Interactions Between Erwinia herbicola and E. amylovora on the Stigma of Hawthorn Blossoms

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ABSTRACT


Erwinia herbicola HL9N13 is an effective biological control agent of fire blight disease of hawthorn. The interactions between E. herbicola and E. amylovora on the stigma of hawthorn blossoms were examined to assess the possible roles of competition and antibiotic in the mechanism of biological control of blight. Preemptive and competitive colonization of the stigma by the biological control agent reduced the pathogen growth rate and final population. Scanning electron microscopy indicated that E. herbicola colonized the same sites on the stigmatic surface as E. amylovora in its epiphytic phase of development. The competitive advantage exhibited by E. herbicola may have resulted from antibiosis. Although E. herbicola HL9N13 produced a broad-spectrum antibiotic on potato-dextrose agar, it was not determined whether this antibiotic was produced in planta. The results suggest that stigma colonization by E. amylovora is prevented by preemptive or competitive occupation of colonization sites by E. herbicola and by the reduction in availability of a resource required by the pathogen for growth at these sites.

There have been several reports of biological control of fire blight, caused by Erwinia amylovora, using E. herbicola (5,10,19,28); however, the mechanisms of disease suppression remain uncertain. Recent investigations into mechanisms have focused primarily on the production of inhibitory compounds by E. herbicola. Several E. herbicola strains produce bacteriocin-like substances in vitro, and it was thought that bacteriocins played a role in biological control (6,9). It is now apparent that there is no correlation between in vitro bacteriocin production and the suppression of fire blight (2-4). There is, however, a correlation between antibiotic production on glucose-asparagine medium and control of fire blight in the orchard (31,32). In vitro E. herbicola produces at least two types of antibiotic with antibacterial activity (11,12,32), but the occurrence of nonantibiotic-producing E. herbicola strains that effectively suppress disease (7,28) and the use of antibiotic-resistant mutants of E. amylovora (13), or Tn5-derived antibiotic-deficient mutants of E. herbicola (23,24), all suggest that, in addition to antibiotics, other mechanisms may be involved in disease suppression in planta.

In the study of mechanisms of disease suppression, relatively little attention has been paid to competition between the pathogen and the biological control agent in planta. In the epidemiology of fire blight, the stigmatic surface plays an important role as an infection site for E. amylovora in apple blossoms (20,21) and as a site of inoculum buildup in pear (22) and hawthorn (27). Population studies by Rundel and Beer (21) indicated that in apple blossom the stigma was the site of interaction between E. herbicola and E. amylovora. Hattingh et al (8) showed by means of scanning electron microscopy (SEM) that E. herbicola Eh252 colonized the same sites as E. amylovora Ea273 on the stigmatic surface of apple and suggested that prior colonization of the stigma by the antagonist would prevent the pathogen from entering these sites.

This paper reports the interactions between E. amylovora Ea519Rif, the pathogen, and E. herbicola HL9N13, an effective biological control agent of fire blight (28), on the stigma of the pistils of hawthorn (Crataegus monogyna Jacq.). Population data are combined with results from SEM to give a comprehensive evaluation of the interaction between the pathogen and biological control agent.

MATERIALS AND METHODS

Source of E. herbicola and E. amylovora strains. E. herbicola HL9 was isolated from symptomless hawthorn leaves and was shown to be an effective control agent of both blossom-blight and shoot-blight phases of fire blight in hawthorn (28). A spontaneous mutant of E. herbicola HL9 resistant to 50 μg/ml of nalidixic acid, designated E. herbicola HL9N13, was determined to be as effective in biological control of blossom blight of hawthorn as the wild-type, parental strain (26). E. amylovora Ea519 was isolated from an infected hawthorn shoot in Kent, UK, in 1986. A spontaneous mutant of E. amylovora Ea519 resistant to 100 μg/ml of rifampin, designated Ea519Rif, was determined to be as virulent as the wild-type, parental strain (29). Bacterial strains were maintained in a freeze-dried state in the culture collection at the University of Manchester, Manchester, UK.

Population studies. Flowering branches of hawthorn were collected from an area where fire blight had not been observed. In the laboratory the branches were placed in bottles of distilled water. Branches were held in a growth chamber at high relative humidity (85-90%), a day length of 17 h, and a day/night temperature regime of 20/10 C. Buds and blossoms with senescent stigmas were removed before inoculation, leaving only freshly opened blossoms with noncollected stigmas.

In the population studies, blossoms were inoculated with a biological control agent either 24 h in advance of the pathogen at a tenfold numerical advantage (preinoculation) to reproduce the conditions under which successful control of blossom blight was observed (28), or at the same time and concentration as the pathogen (coinoculation). Inoculum was prepared by suspending bacteria from a yeast-peptone-sucrose-agar (YPSA) slant cultured for 18 h at 25 C in sterile citrate-phosphate (C-P) buffer (0.05 M, pH 6.5). The suspension was adjusted turbidimetrically to the appropriate cell concentration. In the preinoculation experiment, a microapplicator (Burkard Scientific, Rickmansworth, UK) was used to apply 0.5 μl of the following suspensions to the stigma of the pistils of 50 hawthorn blossoms (10 blossoms on each of five replicate branches): E. herbicola HL9N13 (10⁷ cells per milliliter), C-P buffer followed by E. amylovora Ea519Rif.
(10³ cells per milliliter) 24 h later, or *E. herbicola* HL9N13 (10³ cells per milliliter) followed *E. amylovora* Ea519Rif (10³ cells per milliliter) 24 h later. Flowering branches were arranged randomly in the growth chamber for incubation. This experiment was performed twice. In the co-inoculation experiment, a microapplicator was used to apply 0.5 μl of the following suspensions to the stigmas of the pistils of 50 hawthorn blossoms (10 blossoms on each of five replicate branches): *E. herbicola* HL9N13 alone (10³ cells per milliliter), *E. amylovora* Ea519Rif alone (10³ cells per milliliter), or *E. herbicola* HL9N13 plus *E. amylovora* Ea519Rif (each at 10³ cells per milliliter). Flowering branches were arranged randomly in the growth chamber for incubation. This experiment was performed once.

Five flowers, one from each replicate branch, were sampled from each treatment at approximately 12-h intervals up to 84 h after inoculation. Viable counts were obtained by homogenizing each flower individually in 5 ml of sterile C-P buffer. The homogenates were serially diluted, and the dilutions were plated on either YPSA containing 50 μg/ml each of nalidixic acid and cycloheximide or YPSA containing 100 μg/ml of rifampicin and 50 μg/ml of cycloheximide to detect *E. herbicola* HL9N13 and *E. amylovora* Ea519Rif, respectively. Colony counts were made after 72-h incubation at 25 C. Uninoculated control blossoms were homogenized in sterile C-P buffer, serially diluted, and plated on YPSA containing, in addition to 50 μg/ml of cycloheximide, either 100 μg/ml of rifampicin or 50 μg/ml of nalidixic acid to determine the absence of naturally occurring rifampinc-resistant and nalidixic acid-resistant microorganisms, respectively.

Population means were derived from log₁₀-transformed populations of five replicate blossoms. Statistically significant differences were determined using the Student's t test. Population doubling times were estimated from the slope of the regression of logₐ (population size) against time in the phase of most rapid population growth.

**Microscopy.** Five flowers inoculated only with *E. herbicola* HL9N13 from the co-inoculation experiment were sampled at 24 and 48 h after inoculation. The pistils of the five blossoms were prepared by critical-point drying for SEM using the methods of Wilson et al (29).

**In vitro antibiotic production by *E. herbicola* HL9N13.** Bacteria from a potato-dextrose agar (PDA) slope of *E. herbicola* HL9N13, cultured for 18 h at 25 C, were suspended in C-P buffer and adjusted to 10⁶ cells per milliliter. Aliquots of 10 μl of the suspension were spotted onto the center of surface-dry PDA plates. The plates were incubated for 72 h at 25 C. Suspensions of the test organisms, *E. amylovora* Ea519Rif, *Pseudomonas syringae*, *Escherichia coli*, *Serratia marcescens*, *Proteus vulgaris*, *Bacillus subtilis*, *B. cereus*, and *Staphylococcus aureus*, at a concentration of 10⁶ cells per milliliter in C-P buffer, were atomized over the surface of the plates. Plates were dried for 5 min in a laminar flow unit and then incubated for 48 h at 25 C, after which time they were examined for the presence or absence of inhibition zones.

**RESULTS**

**Population studies.** The biological control agent *E. herbicola* HL9N13 effectively colonized the stigmatic surface of the pistil of the hawthorn blossom in all three experiments. The population increased rapidly in the first 36 h, with a mean estimated doubling time of 3.9 h (standard error ±0.2 h), and reached a mean final population of 1.1x10⁸ cfu per blossom.

In the first preinoculation experiment (data not shown), the population of *E. herbicola* HL9N13 was not significantly reduced in the presence of *E. amylovora* Ea519Rif. Although the population of *E. amylovora* Ea519Rif was reduced in the presence of *E. herbicola* HL9N13, the reductions were not significant. The doubling time of *E. amylovora* Ea519Rif was longer in the presence of *E. herbicola* HL9N13 than in its absence (15.7 and 4.8 h, respectively).

In the second preinoculation experiment (Fig. 1), the population of *E. herbicola* HL9N13 was not significantly reduced in the presence of *E. amylovora* Ea519Rif (Fig. 1A). The population of *E. amylovora* Ea519Rif was significantly reduced in the

![Fig. 1](image.jpg)

**Fig. 1.** Inoculation of *Erwinia herbicola* HL9N13 on the stigma of the pistil of hawthorn blossom 24 h before *E. amylovora* Ea519Rif. A. Population of *E. herbicola* HL9N13 when inoculated alone (■) and when inoculated 24 h before *E. amylovora* Ea519Rif (▲). B. Population of *E. amylovora* Ea519Rif when inoculated alone (●) and when inoculated 24 h after *E. herbicola* HL9N13 (▲). Bars represent one standard error of the mean.
presence of *E. herbicola* HL9N13 at all time points from 48 h after inoculation onward (*P* = 0.001 to 0.02) (Fig. 1B). The doubling time of *E. amylovora* Ea519Rif was longer in the presence of *E. herbicola* HL9N13 than in its absence (6.2 and 3.8 h, respectively).

In the co-inoculation experiment (Fig. 2), the population of *E. herbicola* HL9N13 was not significantly reduced in the presence of *E. amylovora* Ea519Rif (Fig. 2A). The population of *E. amylovora* Ea519Rif was significantly reduced in the presence of *E. herbicola* HL9N13 at all time points (except 48 h) from 36 h after inoculation onward (*P* = 0.001 to 0.05) (Fig. 2B). The doubling time of *E. amylovora* Ea519Rif was longer in the presence of *E. herbicola* HL9N13 than in its absence (6.0 and 3.4 h, respectively).

SEM. Examination of the inoculated stigmas by SEM showed that *E. herbicola* HL9N13 effectively colonized the stigmatic surface of the hawthorn blossom. At 24 h after inoculation, cells of *E. herbicola* were observed both on the surface of the papillae and in the intercellular spaces between the papillae. By 48 h after inoculation, the number of *E. herbicola* cells in the intercellular spaces between the papillae had increased and some of the papillae had collapsed (Fig. 3).

**Spectrum of antibiotic activity.** The antibiotic(s) produced by *E. herbicola* HL9N13 on PDA gave bactericidal inhibition zones when sprayed with *E. amylovora* Ea519Rif. The antibiotic(s) were also active against *P. syringae, E. coli*, *S. marcescens*, *P. vulgaris*, *B. subtilis*, *B. cereus*, and *S. aureus*.

**DISCUSSION**

The biological control agent *E. herbicola* HL9N13 was antagonistic to the development of the pathogen *E. amylovora* Ea519Rif on the stigma of the pistil of the hawthorn blossom. Both preemptive and competitive colonization of the stigma by the biological control agent reduced the pathogen growth rate and final population. Hattingh et al. (8) suggested that preemptive exclusion of *E. amylovora* Ea273 by *E. herbicola* Eh252 on the apple stigma occurred because the two strains occupied a similar ecological niche and that prior colonization of stigmatic sites by *E. herbicola* Eh252 prevented occupation of those sites by *E. amylovora* Ea273. Preemptive and competitive exclusion of *E. amylovora* Ea519Rif by *E. herbicola* HL9N13 on the hawthorn stigma appear to involve at least two factors, including competition for a growth-limiting resource and either antibiosis or some form of habitat modification.

The stigmatic surface of the hawthorn pistil appears to exhibit a consistent carrying capacity for an epiphytic bacterial population of approximately $10^6$ cfu per blossom. *E. herbicola* HL9N13 colonized the intercellular spaces between the stigmatic papillae up to a population of approximately $10^6$ cfu per blossom. *E. amylovora* Ea519Rif also colonized the sites between the stigmatic papillae in its epiphytic phase of development and reached a population of approximately $10^6$ cfu per blossom (27). These observations suggest that the growth of an epiphytic bacterial population colonizing the intercellular spaces between the stigmatic papillae is resource-limited.

Occupation of the same colonization sites by two different epiphytic populations does not imply growth limitation by the same resource; hence, two alternative explanations of the interaction between *E. herbicola* HL9N13 and *E. amylovora* Ea519Rif are possible. In the first, the populations of *E. herbicola* HL9N13 and *E. amylovora* Ea519Rif occupied the same sites but were limited by different resources, and the production of an inhibitory compound or other habitat modification gave *E. herbicola* HL9N13 a competitive advantage in acquisition of that resource. The results from the pre inoculation experiments are consistent with either explanation. The results from the coinoculation experiment, however, suggest that *E. herbicola* HL9N13 and *E. amylovora* Ea519Rif were limited by the availability of the same resource, because both populations

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**Fig. 2.** Coinoculation of *Erwinia herbicola* HL9N13 and *E. amylovora* Ea519Rif on the stigma of the pistil of hawthorn blossom. A. Population of *E. herbicola* HL9N13 when inoculated alone (●) and when coinoculated with *E. amylovora* Ea519Rif (▲). B. Population of *E. amylovora* Ea519Rif when inoculated alone (●) and when coinoculated with *E. herbicola* HL9N13 (▲). Bars represent one standard error of the mean.

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ceased to increase at 36 h, presumably when the mutually required growth-limiting resource had been partitioned between the two competing populations.

The conclusion that resource competition is one factor involved in the interaction between _E. herbicola_ and _E. amylovora_ is consistent with observations of Vanneste et al. (23, 24) that antibiosis alone was insufficient to explain the inhibition of _E. amylovora_ by _E. herbicola_. Although the nature of the resource for which _E. herbicola_ HL9N13 and _E. amylovora_ Ea519Rif competed remains unknown, the data from SEM and light microscopy (27) suggest that space was probably not the growth-limiting resource at these sites. In similar competitive interactions between isogenic _P. syringae_ strains growing epiphytically on leaf surfaces, the populations usually multiplied independently until the carrying capacity was reached (13–17). These isogenic _P. syringae_ strains competed for a growth-limiting nutritional resource that was partitioned equally between the two populations.

In the coinoculation experiment, the growth rate of _E. amylovora_ Ea519Rif was reduced in the presence of the biological control agent, giving _E. herbicola_ HL9N13 a competitive advantage in the acquisition of the mutually required growth-limiting resource. Although this effect of _E. herbicola_ HL9N13 on _E. amylovora_ Ea519Rif may have resulted from habitat modification, the production of an antibiotic in planta is a possible explanation because antibacterial activity was observed for _E. herbicola_ HL9N13 in vitro. The broad spectrum of activity in vitro suggests that the antibiotic produced on PDA was a herbicin rather than a bacteriocin, which by definition are active primarily against organisms closely related to the producer (18, 25). The antibiotic produced by _E. herbicola_ HL9N13 differed from those described by Ishimaru et al. (12) and Wodzinski et al. (32) for yellow-pigmented _E. herbicola_ strains, which were not produced on PDA. However, its spectrum of activity appeared to be similar to that of herbicin O, described by Ishimaru et al. (12). The probable involvement of antibiosis in the interaction between _E. herbicola_ HL9N13 and _E. amylovora_ Ea519Rif is supported by the findings of Ishimaru et al. (12) and Vanneste et al. (23, 24). If antibiosis was involved, then the interaction between _E. herbicola_ and _E. amylovora_ is analogous to that between _E. carotovora_ subsp. _betavasculorum_ and _E. c. carotovora_ in the infection of potato (1), where in situ antibiotic production was the primary factor that enabled _E. c. betavasculorum_ to outcompete _E. c. carotovora_ at the infection site.

The results presented here suggest that _E. herbicola_ HL9N13 and _E. amylovora_ Ea519Rif occupied a similar ecological niche on the hawthorn stigma, colonized the same physical sites, and competed for the same growth-limiting resource. Under these conditions _E. herbicola_ HL9N13 had a competitive advantage over _E. amylovora_ Ea519Rif, which may have resulted in part from antibiotic production. Preemptive or competitive colonization of the stigma by the biological control agent _E. herbicola_ HL9N13 resulted in use of a growth-limiting resource, the lower availability of which reduced the growth of the pathogen _E. amylovora_ Ea519Rif.

**LITERATURE CITED**


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**Fig. 3.** Critical-point dried scanning electron microscope preparations of _Erwinia herbicola_ HL9N13 on the hawthorn stigma. A, Cells of _E. herbicola_ HL9N13 multiplying among collapsed papillae at 48 h after inoculation. B, Cells of _E. herbicola_ HL9N13 multiplying in the intercellular spaces between the papillae. Bars = 2 μm.


