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ORIGINAL ARTICLE

Potential biological control of *Erwinia tracheiphila* by internal alimentary canal interactions in *Acalymma vittatum* with *Pseudomonas fluorescens*

D.C. Roberts, S.J. Fleischer, J.M. Sakamoto and J.L. Rasgon

The Pennsylvania State University, University Park, PA, USA

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Acalymma vittatum, biological control, cucurbit, *Erwinia tracheiphila*, *Pseudomonas fluorescens*.

Correspondence
Dana C. Roberts, The Pennsylvania State University, 519 Ag Science and Industry Bldg, University Park, PA 16802, USA.
E-mails: dcr5101@gmail.com; dcr5101@psu.edu

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Abstract

Aims: We aim to determine if *Pseudomonas fluorescens* is a viable biological control for *Erwinia tracheiphila* within the insect vector, *Acalymma vittatum*.
Methods and Results: *Pseudomonas fluorescens* secreted fluorescein and inhibited growth of *E. tracheiphila* in disc diffusion assays. To determine if this antagonism was conserved within the insect vector, we performed *in vivo* assays by orally injecting beetles with bacterial treatments and fluorescent *in situ* hybridization to determine bacterial presence within the alimentary canal.
Conclusions: *Pseudomonas fluorescens* inhibited the growth of *E. tracheiphila* on a nutrient-limiting medium. *In situ* experiments demonstrated that *P. fluorescens* is maintained within the alimentary canal of the beetle for at least 4 days, and co-occurred with *E. tracheiphila*. When beetles were first presented with *Pseudomonas* and then challenged with *E. tracheiphila*, *E. tracheiphila* was not recovered via FISH after 4 days. These data suggest that *P. fluorescens* has potential as a biological control agent to limit *E. tracheiphila* within the insect vector.
Significance and Impact of the Study: This is a novel approach for controlling *E. tracheiphila* that has the potential to decrease reliance on insecticides, providing a safer environment for pollinators and growers.

Introduction

Acalymma vittatum has a primary relationship with plants in the family Cucurbitaceae: larvae feed on the roots of the plants and adults feed on foliage and fruits (Cuthbert *et al.* 1968; Howe and Zdarkova 1971). In addition to the damage it causes through herbivory, *A. vittatum* is also a vector and overwintering host of *Erwinia tracheiphila* (Smith), the causal agent of bacterial wilt in cucurbits (Haynes and Jones 1975; Bassi 1982; Brust and Rane 1995; Mitchell and Hanks 2009; Sasu *et al.* 2010; reviewed in Saalau Rojas *et al.* 2015). The successful inoculation of *E. tracheiphila* to the next year's crop is achieved through colonization of the alimentary canal of *A. vittatum* (de Mackiewicz *et al.* 1998; Garcia-Salazar *et al.* 2000) and subsequent overwintering.

Erwinia tracheiphila is a Gram-negative rod-shaped bacterium in the Enterobacteriaceae (Breed *et al.* 1957; Naum *et al.* 2008). It is a xylem-limited, biofilm-producing plant pathogen that blocks water and nutrient transport causing subsequent wilting, and eventual death of the infected host plant (Fulton *et al.* 1911). Currently, control strategies for this pathogen rely on systemic and foliar insecticidal treatments for *A. vittatum* (Gould 1944; Brust *et al.* 1996; Pair 1997; Fleischer *et al.* 1998; Egel 2016). Because of the deleterious effects of insecticides on nontarget insects, particularly pollinator insects, there is interest in developing alternative control strategies targeting the tightly interwoven pathogen–vector relationship.

We examined the possibility of using *Pseudomonas fluorescens* (a bacteria common to the rhizosphere and phyllosphere) as a potential control agent against *E. tracheiphila*. Previous attempts at controlling fireblight, *Erwinia amylovora* (Burrill), with *P. fluorescens* were successful at reducing overall disease (Yamamoto *et al.* 2000; Pujol *et al.* 2005; Cabrefiga *et al.* 2007; Silby *et al.* 2009). *Pseudomonas fluorescens* inhibits *E. amylovora* by sequestering free iron, thereby limiting the latter's growth (Cabrera and Durante 2001; Whipps 2001; Stockwell *et al.* 2002; Temple *et al.* 2004; Cabrefiga *et al.* 2007).

Pseudomonas fluorescens EPS62e (which does not produce antibiotics) produces pyoverdin (also known as fluorescein) in low iron environments which functions as a siderophore for iron sequestration from the environment (Lenhoff 1963; Meyer and Abdallah 1978; Crosa 1989; Cabrefiga *et al.* 2007; Couillerot *et al.* 2009). *Pseudomonas fluorescens* has also shown induction of plant growth hormones, and direct elicitation of plant defences (Silby *et al.* 2009).

We hypothesized that *P. fluorescens* should exhibit similar antagonistic characteristics towards *E. tracheiphila* based on the genetic similarity of *E. tracheiphila* to *E. amylovora*. Here, we present the results of in vitro and in vivo experiments examining the interaction between *P. fluorescens* and *E. tracheiphila.*

Materials and methods

Biological material

Bacterial strains

We acquired USDA APHIS permits to receive shipments of the *E. tracheiphila cucumis* strain TPINCu1, and a rifampicin-resistant strain TedCu10^{Rif}, from Iowa State University. We obtained an isolate of *P. fluorescens* strain 55 from Dr. Tim McNellis (Penn State University). *P. fluorescens* strain 55 was cultured on King's B medium, a low iron content, semiselective medium designed to induce fluorescein production in fluorescent pseudomonads (King *et al.* 1954). Because both *E. tracheiphila* and *P. fluorescens* can metabolize glycerol (the carbon source used in King's B), this medium was ideal for studying the interactions between the two bacterial species *in vitro*. *Erwinia tracheiphila* strain TPINCu1 was used for *in vitro* experiments only. In *in situ* experiments we used rifampicin-resistant *E. tracheiphila* strain TedCu10^{Rif} that was cultured on nutrient broth amended with 0·75 μg ml^{-1} rifampicin.

Acalymma vittatum

The experimental colony was established from field-collected beetles from Rock Springs and State College, PA with additional beetles included throughout the season to maintain the population. Adult beetles were placed in a 30·2 cm × 30·2 cm × 30·2 cm (12 in × 12 in × 12 in) pop-up mesh cage (Bioquip, Rancho Dominguez, CA) with a plastic sandwich container of moist soil for oviposition in a greenhouse. Rearing conditions were: 16 h light : 8 h dark cycle and a temperature range of 22–28°C, following the protocols in Cuthbert *et al.* (1968), Howe and Zdarkova (1971), and Shapiro (2012). Three times a week cucumber (*Cucumis sativus:* Northern Pickling) leaves and flowers were given to adults to feed *ad libitum*. Once a week, soil from the adult cages was collected and placed in a 5-l plastic bin with squash seedlings (*Cucurbita pepo*: Yellow Crookneck), which were provided as needed. After 4–6 weeks, newly emerged adult beetles were collected and placed into a mesh cage with other beetles from the colony. Each field season the colony was supplemented with newly collected beetles to maintain genetic diversity. To minimize *E. tracheiphila* contamination within the colony, the field-collected beetles were kept separate from all other beetles.

Probe design

We used fluorescence *in situ* hybridization (FISH) to observe the colocalization between *E. tracheiphila* and *P. fluorescens* within the *A. vittatum* alimentary canal. *Pseudomonas fluorescens* was visualized using probe Pseudo120 labelled with a 6-FAM fluorophore (Saha *et al.* 2012). To visualize *E. tracheiphila*, we designed a species-specific probe. We aligned representative 16S RNA sequences published in the National Center for Biotechnology Information (NCBI) nucleotide database (as of September 2014) for 11 *Erwinia* isolates and 3 *E. coli* reference sequences in MEGA6 software (Tamura *et al.* 2013) to determine hypervariable regions (Kwon *et al.* 1997; Hauben *et al.* 1999; Chakravorty *et al.* 2007; Naum *et al.* 2008). We selected *E. coli* reference sequences J01859.1, NR_074891.1, and NR_102804.1 to capture strain variations or sequencing errors. *Erwinia* isolate sequences used included five species (*amylovora, aphidicola, billingiae, pyrifoliae*, and *tasmaniensis*) in addition to *E. tracheiphila* (AF140338.1, NR_117000.1, AM055711.1, NR_119255.1, NR_104932.1, JN175337.1, KC817809.1, NR_074869.1, NR_044924.1, EU490598.1, Y13250.1). We selected short nucleotide sequences (15–30 base pairs) within the hypervariable regions where *E. tracheiphila* sequences differed from all other *Erwinia* species sequences. We confirmed each candidate probe sequence using TestProbe (http://www.arb-silva.de/search/testprobe/), which utilizes the SILVA ribosomal RNA database to compare nucleotide sequences to all published sequences for matches (Klindworth *et al.* 2013; Quast *et al.* 2013; Yilmaz *et al.* 2014). Of the possible sequences, we identified only one probe that matched exclusively to *E. tracheiphila* (EtPennState 5′-CAC-AGA-ACT-TAG-CAC-AG-3′). We used this probe labelled with a green (5′-6FAM) or red (5′-Rhodamine or 5′-Cy5) (Integrated DNA Technologies, Skokie, IL, USA). To minimize cost, we used *E. tracheiphila*-6FAM (green) for samples when only *E. tracheiphila* was the target, and *E. tracheiphila*-Rho (red) for colocalization with *Pseudomonas* when using the Pseudo120-6FAM (green) probe for assays where we needed to distinguish between the two species. We verified specificity of this probe for *E. tracheiphila* by performing fluorescent *in situ*

hybridization on bacterial cells isolated from plates and in *A. vittatum* alimentary canals with *E. tracheiphila* as controls. RNase-treated alimentary canals were used as negative control.

Bioassays

The *in vitro* antagonism assays were performed on King's B plates (n = 29 Et lawn Pf disc, n = 7 Pf lawn Et disc) with *E. tracheiphila* strain TPINCu1 and *P. fluorescens* strain 55, grown at 25°C for a minimum of 4 days. Cross-culturing methods were used to test interactions of bacteria, as described by Nguyen & Ranamukhaarachchi (2010), via a modified agar diffusion assay, by lawn plating of one bacterial species with a central inoculation point of the opposing bacteria at the same time. The zone of inhibition (area in mm without opposing bacterial growth) and *P. fluorescens* growth (mm) was measured from the inoculation point daily until the plate was fully colonized, which ranged 4–7 days. The area of *E. tracheiphila* on each plate was calculated as the area of the plate minus the zone of inhibition. The area (mm) with secretion of fluorescein was measured using UV light for visualization.

We tested six treatments *in situ*, each consisting of a pair of doses spaced 2 days apart, to examine bacterial colocalization in the alimentary canal of *A. vittatum* (Table 1). The first three treatments (treatments 1–3) functioned as negative (water) or positive (*E. tracheiphila* or *P. fluorescens*) controls. The next three treatments (treatments 3–6) functioned as challenges of mixtures or sequential dosages of the bacterial species. Each suspension contained 500 µl of water with 5 µl of blue food dye for visualization and copious amounts bacterial growth ($\geq 10^8$) collected from the growing media when necessary. Bacterial doses were created with the aim of overwhelming the alimentary canal to demonstrate colocalization potential. All doses were orally injected with Nanoliter 2010 Injector (World Precision Instruments Item#: NANOLITER2010) with a glass needle. The beetle was placed on a chilling plate set at 11°C to slow its movement. Colony-reared *A. vittatum* were starved for 24 h prior to the first dose of ~100 nl of water, *E. tracheiphila* TedCu10^{Rif} strain, or *P. fluorescens* strain 55. Two days after receiving dose 1, beetles were given ~100 nl of dose 2 (water, *E. tracheiphila* TedCu10^{Rif} strain, or *P. fluorescens* strain 55). One day after the second dose, beetles were placed in acetone for preservation and dehydration.

Following methods outlined by Koga *et al.* (2009); we dissected out the alimentary canals and fixed them overnight in 500 µl Carnoy's solution at room temperature. After fixation, alimentary canals were placed in 100% ethanol in −20°C for storage. The alimentary canals were rehydrated in 1X PBSTx and incubated in hybridization buffer for at least 15 min. After replacing the hybridization buffer with 100 pmol ml^{-1} fluorescent probe in hybridization buffer, we incubated the samples overnight at room temperature. Samples were mounted on slides with Slow-Fade antifade solution with DAPI (ThermoFisher Scientific, Waltham, MA) and imaged using an Olympus Bx41 epifluorescent microscope (Koga *et al.* 2009). Alimentary canals were examined for visual co-occurrence of *P. fluorescens* and *E. tracheiphila*.

Analysis

We used a mixed model and Pearson's correlation to determine the relationship among plates using *P. fluorescens* growth, fluorescein secretion, and zone of inhibition. The influence of *P. fluorescens* growth on *E. tracheiphila* growth was analysed as a mixed model using date as a repeated measure. Treatment effects on the alimentary canal location of each bacterial species and the number of beetles testing positive from FISH bioassays were analysed using Fisher's Exact Test.

Results

In vitro bioassay results

King's B plates inoculated with a lawn of *P. fluorescens* and a central disc of *E. tracheiphila* (n = 7) did not produce zones of inhibition. *Erwinia tracheiphila* did not extend in growth from the central disc and did not inhibit the fluorescein production by *P. fluorescens*. In contrast, King's B plates inoculated with a lawn of *E. tracheiphila* and a central disc of *P. fluorescens* (n = 29) did produce zones of inhibition and secreted fluorescein. There was no effect of plate on zone of inhibition (F = 1·25, df = 28 114, P = 0·2073), fluorescein secretion diameter (F = 0·81, df = 28,114, P = 0·7317) or *P. fluorescens* growth (F = 1·51, df = 28 114, P = 0·0691). *Pseudomonas fluorescens* growth was highly correlated with fluorescein secretion diameter (r = 0·74, n = 143, $P < 0·0001$) and with the zone of inhibition (r = 0·49, $P < 0·0001$). *Pseudomonas fluorescens* growth reduced the area of *E. tracheiphila* on the plates (F = 45·25, df = 1141, $P < 0·0001$).

In situ bioassay results

Fluorescent in situ hybridization of *E. tracheiphila* probe specificity

Initial trials of the *E. tracheiphila* 16S probe viability were performed on slide-mounted bacterial cells of the *E. tracheiphila* strains (Fig. 1). These tests confirmed specificity to the *E. tracheiphila* strains and no cross-reaction with *P. fluorescens* strain 55. RNase-treated alimentary canal

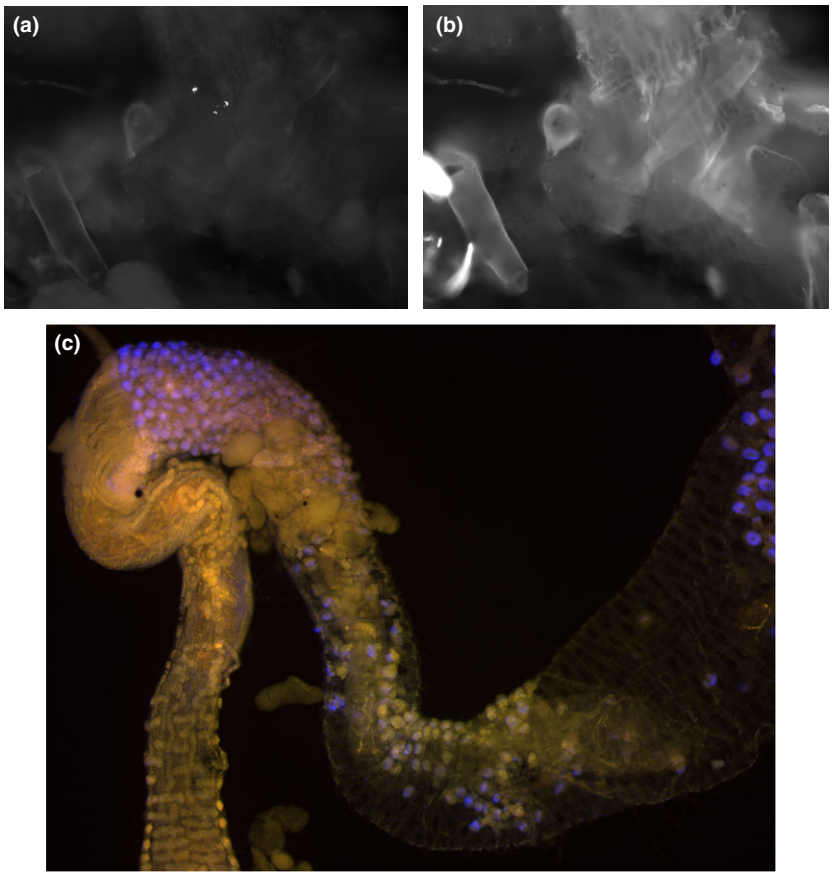

Figure 1 *Acalymma vittatum* hindgut of the alimentary canal stained with DAPI for nuclei (a, 40×) and stained with 6FAM for *Erwinia tracheiphila* (b, 40×) same image exposed under different light for each fluorophore. RNase-treated alimentary canal of an *A. vittatum* stained with 6FAM *E. tracheiphila* 16s probe (4×) as shown in (c).

controls had no binding of the probe, confirming specificity for *E. tracheiphila in situ*.

Bacterial colocalization in the alimentary canal

Alimentary canals were analysed to determine if bacterial presence varied between the mid and hindgut. Presence of *E. tracheiphila* in both the mid and hindgut for all treatments did not differ (treatment 1 P = n/a all zero, treatment 2 $P = 0{\cdot}2859$, treatment 3 P = n/a all zero, treatment 4 $P = 1{\cdot}00$, treatment 5 $P = 0{\cdot}6662$, treatment 6 P = n/a all zero). However, *P. fluorescens* had a significant gut location effect ($P = 0{\cdot}041$) when *E. tracheiphila* was followed by *P. fluorescens* (treatment 4). All other treatments were not significantly different between gut locations for *P. fluorescens*. Due to the significant effect of gut location on *P. fluorescens* in treatment 4, we analysed treatment effects for each gut location separately.

Controls

The negative control (treatment 1, where only water was fed to the beetles) resulted in no detection of *E. tracheiphila* in either the midgut or hindgut. There was, however, a background detection of the *Pseudomonas* genus in 19% of midguts and 6% of hindguts of beetles in the water control (Table 1), which may reflect the lack of species specificity of our *Pseudomonas* detection probe.

As expected, the positive control for *E. tracheiphila* (treatment 2, where *E. tracheiphila* was fed on day 1, followed by water on day 2), resulted in high rates of recovery of *E. tracheiphila* in the midgut and hindgut (42 and 29% respectively; Table 1). However, as in the water control, there were low rates of detection of *Pseudomonas* (6 and 2% in the midgut and hindgut respectively). Recovery rates of *E. tracheiphila* from the positive control were significantly higher than the water control ($P = 0{\cdot}001$ and 0·014 for midgut and hindgut), while the low recovery rates of *Pseudomonas* were no different than the water control ($P = 0{\cdot}16$ and 0·441 for midgut and hindgut; Table 1).

Also as expected, the positive control for *P. fluorescens* (treatment 3) resulted in high recovery rates of *Pseudomonas* (41 and 32% in the midgut and hindgut respectively) and no recovery of *E. tracheiphila* (Table 1). However, these high rates of *Pseudomonas* recovery were

Table 1 Number and percentage of samples testing positive for *Erwinia tracheiphila* or *Pseudomonas* spp. following six treatment regimens given to *Acalymma vittatum*. Dose 1 was provided to beetles after 24 h of without food, and dose 2 provided 24 h after dose 1. TedCu10^{Rif} = *E. tracheiphila* strain and *P. f.* = *P. fluorescens* strain 55

Treatment regime					Presence of *E. tracheiphila*				Presence of *Pseudomonas* spp.			
Treatment					Midgut		Hindgut		Midgut		Hindgut	
Name	Number	Dose 1	Dose 2	Sample size	Count	%	Count	%	Count	%	Count	%
Water Control	1	Water	Water	16	0	0	0	0	3	19	1	6
E.t. Control	2	*E.t.*	Water	48	20	42	14	29	3	6	1	2
P.f. Control	3	*P.f.*	Water	63	0	0	0	0	26	41	20	32
Mix	4	*E.t.* + *P.f.*	Water	29	5	17	6	21	9	31	7	24
E.t. → *P.f.*	5	*E.t.*	*P.f.*	24	4	17	2	8	17	71	9	38
P.f. → *E.t.*	6	*P.f.*	*E.t.*	24	0	0	0	0	12	50	7	29

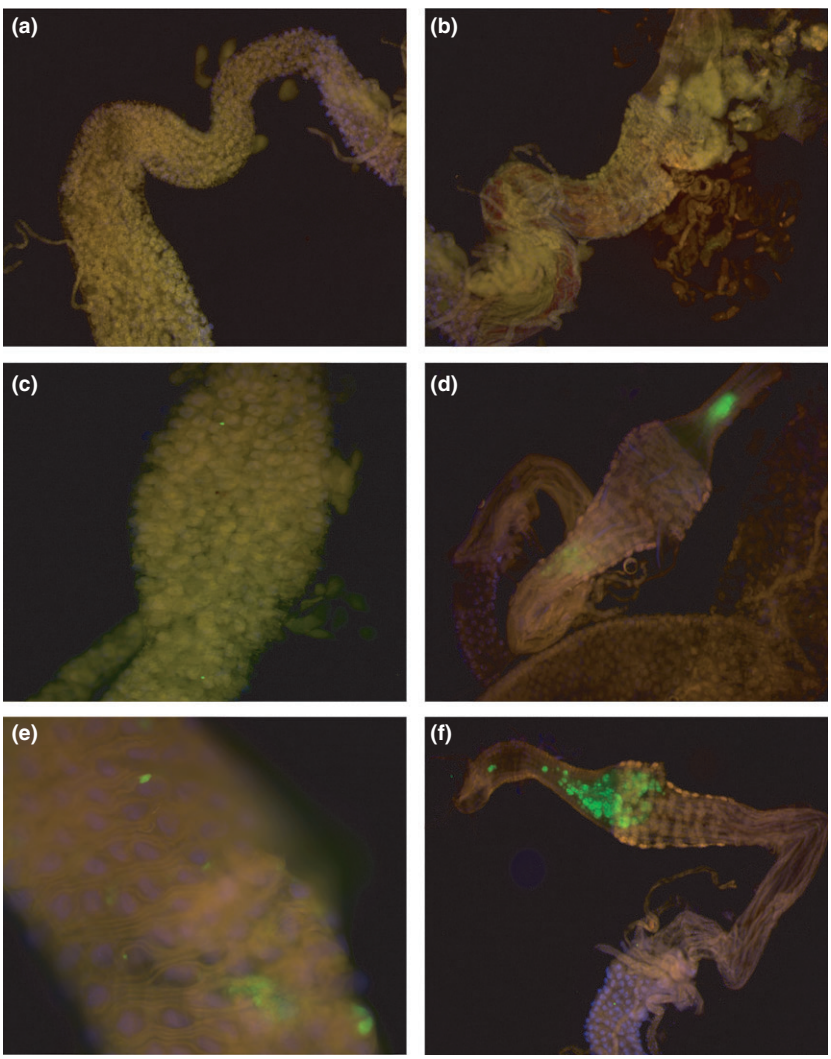

Figure 2 Images of *Acalymma vittatum* alimentary canal control treatments, all were stained with DAPI (blue) for nuclei. Water control (treatment 1) *A. vittatum* midgut at 4× (a) and hindgut at 4× (b) stained with Pseudo120 6FAM (green) for *Pseudomonas* spp. and Et16s Rhodamine (red) for *Erwinia tracheiphila*. *Erwinia tracheiphila*-positive control (treatment 2) *A. vittatum* midgut at 4× (c) and hindgut at 4× (d) stained with Et16s 6FAM (green) for *E. tracheiphila*. *Pseudomonas fluorescens*-positive control (treatment 3) *A. vittatum* midgut at 20× (e) and hindgut at 4× (f) stained with Pseudo120 6FAM (green) for *P. fluorescens*.

not significantly different than the background recovery rates of *Pseudomonas* in the water control (P = 0·146 and 0·056 in the midgut and hindgut respectively) (Table 1).

Alimentary canal images of the mid and hindgut using fluorescent *in situ* hybridization illustrate the localization of the bacteria in Fig. 2.

Table 2 Fisher's exact test *P*-values comparing number of beetles testing positive for *Erwinia tracheiphila* or *Pseudomonas* spp. among varying treatment regimens (data in Table 1, treatment regime given in parenthesis). n/a refers to inability to compare due to zero counts. Bold indicates significance.

			Presence of *Erwinia tracheiphila*		Presence of *Pseudomonas fluorescens*	
Control	*vs*	Treatment	Midgut	Hindgut	Midgut	Hindgut
Water Control (1)		*E.t.* control (2)	**0·001**	**0·014**	0·16	0·441
		P.f. control (3)	n/a	n/a	0·146	0·056
		Mix (4)	0·144	0·075	0·491	0·227
		E.t. → *P.f.* (5)	0·136	0·508	**0·003**	**0·032**
		P.f. → *E.t.* (6)	n/a	n/a	0·056	0·114
E.t. Control (2)		Mix (4)	**0·043**	0·592	**0·007**	**0·004**
		E.t. → *P.f.* (5)	**0·038**	0·07	**<0·0001**	**1E-04**
		P.f. → *E.t.* (6)	**<0·0001**	**0·003**	**<0·0001**	**0·001**
P.f. Control (3)		Mix (4)	**0·002**	**0·001**	0·368	0·623
		E.t. → *P.f.* (5)	**0·005**	0·074	**0·017**	0·62
		P.f. → *E.t.*(6)	n/a	n/a	0·479	1

Treatment with a mix

The next three treatments functioned as challenges of a mixture (treatment 4) or sequential dosing (treatments 5 and 6) of the bacterial species. When *A. vittatum* was orally injected with a mix of both *E. tracheiphila* and *P. fluorescens* on day 1, followed by water on day 2 (treatment 4), both *E. tracheiphila* and *Pseudomonas* were detected, at rates that ranged from 5 to 21% for *E. tracheiphila* and 9 to 31% for *Pseudomonas* (Table 1). When compared to the positive control for *E. tracheiphila*, detection rates of *E. tracheiphila* were lowered in the midgut (from 42 to 17% ($P = 0{\cdot}043$)) but not the hindgut ($P = 0{\cdot}592$; Table 2), while detection rates of *Pseudomonas* increased (from 6 to 31% in the midgut, $P = 0{\cdot}007$, and 2 to 24% in the hindgut, $P = 0{\cdot}004$; Tables 1 and 2). In contrast, when compared to the positive control for *Pseudomonas*, *E. tracheiphila* detection rates increased in both gut locations (midgut $P = 0{\cdot}002$, hindgut $P = 0{\cdot}001$), which simply reflects the absence of *Erwinia* in the *Pseudomonas*-positive control, and the addition of *Erwinia* from the mix treatment. There was no significant change in *Pseudomonas* detection rates between the *P. fluorescens* control and the mix treatment.

Treatment with a sequence

Beetles dosed first with *E. tracheiphila* and challenged with *P. fluorescens* 1 day later (treatment 5) had both bacteria in the mid and hindguts (Table 1). However, the plant pathogenic species, *E. tracheiphila* was less prevalent in the mid and hindguts (17% and 8% respectively) than the *E. tracheiphila*-positive controls (42% of midguts and 29% of hindguts), and this reduction was significant in the midgut ($P = 0{\cdot}038$) and marginally significant in the hindgut (0·070). This sequential treatment also resulted in the highest rates of *Pseudomonas* detection in both gut locations (midgut 71%, hindguts 38%, Table 1), a significant increase compared to the *Erwinia*-positive controls ($P < 0{\cdot}0001$ for both gut locations), and surprisingly also for the midgut of the *Pseudomonas*-positive control ($P = 0{\cdot}017$) (Table 2).

Beetles dosed first with *P. fluorescens* and challenged 1 day later with *E. tracheiphila* (treatment 6) had only *P. fluorescens* in the mid and hindguts (50 and 29% respectively). *Erwinia tracheiphila* was not found in the alimentary canal of any these beetles (Table 1). Both the elimination of *E. tracheiphila* and the increase in *Pseudomonas* was significant when compared to the *Erwinia*-positive controls ($P < 0{\cdot}001$). There was no significant change in the rates of *Pseudomonas* detection compared to the *P. fluorescens*-positive control. Example alimentary canal images of the mid and hindgut using fluorescent *in situ* hybridization illustrate the colocalization of the bacteria in Fig. 3. Each beetle treated with both bacteria were analysed for bacterial presence in both the mid gut and hind gut for *E. tracheiphila* and/or *Pseudomonas* (Table 2). Specific gut locations, either in the midgut or the hindgut, for each bacterium were counted in the mix (treatment 4) and the sequential dosing (treatments 5 and 6). This data illustrates the presence of no, either, or both bacteria for co-localization potential (supplemental table).

Discussion

In vitro interactions of *P. fluorescens* and *E. tracheiphila*

Based on studies of *P. fluorescens* inhibition of *E. amylovora,* we hypothesized that *P. fluorescens* would likewise inhibit *E. tracheiphila*. We used King's B medium (a semiselective medium that supports both bacterial species) to study

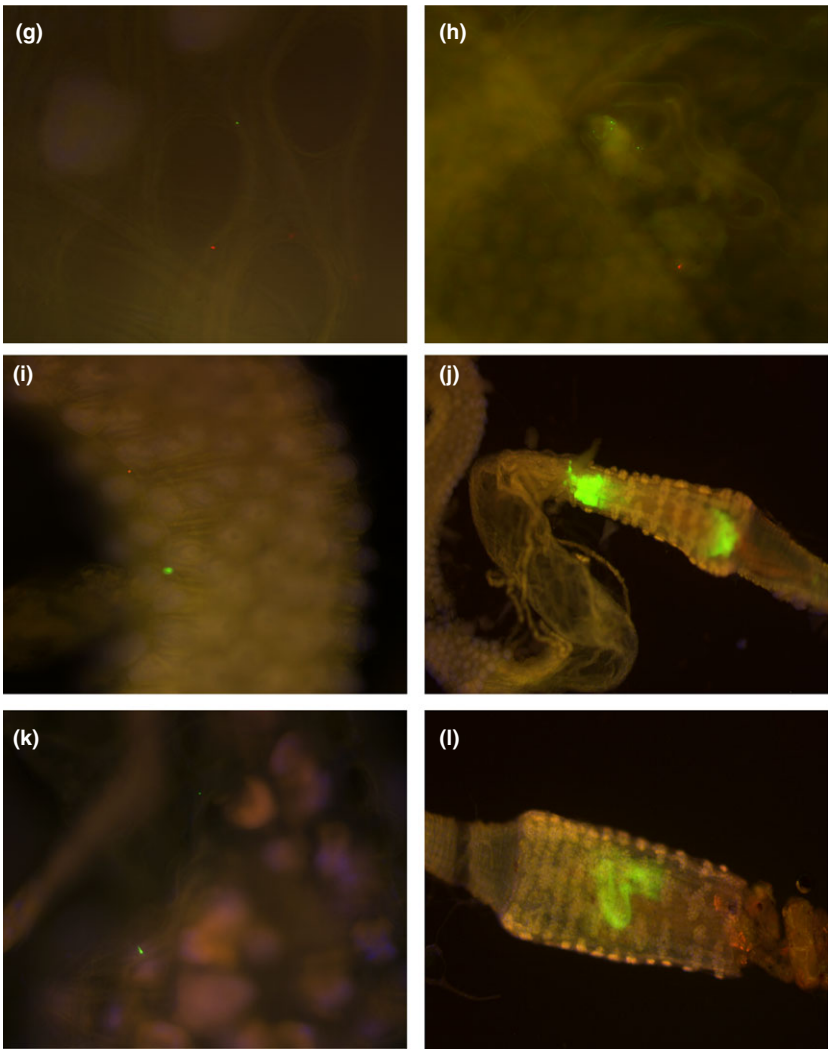

Figure 3 Images of *Acalymma vittatum* alimentary canal control treatments, all were stained with DAPI (blue) for nuclei, Pseudo120 6FAM (green) for *Pseudomona fluorescens* and Et16s Rhodamine (red) for *Erwinia tracheiphila*. *Erwinia tracheiphila* with *P. fluorescens* mix (treatment 4) *A. vittatum* midgut at 20× (g) and hindgut at 20× (h). *Erwinia tracheiphila* challenged with *P. fluorescens* (treatment 5) *A. vittatum* midgut at 20× (i) and hindgut at 4× (j). *Pseudomonas fluorescens* challenged with *E. tracheiphila* (treatment 6) *A. vittatum* mid gut at 20× (k) and hindgut at 4× (l).

heterospecific bacterial interaction using a modified agar disc diffusion assay. *Pseudomonas*-specific production of fluorescein on King's B was the growth characteristic used to distinguish between *P. fluorescens* and *E. tracheiphila*.

In the *in vitro* experiments, inhibition of growth rate and clearing (zone of inhibition) between resident and challenging bacterial species were used to assess antagonism. When a lawn of *P. fluorescens* was challenged from a central disc of *E. tracheiphila*, *E. tracheiphila* did not induce a zone of inhibition in the resident *P. fluorescens* lawn, nor was fluorescein production by *P. fluorescens* inhibited. In contrast, when the resident lawn of *E. tracheiphila* was challenged with *P. fluorescens*, *P. fluorescens* induced a zone of inhibition against *E. tracheiphila* in 23 of 29 plates. This indicates that growth of *P. fluorescens* was necessary to inhibit *E. tracheiphila*. Once a zone of inhibition was present, *P. fluorescens* maintained the inhibition of *E. tracheiphila* over 7 days. This could be explained by the increase in growth of the *P. fluorescens* from the central disc as nutrients became less available in the immediate area of inoculation based on the general increase in diameter of the fluorescein secreted and the zone of inhibition. With these data, we conclude that in a restricted nutrient environment, *P. fluorescens* prevents the growth of *E. tracheiphila*. We cannot state whether this was caused by antibiosis or siderophore excretion. *Pseudomonas fluorescens* did, however, secret fluorescein into the media in all replicate plates and the diameter of the fluorescein increased over time. Fluorescein production, however, was not always indicative of a zone of inhibition against the *E. tracheiphila*. There could have been some selection for specific pathways being turned on or off, such as fluorescein production in *P. fluorescens* and biofilm production in *E. tracheiphila*.

In situ analysis of *P. fluorescens* and *E. tracheiphila*

To investigate if *P. fluorescens* may be a potential biological control candidate within the beetle, we used a colocalization FISH assay for *E. tracheiphila* and *P. fluorescens* to

determine whether they would (i) colonize the same region of the alimentary canal of the beetle and (ii) whether *P. fluorescens* would inhibit retention of *E. tracheiphila* in the alimentary canal. We demonstrated that the *E. tracheiphila*-specific probe hybridizes with the strains of *E. tracheiphila* and will also work within the alimentary canal of *A. vittatum*. The probe was also tested for specificity with RNase-treated alimentary canals to determine if other DNA segments could provide a non-specific binding target, upon which there was no reaction.

Erwinia tracheiphila was not detected in any of the water controls (treatment 1). A small percentage of beetles, however, was positive for *Pseudomonas* species. The Pseudo120 probe is specific for Group I *Pseudomonas,* thus we cannot be certain what species was detected, and we did not identify this naturally occurring isolate. If the unidentified *Pseudomonas* species was not *P. fluorescens*, it may be another candidate for biological control since it resides in the same location of the beetle alimentary canal as *E. tracheiphila*.

We have shown that *E. tracheiphila* can survive and be retained in the alimentary canal of *A. vittatum* 4 days after inoculation. This is consistent with prior vector competence and bacterial retention studies (Shapiro *et al.* 2014).

We confirmed that *P. fluorescens* could be detected in both the mid and hindgut of the alimentary canal of *A. vittatum*. *Pseudomonas fluorescens* was retained for 4 days, which indicates that the alimentary canal is a micro-aerobic environment and provides enough oxygen for the bacteria. This gives support for the possibility of these bacteria interacting within the alimentary canal due to their presence in the same regions of the alimentary canal. Future studies on *P. fluorescens* colonization and survival rates within the alimentary canal are warranted. Several studies have monitored *P. fluorescens* survival on roots, flowers and leaves (Ganeshan and Kumar 2005). *Pseudomonas fluorescens* epiphytic survival in greenhouse and field trials are also needed to determine if *A. vittatum* would reasonably interact with the bacteria.

We demonstrated that when we fed *A. vittatum* a mix of both bacteria simultaneously, *E. tracheiphila* and *P. fluorescens* could be found in the same portion of the hindgut in close proximity. In a field setting, it would be possible for a beetle to ingest both bacteria simultaneously by feeding on a symptomatic plant that had been treated with *P. fluorescens*. Currently, the proportion of beetles harbouring *E. tracheiphila* increases dramatically during the field season (Fleischer *et al.* 1999). Although the *P. fluorescens* did not eradicate the *E. tracheiphila* in this treatment, it did limit the maintenance and retention of *E. tracheiphila* providing partial control that could slow disease progression through a crop.

When *E. tracheiphila* was challenged by *P. fluorescens* in the *A. vittatum* alimentary canal (treatment 5), *E. tracheiphila* was present in fewer beetles compared to the *E. tracheiphila*-positive controls. In contrast, there was about the same number of beetles with *Pseudomonas* as compared to the *P. fluorescens*-positive controls (treatment 3). Taken together, these data suggest that *P. fluorescens* shows promise as a potential inhibitor of *E. tracheiphila* maintenance in beetles.

When *A. vittatum* was first inoculated with *P. fluorescens* and challenged with *E. tracheiphila* (treatment 6), we did not detect *E. tracheiphila* in the alimentary canal. *Pseudomonas fluorescens* was retained at approximately the same rate as the *P. fluorescens*-positive control. This indicates that *P. fluorescens* can prevent *E. tracheiphila* from being retained in the alimentary canal and potentially inhibit transmission/maintenance by the beetle vector. If these findings hold true under field conditions, a foliar spray of *P. fluorescens* may provide an effective treatment for newly emerged beetles that have not been inoculated with *E. tracheiphila* and thus disrupt the overwintering stage of the disease cycle. A commercially available *P. fluorescens* foliar spray has been used to disrupt the transmission of *E. amylovora* and these data provide evidence of the possibility of managing the plant pathogen using similar application methods to target gut interactions within *A. vittatum* to decrease vector capacity of the beetle.

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Conflict of Interest

Dana C. Roberts none, Shelby J. Fleischer none, Joyce Sakamoto none, Jason Rasgon none.

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