BACTERIOPHAGE OF *ERWINIA AMYLOVORA* AND THEIR POTENTIAL FOR BIOCONTROL

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Abstract

Three species of bacteriophage specific to *Erwinia amylovora* were isolated from Michigan apple orchards and tested for their ability to control fire blight. In liquid culture, individual phage were ineffective at controlling the growth of *E. amylovora*; but a mixture of the three phage kept bacterial populations at about 1% of the level reached by cultures grown without phage. Apple blossoms in the field were inoculated with the three-phage mixture and a marked strain of *E. amylovora*, and their population dynamics monitored through the bloom period. Populations of phage gradually declined on blossoms not inoculated with *E. amylovora*; in the presence of *E. amylovora*, however, phage populations remained high for the duration of the experiment. There was significantly less fire blight on inoculated blossom clusters treated with phage resulting in 26% to 37% control. Because high populations of phage were dependent on the presence of *E. amylovora*, strategies need to be found for maintaining phage populations during the bloom period.

1. Introduction

Fire blight, caused by *Erwinia amylovora*, is one of the most serious diseases limiting apple and pear production in Michigan and throughout North America. The use of streptomycin has been the primary means for suppressing populations of *E. amylovora* during the bloom period. However, streptomycin-resistant *E. amylovora* have been detected in many fruit producing regions of the United States. In the western United States the biological control agents *Pseudomonas fluorescens* strain A506 and *Erwinia herbicola* (*Pantoea agglomerans*) strain C9-1 are used to complement streptomycin during bloom (Lindow *et al.*, 1996, Stockwell *et al.*, 1996).

Bacteriophage of *E. amylovora* are common in Michigan apple orchards (Ritchie and Klos, 1977), but little is known about their role in regulating field populations of *E. amylovora* or their potential for biological control of fire blight. The objectives of this study were to assess the ability of phage isolated from apple orchards to inhibit the growth of *E. amylovora* in liquid cultures and, following the selection of superior phage, their ability to survive and suppress *E. amylovora* under field conditions.

2. Materials and methods

PEa1, PEa116B, and PEa116C were naturally occurring phage isolated from fire blight infected Michigan apple orchards. They were stored in SM buffer with 5% chloroform.

Each phage isolate was tested for its effectiveness in inhibiting the growth of *E. amylovora* strain Ea110 in LB medium. The phage (10^7 pfu) were added to liquid cultures of bacteria (10^8 cfu), then bacterial cell densities were monitored over time. Mixtures of phage were also tested in different combinations for their ability to suppress the growth of *E. amylovora* in LB medium.

The effectiveness of the three-phage mixture for suppressing *E. amylovora* and for controlling fire blight was evaluated on 26-year-old Jonathan apple trees. A completely

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randomized experimental design was used with six replicates per treatment. Blossom clusters on individual branches were atomized with rifampicin-resistant *E. amylovora* strain Ea110 (10^8 cfu/ml), phage (10^{10} pfu/ml), or a combination of Ea110 and phage at 75-90% bloom to give five treatments (Table 1).

Blossoms and fruitlets (10/replication) were sampled 12 times, starting on the day of inoculation, to estimate phage and bacterial populations. The samples were washed in 0.02M potassium phosphate buffer and spiral-plated onto LB agar amended with rifampicin and cycloheximide. To test for the presence of phage, aliquots of washings from samples were mixed with Ea110 and plated on nutrient agar amended with 0.5% glucose (NAG) using the double layer agar technique. Plaques appearing on the lawn were counted.

Disease incidence was determined 15 and 22 days after inoculation. The number of healthy and symptomatic blossom clusters was counted on each branch and the results presented as the percentage of blossom clusters infected.

3. Results and discussion

Phage PEa1 produced large plaques and phage PEa116B and PEa116C produced small plaques after 24 h incubation with propagation host *E. amylovora* Ea110. Although each phage species lysed *E. amylovora* during the early stages of growth in LB medium, bacterial cell densities after 18 h were 50-110% of the density of cultures without added bacteriophage (Fig. 1), and after an additional 72 h densities for inoculated cultures were similar to those of non-inoculated cultures. In the case of PEa1, survival of the bacteria was probably due the removal of phage binding sites from the surface of the bacterium by a phage-encoded polysaccharide depolymerase rendering it resistant to infection (Hartung *et al.*, 1988; Ritchie and Klos, 1977).

Pairs of phage were more effective then any of the phage alone and the effect of all three together was quite dramatic (Fig. 1). The three-phage combination was the only treatment to suppress the growth of *E. amylovora* Ea110 after an additional 54 h incubation.

In the orchard, initial phage populations following phage application were about 10^8 plaque-forming units (pfu) per blossom. Phage populations in the absence of *E. amylovora* declined to about 10^4 pfu per blossom over the first 6 days and then remained static (Fig. 2). Phage populations remained high for the duration of the experiment on blossoms inoculated with *E. amylovora*.

Populations of *E. amylovora* Ea110 were about 10^6 colony forming units (cfu) per blossom 1.5 h after inoculation (Fig. 2). In general, populations of *E. amylovora* on blossoms declined for a few days following inoculation, increased to their maximum level by day 8 and 9, and then remained relatively static. Between days 3 and 8, *E. amylovora* populations were significantly lower on phage-treated blossoms than on non-treated blossoms (Fig. 2). No *E. amylovora* Ea110 were detected on blossoms treated with phage alone nor on blossoms from non-inoculated branches, indicating that the bacteria had not moved onto non-inoculated blossoms.

Fire blight symptoms began to appear one week after inoculation. There was significantly less fire blight on inoculated blossom clusters treated with phage (Table 1). The incidence of fire blight on phage-treated blossoms was reduced by 12.7 to 18.1% and by 10.1 to 18.2% at 15 and 22 days after inoculation, respectively. Applying the phage treatment on the day of inoculation reduced fire blight more than applying it one day after inoculation. The phage treatments exhibited 26% to 37% control of blossom blight on day 15 and 17 to 31% control on day 22.

It was clear that the maintenance of high populations of *E. amylovora*- specific phage on apple blossoms was dependent on the continued presence of *E. amylovora*. Since the phage are specific for *E. amylovora*, we need to evaluate strategies for supporting phage populations without having to inoculate trees with virulent strains. Avirulent mutants of

E. amylovora may be useful for this purpose. Besides supporting populations of phage, they should also provide some control of fire blight (Tharaud *et al.*, 1997).

The level of blossom blight control obtained in this experiment with phage was at the low range of control levels reported for bacterial antagonists (Lindow *et al.*, 1996; Stockwell *et al.*, 1996). Unlike many experiments with bacterial antagonists, phage-treated blossoms were challenged with high levels of *E. amylovora* rather than with low to moderate levels of the pathogen. Phage may provide a higher level of fire blight control when blossoms are challenged with fewer bacteria.

References

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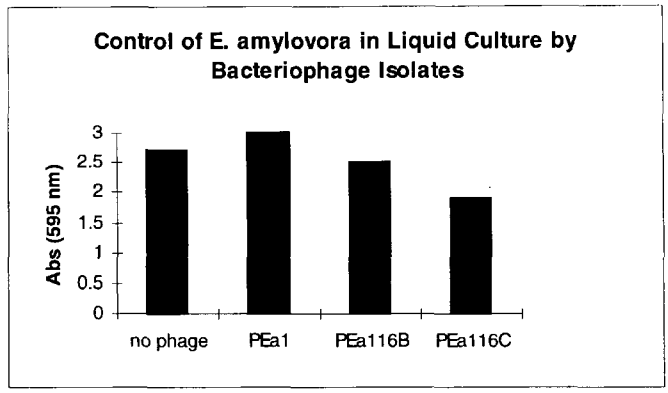
Table 1. The percent of bloom clusters on Jonathan trees infected with fire blight 15 and 22 days after inoculation with *Erwinia amylovora* strain Ea110 and a mixture of three bacteriophage.

	Blossom clusters and number infected with fire blight					
	After 15 days			After 22 days		
Treatment	Total (no.)	Infected (no.)	Infected (%)	Total (no.)	Infected (no.)	Infected (%)
Erwinia amylovora strain Ea110	297	145	48.8 a	309	182	58.8 a
Phage mixture	340	0	0*	348	0	0*
Ea110 and phage mixture inoculated on same day	391	120	30.7 b (37.0 %)	401	163	40.6 b (31.0 %)
Ea110 applied one day before phage mixture	380	137	36.1 ab (26.0 %)	390	190	48.7 ab (17.2 %)
Untreated control	355	0	0*	365	0	0*

* Only treatments inoculated with *E. amylovora* Ea110 were included in the statistical analysis. Means followed by the same letter are not statistically different based on LSD at $P<0.05$.
Number in parenthesis is the percent fire blight control provided by the respective treatment.



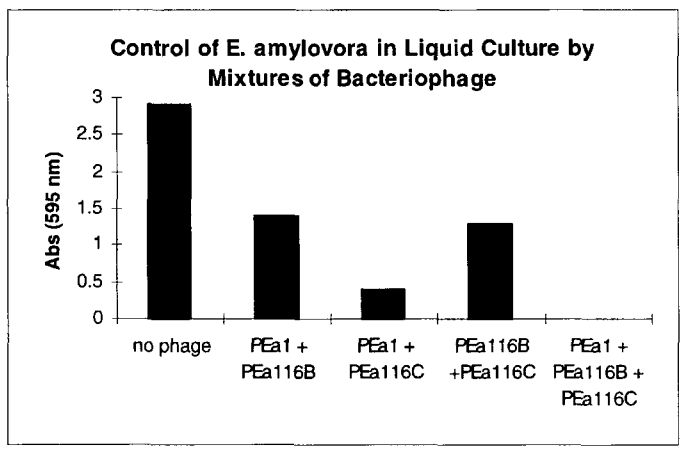


Figure 1. Clearing of cultures of *Erwinia amylovora* strain Ea110 in LB medium after 18 h with individual bacteriophage (top) and with combinations of phage (bottom).

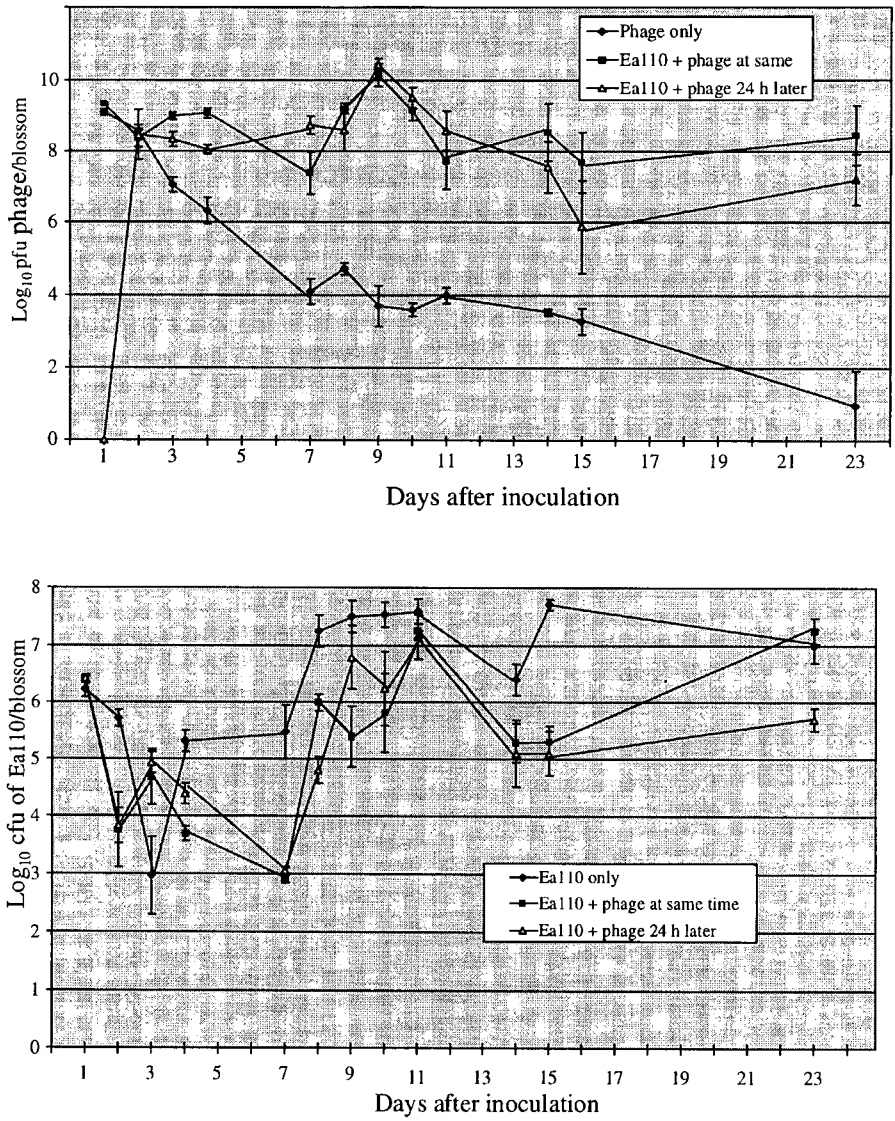


Figure 2. Populations of bacteriophage on apple blooms with and without inoculation with *Erwinia amylovora* strain Ea110 (top) and the corresponding populations of *E. amylovora* Ea110 on these blossoms (bottom). The day of inocuation is labeled as day 1 in both figures.