Interactions Between the Biological Control Agent *Pseudomonas fluorescens* A506 and *Erwinia amylovora* in Pear Blossoms

M. Wilson and S. E. Lindow

Post-doctoral research assistant and professor, Department of Plant Pathology, 147 Hilgard Hall, University of California, Berkeley 94720.
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ABSTRACT

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In the greenhouse, *Pseudomonas fluorescens* strain A506 effectively colonized the pistils of pear blossoms. *P. fluorescens* A506 significantly reduced colonization of pear pistils by *Erwinia amylovora* when the biological control agent was inoculated 72 h in advance of the pathogen, but not when it was coinoculated with *E. amylovora*. *P. fluorescens* A506 probably excluded *E. amylovora* by preemptively utilizing a growth-limiting resource required by the pathogen. A506 also colonized the nectaries of pear blossoms, in which it maintained high populations for 60–72 h. A506 significantly reduced colonization of pear nectaries by *E. amylovora* when it was inoculated 72 h in advance of the pathogen. Although the inhibition of *E. amylovora* on pear nectaries may involve preemptive utilization of a growth-limiting resource, other factors, such as induced cessation of nectar secretion or accumulation of a host toxin, also may be involved. *P. fluorescens* A506 probably prevents fire blight infection of pear in the field by preventing epiphytic build-up of pathogen inoculum on pistils and by inhibiting the growth of inoculum deposited on nectaries.

Additional keywords: competition.

Fire blight, caused by the bacterium *Erwinia amylovora*, is a disease of major economic importance in pear (*Pyrus communis* L.) growing regions of the western United States. Although the disease can be partially controlled through the use of appropriate cultural measures and repeated applications of antibiotics, such as streptomycin and Terramycin or copper compounds, bactericide resistance is becoming more widespread and may limit control (4,22,25). As a result, biological control of fire blight using epiphytic bacteria is now considered a promising alternative to chemical control. Although *Erwinia herbicola* remains the most frequently studied potential biological control agent of fire blight (1–3,28,29,42), this species is not under development for use on a commercial scale. *Pseudomonas fluorescens* strain A506, however, has been used extensively and effectively in controlling fire blight of pear in California (17,21) and is being tested for control of fire blight of pear in Oregon (13,14,33,34), Washington (23), and Utah (37) and of apple in California, Oregon (13), and Montana. An experimental use permit (EUP 64004-EUP-1) for the large-scale testing of *P. fluorescens* A506 for biological control of fire blight and frost injury of pear and other crops was obtained in 1990.

It is important that the mechanisms of action of biological control agents be understood, not only to identify procedures to enhance their efficacy, but also to improve screening procedures for new strains. To date, the selection of biological control agents for fire blight has been based on in vitro characteristics presumed to be involved in the control mechanism (1,2), disease suppression in the immature pear-fruit assay (42,43), or empirical field data (3,17), not on an understanding of the mechanisms of action of effective agents. The possible mechanisms involved in biological control of fire blight with *E. herbicola* have been studied extensively. Although bacteriocins no longer are considered to play a role in control (1,2), *E. herbicola* also produces several antibiotics (5,10,11), and antibiotic production on certain media has been correlated with control of fire blight in the orchard (47,48). The observation that antibiotic-deficient mutants of *E. herbicola* retain some ability to suppress *E. amylovora* (39,40) suggests that factors other than antibiosis also may be involved. A scanning electron microscopy (SEM) study of the interaction between *E. herbicola* and *E. amylovora* on the stigma of apple (6) suggested that these two species occupy the same sites and that prior colonization of the stigma by the biological control agent prevented the pathogen from gaining effective access to these sites. Rundle and Beer (31) reported that inoculation of the stigma of apple with *E. herbicola* 24 h in advance of inoculation with *E. amylovora* prevented subsequent multiplication by *E. amylovora*. Wilson et al (44) suggested that *E. herbicola* preemptively, or competitively, excludes *E. amylovora* from intercellular spaces between stigmatic papillae by utilizing a growth-limiting resource required by both species. The competitive superiority of *E. herbicola* in this interaction may be the result of antibiosis (44).

In contrast to *E. herbicola*, *P. fluorescens* strain A506 did not exhibit antibiosis against *E. amylovora* on any of several media tested (17). This study, therefore, addressed the hypothesis that *P. fluorescens* A506 controls fire blight through some mechanism other than antibiosis (i.e., competitive or preemptive exclusion of the pathogen).

MATERIALS AND METHODS

Source of bacterial strains. *P. fluorescens* strain A506 was isolated from asymptomatic leaves of pear trees from a commercial orchard near Healdsburg, CA. A spontaneous mutant of *P. fluorescens* A506, resistant to 100 μg per milliliter of rifampicin, was selected; this mutant colonized plants as effectively as the wild-type parental strain (M. Wilson, *unpublished data*). *E. amylovora* strain Ea8 was isolated from an infected pear tree near Sacramento, CA. (This strain is not related to the avirulent strain E8 used by C. G. Suhayda and R. N. Goodman [35]). Through inoculation into immature pear fruit and through spraying onto newly opened pear blossom (M. Wilson, *unpublished data*), a spontaneous mutant of *E. amylovora* Ea8, designated Ea8R, resistant to 100 μg per milliliter of rifampicin, was determined to be as virulent as the wild-type parental strain. A spontaneous mutant of *E. amylovora* Ea8R, designated Ea8RN, resistant to 50 μg per milliliter of nalidixic acid, also was determined to be as virulent as the wild-type parental strain (M. Wilson, *unpublished data*).

Population studies. Pear trees (cv. Comice) about 2-m tall were grown in containers on dwarfing quince rootstock and were

overwintered outdoors at Berkeley, CA. The trees were placed in a cold room held at 6 C during February. Trees were removed from the cold room as required and placed in a greenhouse (average minimum and maximum temperatures were 18 and 25 C, respectively; average minimum and maximum relative humidities [RH] were 21 and 45%, respectively) to stimulate bloom. Trees were inoculated at about 75% bloom. Freshly opened blossoms (those with pink, undehisced anthers) were tagged for identification. Inoculum was prepared by suspending bacteria from plates of King's medium B (KB), which were cultured for 18 h at 25 C, in sterile phosphate buffer (0.01 M, pH 7.0). The suspension was adjusted turbidimetrically to the appropriate cell concentration. Bacterial suspensions were sprayed until run-off on 50 or more tagged pear blossoms.

Blossoms were inoculated either with *P. fluorescens* A506 alone, with *E. amylovora* alone, or with *P. fluorescens* and *E. amylovora* in combination. Some blossoms remained uninoculated for control purposes. When blossoms were inoculated with both the biological control agent and the pathogen, the two strains were inoculated either simultaneously at the same concentration of 10^7 cells per milliliter (coinoculation) or the biological control agent, at 10^8 cells per milliliter, was inoculated 72 h in advance of the pathogen, at 10^7 cells per milliliter (preinoculation). Each treatment was applied to three replicate trees. Branches bearing inoculated blossoms either remained uncovered at the ambient RH or were covered with plastic bags (high RH). Coinoculation experiments were performed twice at high RH. Preinoculation experiments were performed once at high RH and three times at ambient RH.

After inoculation, 10 flowers from each treatment were sampled at either 12 or 24 h intervals. The pistil was dissected from each blossom and homogenized in 1.2 ml of sterile phosphate buffer in a plastic microfuge tube, using a plastic pestle. The rest of the blossom (subsequently referred to as the nectary) was homogenized in 5.0 ml of sterile phosphate buffer, using a ceramic mortar and pestle. The homogenates were serially diluted, and the dilutions were plated on KB containing 100 μg per milliliter of rifampicin (KBR). All enumeration media also were amended with 50 μg per milliliter each of cycloheximide and benomyl to inhibit fungal growth. Colony counts were made after 48–72 h incubation at 25 C. Colonies of A506 and Ea8R were distinguished on the basis of colony morphology and/or fluorescence of A506 under ultraviolet illumination. In the experiment using mixtures of Ea8RN and A506, appropriate dilutions of macerates were plated on KBR to enumerate A506 and were plated on KB containing 50 μg per milliliter of nalidixic acid (KBN) to enumerate Ea8RN. Uninoculated control blossoms were homogenized in sterile phosphate buffer, were serially diluted, and were plated on KBR and KBN to document the absence of naturally occurring rifampicin-resistant and nalidixic acid-resistant microorganisms, respectively.

Disease assessment. The effectiveness of biological control achieved by *P. fluorescens* A506 after coinoculation and preinoculation was determined. Four replicate trees were inoculated with the pathogen alone (10^7 cells per milliliter); with the biological control agent (10^8 cells per milliliter) and the pathogen (10^7 cells per milliliter) simultaneously; and with the biological control agent (10^8 cells per milliliter) 72 h in advance of the pathogen (10^7 cells per milliliter). The proportion of infected blossoms was assessed 6 days after pathogen inoculation.

Statistical methods. Population means were derived from $\log_{10}$-transformed population estimates from 10 individual blossoms. The statistical significance of differences was determined using the Student's t test. Population doubling-times were estimated from the slope of the regression of $\log_2$ (population size) against time in the most rapid phase of population growth.

RESULTS

Coinoculation studies. In coinoculation experiments in which *P. fluorescens* A506 and *E. amylovora* Ea8R were simultaneously inoculated into blossoms, the biological control agent had no significant effect on the pathogen population size on either pistils or nectaries at any time after inoculation (Fig. 1). The population of A506 on pistils increased to a peak size of 1.2×10^6 colony-forming units (cfu) per pistil (Fig. 1A). Although the population size of A506 on pistils was reduced significantly ($P = 0.01$) by the presence of Ea8R (Fig. 1A), the population size of Ea8R was not reduced significantly ($P > 0.05$) by the presence of A506 (Fig. 1B). The population size of A506 on nectaries at first was enhanced significantly ($P = 0.01$) by the presence of Ea8R but later was inhibited (Fig. 1C). The population size of Ea8R, however, was not affected significantly ($P > 0.05$) by the presence of A506 (Fig. 1D) when coinoculated. Similar results were obtained in a replicate experiment (data not shown).

Preinoculation studies. When *P. fluorescens* A506 was inoculated into blossoms 72 h in advance of *E. amylovora* Ea8R and the blossoms were incubated either at high or ambient RH, the biological control agent significantly reduced the pathogen population size on both pistils and nectaries in all experiments. When experiments were performed at high RH, the population size of A506 on pistils increased, with an estimated doubling-time of 12.2 h, to a peak size of 1.7×10^6 cfu per pistil (Fig. 2A). Compared to controls, the population size of A506 on pistils was reduced slightly ($P > 0.05$) by the presence of Ea8R (Fig. 2A). In contrast, the final population size of Ea8R was reduced by over 1,900-fold (Fig. 2B) after inoculation of blossoms that supported an A506 population of $\sim 5.5 \times 10^5$ cfu per pistil. The population size of A506 on nectaries was maintained for ~72 h but then began to decrease (Fig. 2C). The population size of A506 on nectaries 120 h after inoculation was enhanced significantly ($P = 0.01$) by the presence of Ea8R (Fig. 2C). The final mean population size of Ea8R on nectaries was reduced significantly ($P = 0.001$), by 85-fold (Fig. 2D), after inoculation of blossoms that supported an A506 population of $\sim 2.4 \times 10^6$ cfu per nectary.

Results of preinoculation experiments performed at ambient RH (21–45%), considered to be closer to field conditions in California, were very similar to experiments conducted at high RH. In a typical preinoculation experiment, the population of A506 on pistils increased, with an estimated doubling-time of 12.7 h, to a peak size of 2.1×10^6 cfu per pistil (Fig. 3A). The population size of A506 on pistils was not reduced significantly ($P > 0.05$) by the presence of Ea8R (Fig. 3A). The amount of Ea8R inoculum initially detectable on pistils, however, was significantly ($P = 0.02$) lower than in controls, and the final mean population size of Ea8R on pistils was reduced significantly ($P = 0.05$), by 36-fold (Fig. 3B), after inoculation of blossoms that supported an A506 population of $\sim 9.7 \times 10^5$ cfu per pistil. The population size of A506 on nectaries was maintained with little change for ~60 h, but it then decreased (Fig. 3C). The population size of A506 was not affected significantly ($P > 0.05$) by the presence of Ea8R (Fig. 3C). The amount of Ea8R inoculum initially detectable on nectaries, however, was significantly ($P > 0.05$) lower than in controls, and its population size continued to decrease for at least 24 h before beginning to increase (Fig. 3D). The population size of Ea8R at 120 h after inoculation was reduced significantly ($P = 0.001$), by 194-fold (Fig. 3D), after inoculation of blossoms that supported an A506 population of $\sim 1.3 \times 10^6$ cfu per nectary.

In other preinoculation experiments with A506 and Ea8R and with A506 and Ea8RN, both conducted at ambient RH, the results were very similar to those described above, indicating that the interactions are reproducible. The data obtained with A506 and Ea8RN, in which the two strains were enumerated on different media, and with A506 and Ea8R, in which the two strains were enumerated on the same medium and were distinguished by colony morphology, were similar. This observation, together with the fact that A506 does not exhibit antibiosis against Ea8R on KB, suggests that the enumeration of both strains on the same medium was accurate.

The main difference observed between experiments conducted at ambient RH and at high RH was that the population of Ea8R on nectaries in the presence of A506 decreased by 0.5–1.0 $\log_{10}$

unit before increasing under conditions of ambient RH.

Disease assessment. The efficacy of control achieved by *P. fluorescens* A506 was determined after coinoculation and preinoculation. The percentage of blossom infection observed when the biological control agent was inoculated 72 h in advance of the pathogen (17%) was significantly less than the percentage of infection observed when the biological control agent and pathogen were coinoculated (50%), which in turn was significantly less than the percentage observed on the pathogen-only control (71%). While not quantified, the incidence of infection in blossoms used for population studies in which *P. fluorescens* and *E. amylovora* were coinoculated was less than in the pathogen-only control but was still far more than the incidence observed when *P. fluorescens* was preinoculated 72 h in advance of *E. amylovora*.

DISCUSSION

P. fluorescens A506 was a very effective colonist of pear pistils in these experiments, and it also has been shown to effectively colonize pistils in the field (33,34). Under the environmental conditions of these greenhouse experiments, A506 multiplied with a mean estimated doubling-time of 13.7 h to a peak population of 1.9×10^6 cfu per pistil (SE 1.0×10^5 cfu per pistil) within 100–120 h after inoculation. The A506 population increased at a similar rate and reached a similar size under both high and ambient RH conditions, a fact that may be attributable to a higher RH at the stigmatic surface even when the ambient RH is low (36). The population sizes achieved by A506 on pistils were comparable to those achieved by *E. herbicola* on pistils of pear

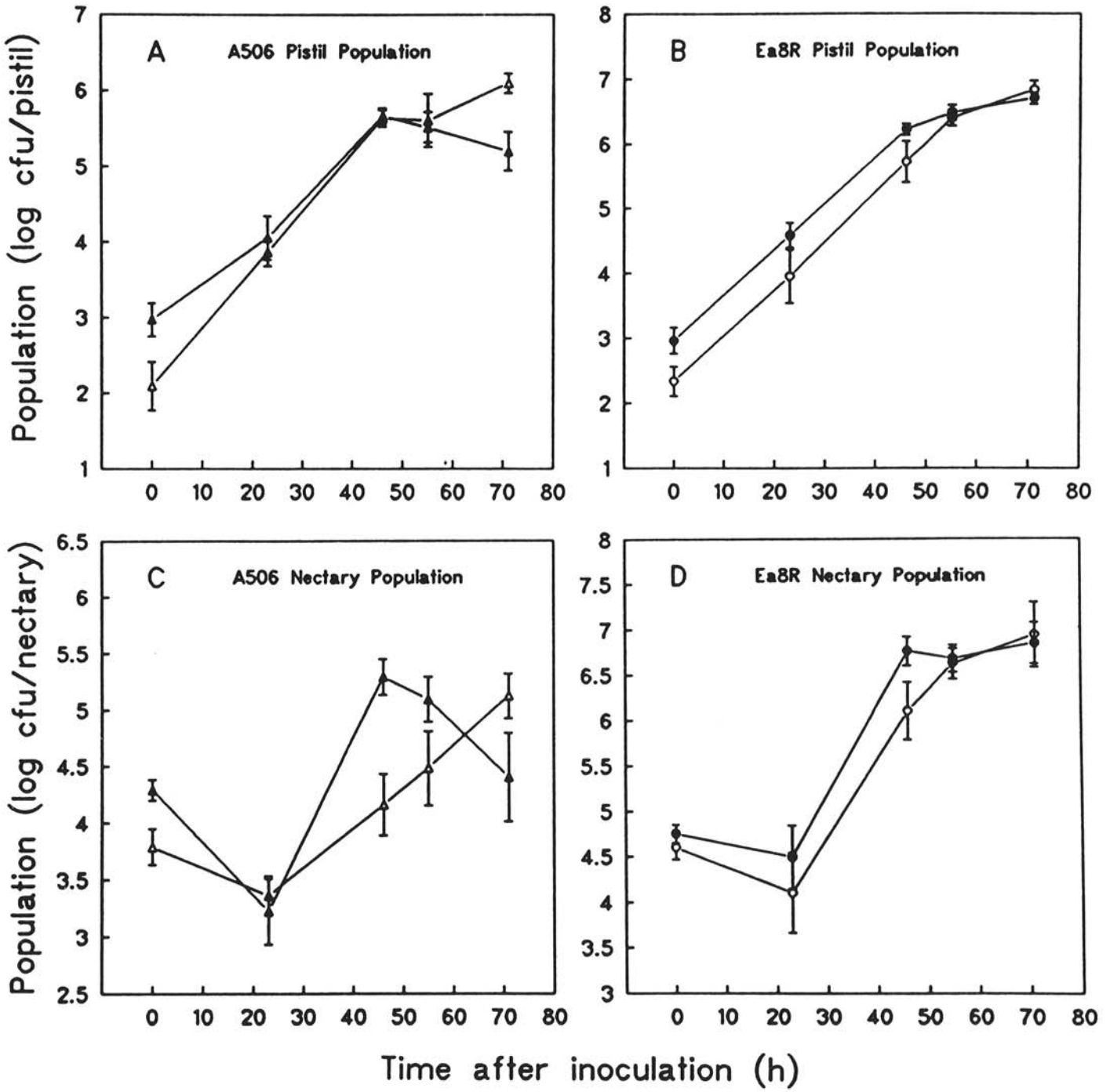

Fig. 1. Population dynamics of the biological control agent *Pseudomonas fluorescens* A506 and the pathogen *Erwinia amylovora* Ea8R, alone and in combination, on pear blossoms. Blossoms were coinoculated with A506 and Ea8R, both at 10^7 cells per milliliter, and were incubated at high-relative humidity. This experiment was performed twice. **A** and **C,** A506 population on pistils and nectaries, respectively, when inoculated alone (open triangles) and coinoculated with Ea8R (filled triangles). **B** and **D,** Ea8R population on pistils and nectaries, respectively, when inoculated alone (open circles) and when coinoculated with A506 (filled circles). Bars represent one standard error of the mean.

(M. Wilson, *unpublished data*) and hawthorn (44). These data support the hypothesis of Wilson et al (44) that the stigmatic surface exhibits a finite carrying capacity for an epiphytic bacterial population. In the case of *P. fluorescens* A506 on the pistil of pear (cv. Comice), this carrying capacity was $\sim 2 \times 10^6$ cfu per pistil. To explain such a carrying capacity, Wilson et al (44) hypothesized that an epiphytic bacterial population multiplying in the intercellular spaces between the stigmatic papillae is limited by the resources available for growth.

Although preinoculation of blossoms with *P. fluorescens* A506 before inoculation with *E. amylovora* Ea8R greatly reduced subsequent Ea8R population growth, coinoculation of A506 with Ea8R had no significant effect on development of the pathogen population. In the presence of low numbers of A506, Ea8R continued to multiply at the same rate and continued to achieve the same final population size on pistils. Although the pathogen and biological control agent multiplied at a similar rate on pistils in this experiment, A506 apparently was unable to competitively acquire a sufficiently high proportion of the growth-limiting resources to prevent *E. amylovora* from reaching the population levels necessary to invade stigmatic tissue (41). In contrast, coinoculation of *E. herbicola* with *E. amylovora* significantly reduced the pathogen-growth rate and final population size (44). Production of antibiotics by *E. herbicola*, it was hypothesized, reduced the growth rate of *E. amylovora*, enabling *E. herbicola* to competitively exclude *E. amylovora* by acquiring a greater

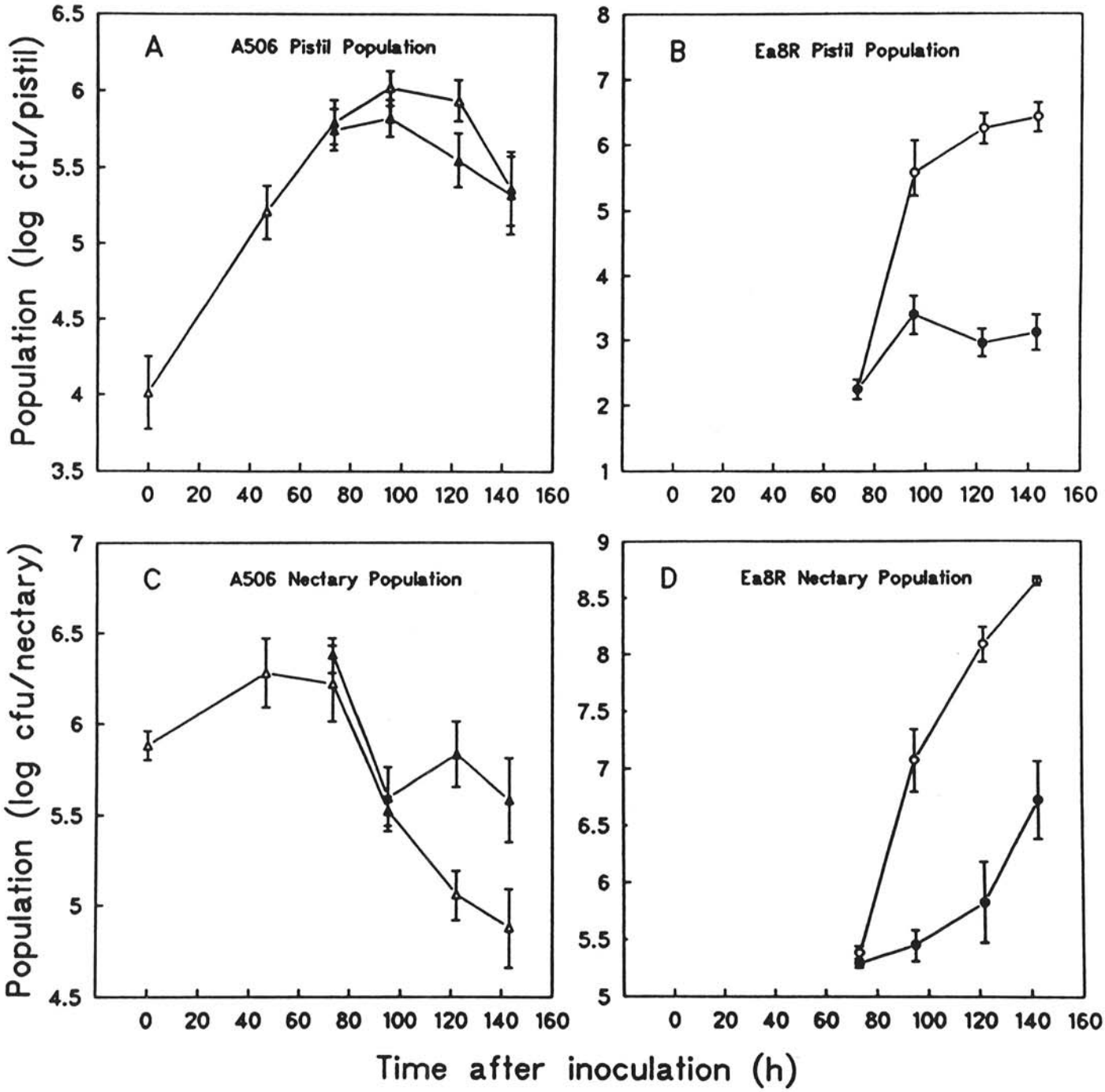

Fig. 2. Population dynamics of the biological control agent *Pseudomonas fluorescens* A506 and the pathogen *Erwinia amylovora* Ea8R, alone and in combination, on pear blossoms. Blossoms were preinoculated with A506 (10^8 cells per milliliter) 72 h in advance of Ea8R (10^7 cells per milliliter) and incubated at high-relative humidity. This experiment was performed once. **A** and **C**, A506 population on pistils and nectaries, respectively, when inoculated alone (open triangles) and when inoculated with Ea8R (filled triangles). **B** and **D**, Ea8R population on pistils and nectaries, respectively, when inoculated alone (open circles) and when inoculated with A506 (filled circles). Bars represent one standard error of the mean.

portion of the mutually required, growth-limiting resources (44).

Preinoculation of the biological control agent, *P. fluorescens* A506, 72 h in advance of the pathogen, *E. amylovora* Ea8R, significantly reduced pathogen colonization of pistils in all experiments. The population size of *E. amylovora* only increased 10- to 30-fold and then stopped. Although this would be consistent with some form of habitat modification mediated by A506 or accumulation of a bacteriostatic antibiotic, the absence of any effect when A506 and Ea8R were coinoculated strongly suggests that A506 exhausted resources required for the growth of *E. amylovora* Ea8R. Preemptive utilization of a mutually required, growth-limiting resource also played a role in the antagonism observed between *E. herbicola* and *E. amylovora* (44) and in the preemptive exclusion of Ice^+ *Pseudomonas syringae* by isogenic Ice^- *P. syringae* on leaf surfaces (16,18–20,45).

P. fluorescens A506 did not colonize pear nectaries as effectively as pistils. The A506 population was maintained at inoculation levels for only 60–72 h after inoculation. The drop in nectary populations of A506 after 72 h did not appear to be attributable to blossom age per se, because inoculation of 5-day-old blossoms and incubation at high RH resulted in A506 population sizes similar to those observed after inoculation of recently opened blossoms (M. Wilson, *unpublished data*). The population decline may have resulted from depletion of nutrients, accumulation of toxic metabolites, or some other form of habitat modification.

P. fluorescens A506 significantly reduced the population size

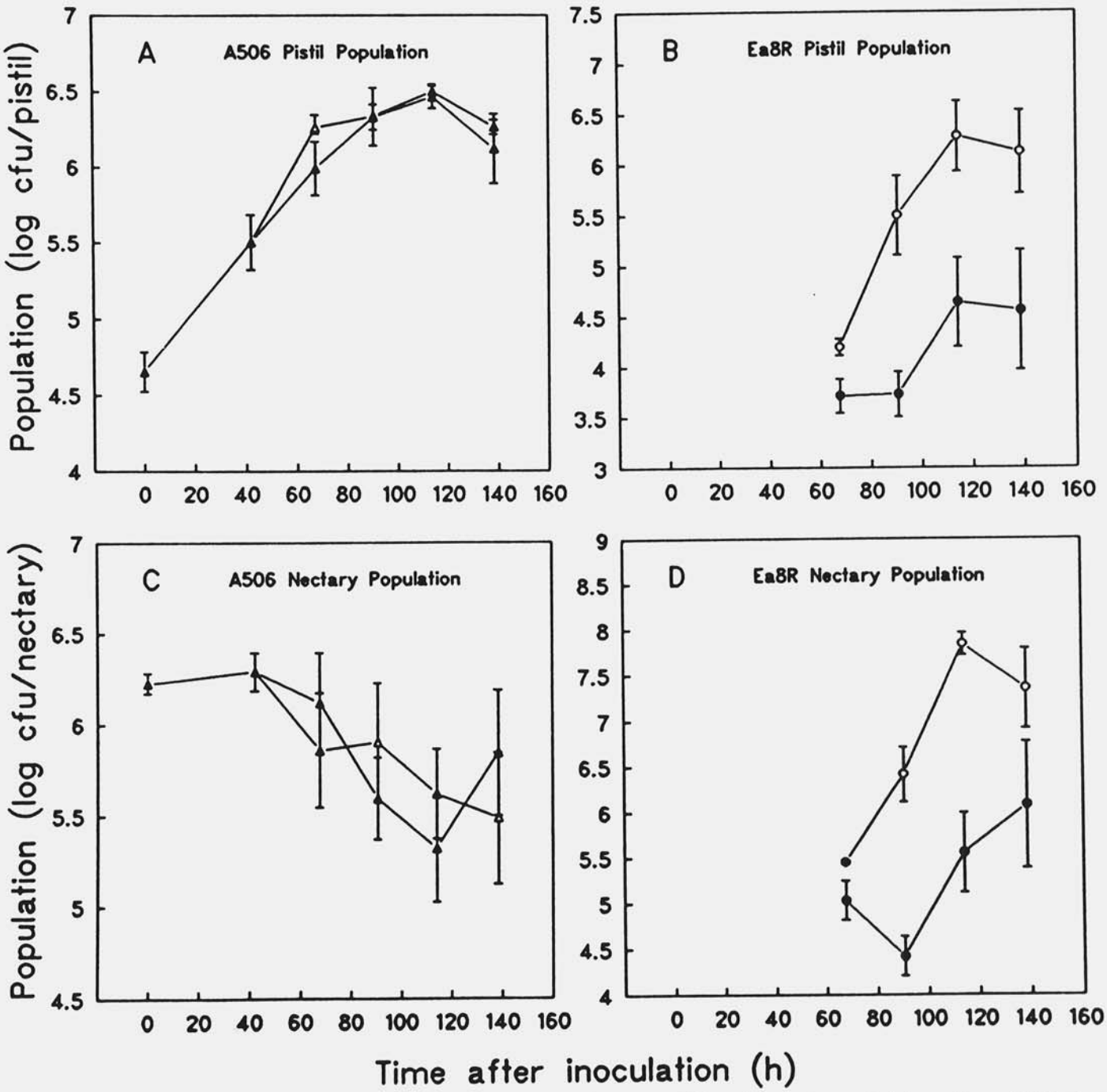

Fig. 3. Population dynamics of the biological control agent *Pseudomonas fluorescens* A506 and the pathogen *Erwinia amylovora* Ea8R, alone and in combination, on pear blossoms. Blossoms were preinoculated with A506 (10^8 cells per milliliter) 72 h in advance of Ea8R (10^7 cells per milliliter) and incubated at ambient relative humidity. This experiment was performed twice and was repeated for strain Ea8RN. **A** and **C**, A506 population on pistils and nectaries, respectively, when inoculated alone (open triangles) and when inoculated with Ea8R (filled triangles). **B** and **D**, Ea8R population on pistils and nectaries, respectively, when inoculated alone (open circles) and when inoculated with A506 (filled circles). Bars represent one standard error of the mean.

of *E. amylovora* Ea8R on the nectary in all preinoculation experiments. In contrast to interactions on the stigma, however, the mechanism of action on the nectary probably involves factors in addition to preemptive utilization of nutritional resources. We consistently observed that nectar secretion was considerably reduced in pear blossoms ~72 h after inoculation with *P. fluorescens* A506 compared to blossoms not inoculated with A506, in which nectar was abundant for, at least, a further 48 h. The effect of prior colonization of nectaries by A506 on Ea8R inoculum depended on the RH after pathogen inoculation. At ambient RH levels, the detectable population of Ea8R decreased before increasing, while at high RH levels, the *E. amylovora* population started to increase immediately after inoculation. We speculated that the reduced secretion of nectar, caused by the presence of A506, led to the death of Ea8R inoculum under ambient RH conditions, because the remaining nectar probably reached osmotic concentrations lethal to *E. amylovora* cells (12). *E. amylovora* exhibited a similar death phase or lag prior to multiplication on the hawthorn nectary at ambient RH levels (46).

In addition to the reduced nectar secretion, we also noticed that the nectarial surface of blossoms colonized by A506 exhibited a purple/red pigmentation, which increased in intensity from about 72 h on. This effect could result from the accumulation of an inhibitory compound in the host tissues that may have contributed to the lag in population growth observed even at high RH levels. Although the oxidative (27) and hydrolytic (7,8,32) catabolism of the glycoside arbutin, which is found in pear tissues, including the pear nectary (8,9), has been correlated with antibiotic activity against *E. amylovora*, the pear nectary ordinarily exhibits only low levels of antibiosis (9,15). It is possible that *P. fluorescens* A506 hydrolyzed or oxidized arbutin or another phenolic compound to a toxic product, which then contributed to the inhibition of *E. amylovora*.

Epiphytic populations of *E. amylovora* have been reported in asymptomatic pear blossoms under California growing conditions (24,36,38). These populations appear to be limited to the pistil (36). Thomson (36) proposed that epidemics of fire blight occur when epiphytic populations on pistils are transferred to the nectary in high numbers by rain or dew. Preemptive exclusion of *E. amylovora* from the pear pistil appears to be a major effect of *P. fluorescens* A506 as a biological control agent. In addition, colonization of nectaries by *P. fluorescens* A506 apparently delays pathogen multiplication on the nectaries at high RH and can cause death of inoculum at ambient RH. A reduction in the viable inoculum would reduce the probability of successful infection (30). Moreover, delaying the development of large pathogen populations may further reduce the likelihood of infection because the susceptibility of blossoms to infection declines as they age (26).

P. fluorescens strain A506 apparently effects the biological control of fire blight through more than one mechanism. More studies are required to determine which of these mechanisms is most important in reducing infection of blossoms by *E. amylovora* under field conditions. This knowledge may facilitate the identification of bacterial strains with biological control potential superior to, or complementary to, *P. fluorescens* A506.

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