the flowers were open before being inoculated. Because strain A506 was inoculated onto pear trees at different phenological stages and because many different cohorts of flowers were tagged prior to spraying, it was possible to determine the efficiency with which strain A506 colonized flowers that were open for various periods of time before spraying. Because the population dynamics of strain A506 in both plots were similar in a given year, results from both trials could be combined to yield a more comprehensive data set to address this issue. In each year of the study, a nonlinear relationship was found between flower age at the time of spray and the maximum population size of strain A506 that developed within 3 to 7 days after inoculation of open flowers (Fig. 5). Flowers that were open for 7 days or less usually developed similarly high population sizes of strain A506 (approximately 10^6 cells per flower). The population size of strain A506, however, decreased progressively with increasing flower age at the time of inoculation for flowers older than about 5 days (Fig. 5). The decreased efficiency of colonization of older flowers could be due either to the occurrence of indigenous bacterial populations in old flowers that compete against the antagonist or reduced flower receptivity associated with aging of the flower. To assess the first possibility, the

population size of strain A506 that developed in flowers of different ages was related to the number of other indigenous bacteria in the flower at the time of inoculation (Fig. 6). The lowest populations of strain A506 were associated with flowers having the

Fig. 4. Frequency distribution of population sizes of *Pseudomonas fluorescens* strain A506 in flowers of pear trees that opened 1 day after spray inoculation with strain A506 (at 20% bloom) when sampled 1 day (top panel), 7 days (middle panel), and 14 days (bottom panel) after treatment in a field trial near Vacaville, CA in 1992. The percentage of individual flowers that harbored the population sizes of strain A506 indicated on the abscissa is shown.

highest populations of indigenous bacteria (which also were the oldest flowers). Conversely, those young flowers that harbored less than about 10^4 cells per flower of indigenous bacteria all supported the colonization by strain A506 of over 10^6 cells per flower. Thus, while it does not prove the first conjecture, competition by indigenous microflora may reduce the colonization of old flowers by strain A506.

The maximum population size of strain A506 that developed in flowers that opened after spray inoculation of trees was strongly influenced by the period of time that elapsed between inoculation and flower opening. Because the day of flower opening had been determined for many different cohorts of flowers, the time that had elapsed between the last spray inoculation of strain A506 and

flower opening was known. The population dynamics of A506 on many such cohorts of flowers was thus determined in the two experimental sites in each of the 2 years of the study to determine how effectively flowers that were not directly inoculated with strain A506 became inoculated and subsequently colonized with this strain. As noted previously, although strain A506 became established on most flowers that emerged after spray inoculation, the average population size on a particular cohort of flowers was greatest when flowers opened shortly after inoculation (Fig. 2). When considered over all cohorts of flowers, a nonlinear relationship was observed between maximum population sizes of strain A506 that developed in flowers as a function of time that had elapsed between spray inoculation and flower opening (Fig. 7). While flowers that opened within about a week after inoculation all achieved high populations of strain A506 ($>10^5$ and 10^4 cells per flower in 1992 and 1993, respectively), the maximum population size of A506 that developed on flowers opening after this time decreased with increasing time (Fig. 7). It should be noted that in 1992, even flowers that opened 3 weeks after spray inoculation developed mean populations of strain A506 of over 10^4 cells per flower. However, flowers that opened more than 30 days after spraying had mean populations of strain A506 of only about 10 to 100 cells per flower, and many flowers lacked detectable A506 populations. The maximum population sizes of strain A506 that developed in unopened flowers in both experimental sites in 1993 were about fivefold lower than the maximum population sizes in 1992 (Fig. 7). Furthermore, the relative population size of strain A506 that developed in flowers that were not directly inoculated tended to decrease more rapidly with time that had elapsed since the last spray inoculation in 1993 than in 1992. For example, in 1992 flowers that opened within about 20 days of the last inoculation with strain A506 eventually developed populations of this strain that were at least 1% that of flowers that opened immediately

Fig. 5. Maximum population sizes of *Pseudomonas fluorescens* strain A506 that were attained 3 to 7 days on flowers that were open for the times shown on the abscissa at the time trees were inoculated with strain A506 in trials in Vacaville and Sacramento in **A**, 1992 and **B**, 1993. Each point represents the mean of log-transformed population sizes of strain A506 from a cohort of 20 individual flowers from plots near Vacaville and Sacramento in a given year.

Fig. 6. Population sizes of *Pseudomonas fluorescens* strain A506 that were attained within 7 days in flowers that had been open for different periods of time before inoculation and had the population sizes of indigenous bacteria at the time of spray inoculation shown on the abscissa. Each symbol represents the mean log population size of strain A506 from 20 individual flowers of a given age group from trees in trials near Vacaville and Sacramento in 1992 (triangles) and 1993 (squares) and the mean log-transformed total bacterial population size on untreated flowers for the same age group.

after inoculation (Fig. 7A). In contrast, only flowers that opened within about 12 days of inoculation developed such a population size in 1993 (Fig. 7B). Since the main pear bloom period normally lasts only about 14 days, a single inoculation early in the bloom period would yield high colonization of all main bloom flowers. However, flowers emerging a month or more after a single inoculation would probably be poorly colonized.

Because the process of colonization of inoculated flowers as well as secondary colonization of flowers not directly inoculated might be influenced by weather conditions or plant factors, such as the number of flowers in a tree at the time of inoculum that would subsequently be available to supply inoculum of strain A506 to other flowers, we inoculated strain A506 onto trees at

different phenological stages and monitored the colonization of flowers of different age groups. Strain A506 was thus sprayed at different times throughout the spring onto trees with different proportions of their flowers open at the time of inoculation. The consequences of application of A506 to flowers at different phenological stages on secondary colonization of flowers in a tree was most easily assessed by comparing the dynamics of this strain on flowers of a given age class. Surprisingly, the population dynamics of strain A506 in a given cohort of flowers that emerged at different relative times before or after spray inoculation was similar, irrespective of the stage of bloom of the trees when strain A506 was inoculated (Fig. 8). For example, the colonization of flowers that emerged 13 days after first bloom by strain A506 was

Fig. 7. Maximum population sizes of *Pseudomonas fluorescens* strain A506 that developed in pear flowers that opened at the various times after spray inoculation of trees with this strain. The mean of log-transformed population sizes of strain A506 from individual flowers of different age groups from trees treated with strain A506 at different times prior to flower opening (shown on abscissa) in field plots near Vacaville and Sacramento, CA in A, 1992 and B, 1993 are shown.

Fig. 8. Population sizes of *Pseudomonas fluorescens* strain A506 on flowers that A, emerged 13 days after first bloom or B, 24 days after first bloom on pear trees treated at 5% bloom ($\square$), 20% bloom ($\diamond$), 50% bloom ($\blacktriangle$), and 90% bloom ($\circ$) with strain A506, as well as on untreated trees ($\blacksquare$) in a field plot near Vacaville, CA, in 1992. The vertical bars represent the standard error of the mean of log-transformed population sizes of these cohorts of flowers at a given sampling time after first bloom.

very similar irrespective of whether A506 was applied at 5% bloom (about 10 days earlier) or at 90% bloom (only 1 day earlier) (Fig. 8A). Mean populations of strain A506 increased rapidly on flowers as they emerged and achieved populations of $>10^5$ cells per flower within about 4 days irrespective of the timing of A506 application (Fig. 8A). Populations on flowers that emerged 10 days before spray inoculation were slightly lower than populations on flowers that emerged closer to the date of inoculation. Similarly, all treatment dates resulted in a similar colonization of flowers that opened 24 days after first bloom (Fig. 8B). Although mean population sizes of strain A506 were lower than those on flowers opening closer to first bloom, the time of spray inoculation had little effect on population dynamics of A506 on these late-opening flowers. As with the cohort of flowers that had opened nearly 2 weeks earlier (Fig. 8A), the population size of strain A506 was generally somewhat higher (<10 -fold) on those flowers that had been inoculated at the later phenological stages than those sprayed closer to first bloom. An analysis of flowers of other age groups revealed a similarity in population dynamics that was largely independent of A506 treatment date (data not shown). Not only did treatment with strain A506 at different dates result in similar mean populations of A506 on a given flower age group, but all resulted in a similarly high frequency of colonization. Over 95% of all flowers that emerged within about 20 days of first bloom and about 80% of those that emerged within 30 days of first bloom (irrespective of the inoculation date of A506 during the main bloom period) had population sizes of A506 that exceeded 10^3 cells per flower (data not shown). Strain A506, therefore, appears to readily migrate to pear flowers after they open. No substantial benefit was observed from multiple applications of A506 because of this fact.

Inoculation of pear trees with strain A506 resulted in large and persistent changes in the total population sizes of bacteria not only in flowers that were directly inoculated but also in flowers that opened after spray inoculation (Fig. 9). The total numbers of bacteria on a given cohort of flowers on trees treated with A506 at

various times was substantially higher than those on untreated control trees at a given sampling time (Fig. 9). For example, the total number of bacteria on flowers that opened 4 days after first bloom increased to over 10^6 cells per flower within approximately 12 days of first bloom, irrespective of whether strain A506 had been applied before or at various times after flowers had opened (Fig. 9). In contrast, the total bacterial populations on this cohort of flowers on untreated trees increased slowly from about 10^4 cells per flower to about $10^{5.5}$ cells per flower over a period of approximately 30 days after opening (Fig. 9). Only after about 30 days after flowers opened did the population size of total indigenous bacteria approach that on flowers treated with strain A506 (Fig. 9). Total bacterial population sizes of this cohort of flowers were from 10- to 100-fold lower on untreated trees than on A506-treated trees at a given sampling date (Fig. 9). Similar differences in total bacterial populations on other cohorts of flowers from treated and untreated trees were observed in all plots in both years of the study (data not shown).

Colonization of pear flowers by strain A506 on trees treated with this strain at different times during the bloom period resulted in reductions in the population size of *E. amylovora*. Although *E. amylovora* was only sporadically isolated on flowers that emerged within about 20 days of first bloom in both plots in both years of the study, much higher populations were observed on late-opening flowers, particularly in 1992 (data not shown). Mean population sizes of *E. amylovora*, particularly on these later emerging flowers, were reduced significantly on flowers from trees treated with strain A506 at 20% bloom but not at 90% bloom. For example, mean populations of *E. amylovora* on late-opening cohorts of flowers sampled in late April and early May were reduced between 10- and 100-fold on A506-treated trees (Table 1). Thus, although populations of strain A506 were low on flowers that emerged in late April and early May, strain A506 had apparently suppressed the growth of *E. amylovora* on earlier flower age groups, thereby reducing the availability of the pathogen to flowers that emerged late in the growing season.

The incidence of fire blight strikes was also reduced significantly on trees treated with strain A506, irrespective of inoculation time, at both the Vacaville and Sacramento plots (Table 2). The incidence of fire blight strikes on trees treated with A506 at different times or at different frequencies did not differ, whereas all treatments differed significantly from untreated control trees (Table 2). The incidence of fire blight infections was reduced between three to fourfold at both sites compared with control trees. It should be noted that the disease control observed in both sites was in addition to that conferred by antibiotics that were periodically applied to all trees. The severity of fruit russet at harvest was also reduced between 30 and 50% on trees at both the Sacramento and Vacaville plots that were treated with strain A506, irrespective of application time or frequency (Table 2).

DISCUSSION

The colonization of secondary flowers, those not directly inoculated with *Pseudomonas fluorescens* strain A506, was impressive in all trials in both years of these studies. Nearly all flowers that opened within a week of spray inoculation of pear trees became colonized by strain A506, and mean populations usually were high, ranging from 10^5 to 10^6 cells per flower (Figs. 2 and 7). Although the initial inoculum on such secondary flowers was often below detection (Figs. 3 and 4), several processes might have contributed to the inoculation of floral parts. Although we did not dissect flowers to ascertain whether strain A506 was found preferentially on stigmatic surfaces as in other studies (7–10, 26,35), we feel it is safe to assume that most of the cells recovered on our intact flowers were in fact on the stigma. Others have found that stigmatic populations of biocontrol agents as well as *E. amylovora* on both inoculated flowers and flowers that, because

Fig. 9. Population sizes of total bacteria on flowers that emerged 4 days after first bloom on pear trees spray inoculated at 5% bloom (○), 20% bloom (▲), and 50% bloom (□) with *Pseudomonas fluorescens* strain A506, as well as on untreated trees (■) in a field plot near Vacaville, CA, in 1992. The vertical bars represent the standard error of the mean of log-transformed population sizes of all culturable bacteria of this cohort of flowers at a given sampling time after first bloom.

they were inoculated with these bacteria via insects, were from 10- to 100-fold higher than that in the hypanthium or on other floral parts, especially when flowers had not experienced rainfall (7–10,26,31). Stockwell et al. (30) also noted that the stigmas of newly-opened flowers seldom harbored indigenous bacteria. Apparently bacteria resident on the surface of flower buds are not efficiently transferred to the stigma upon its exposure during petal expansion. We thus feel that it is unlikely that cells of strain A506 that were usually present only in small numbers, presumably on the surface of unopened flowers, would have had an opportunity to be transferred to the stigmatic surface without some means of physical transfer. Although rain has been demonstrated to move *E. amylovora* and biocontrol agents from the stigma to the hypanthium (26,30), we feel that this mode of transfer was unlikely to have accounted for movement of strain A506 to the stigma. Only a few rain events were encountered during the blossom period in each year of the study. If rain were a primary means of movement of cells into floral parts we might have expected to see effective transfer of inoculum only to those cohorts of newly-opened flowers that had experienced rainfall. In fact, we observed a very consistent temporal pattern of colonization of newly-opened flowers, where colonization efficiency was dictated primarily by the length of time that had elapsed since the last inoculation of earlier-opening flowers in that tree (Figs. 2 and 7). A much more discontinuous process of colonization and one that would vary greatly between different cohorts of flowers might thus have been expected if rain was a primary mobilizer of inoculum of strain A506. It is possible that dew, which frequently occurred in the plot areas in both years, could have allowed movement of strain A506 from exterior locations on flower buds to the interior stigmatic surfaces upon petal expansion. We, however, feel this is unlikely because the stigma is physically separated from exterior floral parts by a considerable distance, making movement by active or passive means in a moisture film torturous and slow. It seems most likely that insect vectors were responsible for secondary movement of strain A506 to newly-opened flowers. Honeybees are effective vectors for moving *E. amylovora* and biocontrol agents between trees, and thus might be expected to be effective for intra-tree movement as well (8,32). Although bees preferentially visit flowers and thus would be expected to be highly efficient vectors of flower colonists, they were unlikely to be the primary vectors in our studies. Honeybees prefer most other flowers over pear as nectar sources and are infrequent visitors of pear flowers when provided a choice of flowers (8). We seldom observed bees in our pear trees but frequently saw large numbers of other flying arthropods that were visiting pear flowers. As with bees, such vectors might be expected to be effective in movement of strain A506 from the stigma of flowers with a high population size to newly-opened flowers lacking populations of strain A506.

The colonization of pear flowers that were open at the time of inoculation of trees was surprisingly strongly influenced by their age at the time of inoculation with strain A506. Although flowers for which petals had expanded for less than about 5 days before inoculation all achieved relatively high eventual populations of

strain A506 ($>10^5$ to 10^6 cells per flower), the final population size of A506 progressively decreased with the time that flowers were open after this point (Figs. 2 and 5). Even though “old” flowers that had been open for 10 days or more had apparently received the same amount of initial inoculum of strain A506 when trees were sprayed as younger flowers, the population sizes on the older flowers did not subsequently increase after inoculation, resulting in maximum populations that were up to 1,000-fold lower than on the younger flowers (Fig. 5). There are several possible reasons why older flowers might not support the growth of a biocontrol agent after direct inoculation. Firstly, after petals expand on flowers, exposing the stigma, indigenous bacterial populations progressively increase in numbers with time (14,30) (Fig. 9). In 1992, total indigenous bacteria on noninoculated flowers approached about 10^5 cells per flower by approximately 2 weeks after petal expansion, whereas in 1993, nearly 10^6 cells per flower were observed in flowers of this age (Figs. 6 and 9). Similar populations in pear flowers in other western states have been reported (5,21,30). We might consider that the “carrying capacity,” the maximum bacterial population size that can be achieved on pear flowers, is approximately 10^6 cells per flower, since that is the number of bacteria most commonly seen on flowers directly inoculated with effective flower colonizing bacteria such as strain A506 (7,19) (Figs. 2, 3, and 5) and *Pantoea agglomerans* strain C9-1 (23,29). It should be noted that the relationship between the logarithm of indigenous bacterial populations on flowers at the time of direct inoculation with strain A506 and the maximum population that this strain attained in such flowers is curvilinear; colonization of flowers is largely independent of indigenous populations less than approximately 10^5 cells per flower, but decreases progressively with larger indigenous populations (Fig. 6). The later relationship is consistent with, but does not unambiguously demonstrate, a process of preemptive competitive exclusion of strain A506 by indigenous bacteria. It would be expected that competitive exclusion of strain A506 would be seen only in such heavily colonized flowers. Stockwell et al. (30) also suggested that indigenous bacterial populations in pear could be sufficiently high to interfere with the establishment of biocontrol agents, but Johnson et al. (10), in less direct analysis of flower populations, discounted such a possibility. Reduced colonization of older pear flowers can also be explained if flowers exhibited only a limited period of time during which they were capable of supporting growth of introduced bacteria. For many plant species the stigmatic surface is moist shortly after flower emergence when they are most receptive to pollination (4). The stigmatic surface obviously has abundant nutrients, principally simple sugars that support not only pollen tube germination, but also abundant bacterial growth. The growth of strain A506, like many plant colonists, is limited by low water availability that might occur in the nectary or perhaps the stigmatic surface when moisture was not available to

TABLE 1. Populations of *Erwinia amylovora* on flowers of pear trees treated with *Pseudomonas fluorescens* strain A506 at different times during the bloom period

Treatment ^y	Bacteria recovered (log cells/flower) ^z	
	29 April	3 May
Control	3.00 a	3.68 a
A506 (20% bloom)	1.51 a	1.68 b
A506 (90% bloom)	3.06 a	3.72 a
A506 (20 + 90% bloom)	2.78 a	1.93 b

^y Flowers sampled from an orchard plot near Sacramento in 1992.

^z Means followed by the same letter at a given sampling date do not differ ($P = 0.05$) according to Fisher's least significant difference test.

TABLE 2. Incidence of fire blight strikes and severity of fruit russet on pear trees treated with *Pseudomonas fluorescens* strain A506 at different phenological stages

Treatment date	Fire blight (no. of strikes/tree) ^y			Fruit russet (% surface) ^y	
	Vacaville	Sacramento		Vacaville	Sacramento
	1992	1992	1993	1992	1993
Control ^z	2.29 a	3.96 a	3.29 a	4.26 a	3.29 a
A506 (5% bloom)	0.46 b	...	...	2.57 b	...
A506 (20% bloom)	0.63 b	1.54 b	1.01 b	2.89 b	2.55 b
A506 (50% bloom)	1.18 b	...	...	2.46 b	...
A506 (90% bloom)	0.52 b	1.05 b	1.53 b	2.78 b	1.48 b
A506 (20 + 90% bloom)	0.69 b	0.79 b	0.86 b	3.16 ab	2.29 ab

^y Means followed by the same letter for a given year do not differ ($P = 0.05$) according to Fisher's least significant difference test.

^z Only streptomycin applied as to all other trees in plot.

dilute these solutes (25,26). This phenomenon might account for a relatively brief period of receptivity of bacterial colonization of crab apple stigmas by *Pantoea agglomerans* and *Pseudomonas fluorescens* strain A506 recently noted in a brief report (27). Our results suggest that, if indeed true, the period of receptivity of pear flowers under typical field conditions in California is approximately 5 to 7 days. It might be expected that both colonization potential of flowers due to presence of indigenous bacterial populations on stigmas as well as stigma receptivity might both decrease with the age of the open flowers so we cannot unambiguously assign the lessened colonization of older flowers to either factor without further study.

The microbial communities in flowers on trees inoculated with strain A506 were very different from that in noninoculated trees. Most obviously, the total bacterial population in most flowers on inoculated trees was from 10- to 100-fold higher than on uninoculated trees. Nearly all of the bacteria in flowers on treated trees, especially of flowers that opened within about 20 days of first bloom, were strain A506 (compare Figs. 2 and 9). An elevation in total bacterial populations in inoculated trees has also been observed in other studies (7,19). This phenomenon suggests that the process of colonization of newly-opened flowers is limited by the number of suitable immigrant bacteria (6,13,17). That is, pear flowers are capable of supporting the multiplication of bacteria such as strain A506 to population sizes of about 10^6 cells per flower (Figs. 2 and 3). Such large population sizes are indeed due to growth because they are attained on directly inoculated flowers that initially had only about 10^4 cells or on indirectly inoculated flowers that had as few as 10 cells (Figs. 2 and 3). Similar to Stockwell et al. (30), we found that many flowers on uninoculated trees remained free of bacteria for a week or more. Even when present on uninoculated flowers, total bacterial populations were usually less than the apparent carrying capacity of 10^6 cells per flower (Fig. 9) (30). Therefore, we conclude that most indigenous colonists of pear flowers are less epiphytically fit than effective biocontrol agents such as *Pseudomonas fluorescens* strain A506. If flowers do indeed have a limited period of receptivity for bacterial colonization, there would be a reduced likelihood that suitable colonists would immigrate to the flower within this "window of opportunity" for colonization. Limited rates of immigration of mostly unfit colonists to flowers with decreasing suitability for colonization could account for the slowly increasing indigenous population sizes observed on untreated trees (Fig. 9) (13). Together, such a scenario suggests that the timing of arrival of immigrant bacteria is critical to the success of flower colonization. Presumably, if *E. amylovora* or other epiphytically fit deleterious bacteria were to arrive at flowers before a biocontrol agent such as *Pseudomonas fluorescens* strain A506, then it could escape competitive exclusion by such an antagonist. Biological control of fire blight is thus probably optimized by introducing an antagonist such as strain A506 into trees early in the bloom period.

The large changes in the microflora of flowers inoculated with strain A506 resulted in substantial control of fire blight and fruit russet. Although a large variation in population sizes of *E. amylovora* among flowers was observed as in other studies (8,10,31), mean populations were reduced in trees treated with strain A506. While not significant, populations of *E. amylovora* as well as incidence of fire blight was consistently lower on trees with the earliest applications of strain A506 (Tables 1 and 2). As is typical of the disease in California, *E. amylovora* was not widely detected in flowers until the main bloom period was nearly over (approximately 20 days after first bloom). Thus, despite the range of phenological stages at which strain A506 was applied to trees during the main bloom period, the last inoculation of trees occurred considerably before *E. amylovora* populations became widely recoverable from late-opening flowers. Given this fact, we were surprised to have observed such a marked effect of A506 treatments on *E. amylovora* populations and disease incidence. Such effects

could be explained if the incidence of *E. amylovora*-colonized flowers increased progressively from a very few flowers inoculated from the limited amounts of inoculum typically present in a commercial orchard. That is, while only a few flowers were colonized early in the bloom period, more would become contaminated by inoculum vectored by insects as the bloom progressed with time. Given that applications of strain A506 during the main bloom resulted in a high incidence of flowers colonized to large population sizes with this strain, such flowers should have been poor recipients for vectored inoculum, and the process of production and movement of secondary inoculum in such trees should have been suppressed compared with control trees. Trees treated early during bloom (e.g., 5% bloom) might have been expected to suppress secondary movement of *E. amylovora* more than those trees treated only later in the bloom period (e.g., 90% bloom). Thus, the control of fire blight on trees treated early in the bloom period (from 70 to 80% control) was similar to that reported previously for this strain (7,19) and for other antagonistic bacteria (7,28).

Fruit russet at harvest was reduced significantly on A506-treated trees as in earlier reports (18). One cause of fruit russet is colonization by a variety of indole-3-acetic acid (IAA)-producing bacteria and the severity of fruit russet is related to the population size of these bacteria on fruit surfaces shortly after bloom (18). Because the majority of flowers in the main bloom period of pear in this study were heavily colonized by strain A506, we conclude that the reduction in fruit russet severity was due to the competitive exclusion of those IAA-producing bacteria that might otherwise have multiplied on flowers after petal expansion. Because the majority of flowers on trees became heavily colonized by strain A506 even when applied as late as 90% bloom, we saw little effect of application time of strain A506 on russet severity.

The insight into the population dynamics of strain A506 in flowers of pear trees under field conditions has several practical implications for fire blight control. Previous work had shown that application of strain A506 to trees at 20% and about 90% bloom were sufficient to achieve fire blight control (10,19). These studies lacked sufficient resolution of the temporal dynamics of flower colonization to understand how blight control had been achieved. Furthermore, variations in biological control of fire blight in earlier reports might be explained by variations in the pattern of flowering and the date of introduction of *E. amylovora* into orchards. Pears, as well as some apple cultivars, usually have a bloom period that can extend over 6 weeks or more. Nonetheless, the majority of flowers usually emerge in a main bloom period within about 2 weeks of first bloom. Although there are thousands of flowers on mature trees in this period, they usually escape infection because weather is more likely to be cool, limiting epiphytic growth and vectoring of *E. amylovora*; suitable weather conditions during the main bloom period result in infrequent but devastating epidemics. Although considerably fewer flowers emerge after the main bloom period, they are more likely to become infected with *E. amylovora*, and hence disease control efforts must continue even after most blossoms have appeared on trees. Our results indicate that antagonists such as strain A506 can be effectively established in a majority of flowers from one or two early season applications. Because secondary movement of strain A506 resulted in heavily colonized flowers that emerged for a week or more after inoculation of a tree (Fig. 7), the majority of main bloom flowers can be protected from colonization by *E. amylovora* by one or two inoculations of an antagonist. In fact, the colonization of flowers by strain A506 and fire blight control was similar irrespective of whether A506 was applied at both 20 and 90% bloom or only once at either 5 or 20% bloom (Fig. 2, Tables 1 and 2). In contrast, late-opening flowers were poorly colonized on trees treated only early in the bloom period (Figs. 2 and 7). This may have been due to either a lower number of flowers that remain in a treated tree to serve as a source of readily vectored

inoculum or perhaps to a reduced period of receptivity of flowers to epiphytic colonization that is associated with warmer weather (27). Together, these results suggest that optimum fire blight control can be obtained by applying antagonist strains only once or twice during the brief main bloom period followed by regular applications at 7- to 10-day intervals to achieve inoculation of delayed blossoms.

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