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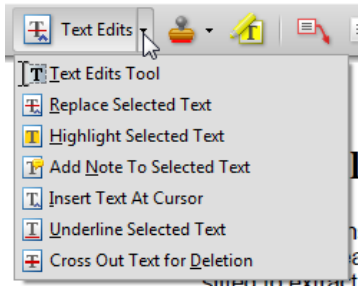
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## RESEARCH ARTICLE

### Two strains of *Pseudomonas fluorescens* bacteria differentially affect survivorship of waxworm (*Galleria mellonella*) larvae exposed to an arthropod fungal pathogen, *Beauveria bassiana*

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Two strains of *Pseudomonas fluorescens* were found contaminating a biopesticide used in a previous study against *Varroa destructor* infestations in honey bee hives. In that study, the biopesticide, a formulation of a fungal pathogen of arthropods, *Beauveria bassiana*, failed to have any negative impact on the mite infestation despite successful results in previous studies using uncontaminated batches of the same biopesticide. The objective of the present research was to determine whether the bacteria may have interfered with the infectivity and/or virulence of *B. bassiana* in a simplified system; positive results in that system would then provide a rationale for further work under more complex conditions. *Galleria mellonella* late instar larvae treated topically with both a bacterial suspension of  $6.8$  to  $7.0 \times 10^7$  cfu/ml and a fungal suspension of  $2.5 \times 10^7$  or  $2.5 \times 10^8$  *B. bassiana* conidia/ml showed, in the case of one of the bacterial strains, significantly increased survivorship compared to larvae treated with just the *B. bassiana* suspension. When larvae were immersed in a bacterial suspension prior to application of *B. bassiana* suspension using a spray tower, a significant positive effect of the same *P. fluorescens* strain on larval survivorship was observed at  $2.5 \times 10^8$  conidia/ml. Neither the bacterial suspensions alone nor blank control solutions had any effect on larval survivorship. These results show that an interaction between the bacteria and the pathogen may explain some of the results from the prior field trial.

**Keywords:** biopesticide; microbial contamination; *Galleria mellonella*; entomopathogenic fungus

#### Introduction

The pathogenic fungus *Beauveria bassiana* (Balsamo) Viullemin (Hypocreales: Cordycipitaceae) has a wide host range among arthropods (Tanada and Kaya 1993) and has been used either as pure conidia or a formulated biopesticide to control many arthropod species, including whiteflies (Islam, Castle, and Ren 2010), mosquitoes (Farenhorst et al. 2009) and tetranychid mites (e.g. Bugeme, Knapp, Boga, Wanjoya, and Maniania 2010), either as pure conidia or as part of a biopesticide formulation. Commercially, conidia of *B. bassiana* are often produced

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45 using large vats holding tens to hundreds of kilograms of growth media, and the occasional presence of microbial contaminants is not uncommon. Jackson, Payne and Odelson (2004) observed that low levels of bacterial contamination in mass-produced conidia of another entomopathogenic fungus *Isaria* (formerly *Paecilomyces*) *fumosoroseus* did not cause significant problems. However, as Jenkins and Grzywacz (2000) argued, higher levels of microbial contamination can interfere with, among other things, biopesticide efficacy. A biopesticide contaminated with bacteria involves a system with at least three components (biocontrol agent, bacteria and target pest), and there is comparatively little information on the effects of contaminating bacteria in such systems.

55 Recently, Meikle, Mercadier, Guermache and Bon (2012a) reported that a *B. bassiana*-based biopesticide contaminated by two strains of *Pseudomonas fluorescens* (Pseudomonadales: Pseudomonadaceae), hereafter PSP1 and PSP2, was ineffective against *Varroa destructor* mite infestations in honey bee hives. Indeed, the observed high incidence of colony death coupled with large *Varroa* populations despite repeated biopesticide applications was at odds with previous field test results using different batches of the same biopesticide (see Meikle, Mercadier, Holst, Nansen, and  
60 AQ4 Girod 2007, 2008, Mercadier, Annas, and Holst 2009). In this case, the *P. fluorescens* contaminants were detected only after the field trial when biopesticide samples were plated on antibiotic-free media. *Pseudomonas* bacteria are commonly found in spoiled food and unclean water (Dogan and Boor 2003, Sacchetti, De Luca, and Zanetti 2007). *P. fluorescens* strains have also been found to reduce the virulence of fungal plant pathogens (de Souza, de Boer, de Waard, van Beek, and Raaijmakers 2003, Haas and D efago 2005), to induce plant defences against insect pests (Bandi and Sivasubramanian 2012) and have even been proposed as components of *B. bassiana*-based biopesticides for use against gelechiid leafminers and collar rot diseases in groundnut agriculture (Senthilraja et al. 2010). In *in vitro* laboratory tests, both bacterial strains slowed or suppressed *B. bassiana* growth on standard media (Meikle et al. 2012a). In trials with caged bees, however, only strain PSP1 had an effect on bee life span: honey bees given food inoculated with PSP1 and kept at 33 C had significantly shorter lifespans compared to bees given food inoculated with *B. bassiana* conidia; the opposite result was observed at 30 C. These results may be linked to how well the bacterial and fungal isolates grow at those temperatures; germination of the *B. bassiana* isolate used in those experiments was markedly slower at 34 C than at lower temperatures (Meikle et al. 2008), but the bacterial isolates grew well even at 35 C (Meikle et al. 2012a).

80 The experiments described here were intended to further examine the possible role of the *P. fluorescens* strains in the problems encountered with the field test of the *B. bassiana*-based biopesticide. In these studies, the waxworm, *Galleria mellonella* L., was used as a test insect because they are common laboratory subjects, easy to maintain and are comparatively sensitive to entomopathogenic fungi (Goettel and Inglis 1997). The ecology of *G. mellonella* in a bee hive is clearly different than that of *V. destructor*, but the focus of the study was on the possible interaction between a fungal pathogen and a bacterium; if such an interaction could be demonstrated in these conditions, that would support the argument that it may occur in other  
85 AQ1 conditions. Whereas the *B. bassiana* isolate used in Meikle et al. (2012) originated in France, the isolate used here, GHA, originated in the United States, has been used extensively in research and is commercially available.

95 These experiments were designed to examine several questions: (1) Does exposure to bacteria affect the longevity of waxworms; (2) Do the bacteria affect the virulence of *B. bassiana* when waxworms are treated with both at the same time; and (3) Does the application method influence the bacterium-fungus-arthropod system? The response variables were *G. mellonella* survivorship and infection rates. In the first experimental series, we examined the infection rate and survivorship of waxworms inoculated with a bacterial suspension, a *B. bassiana* conidial suspension or both, by applying suspensions directly on the cuticle of the waxworms. The second experimental series was designed to simulate spray treatment of insects by *B. bassiana* in an environment previously inoculated with *P. fluorescens*, such as from previous treatments with a contaminated biopesticide. It is possible that these *P. fluorescens* strains colonised the interior of the hive; some *P. fluorescens* strains can survive on honey (Mundo, Olga, Padilla-Zakour, and Worobo 2004) and probably on stored pollen as well.

## Materials and methods

### *Bacterial suspensions*

100 The two bacterial strains PSP1 and PSP2 used in this study were collected and identified as described in Meikle et al. (2012). Bacterial collections are maintained in glycerol at  $-80^{\circ}\text{C}$  at the European Biological Control Laboratory, USDA-ARS, St Gely du Fesc (34), France. A preculture was obtained by plating each strain onto King B agar medium in Petri dishes (10 cm diameter) and incubated overnight at  $26^{\circ}\text{C}$ . Bacterial preculture was transferred using a loop into 10 ml of 50 mM phosphate ( $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ ) buffer, at pH 6.85 in a 15-ml plastic tube. The 10-ml suspension was agitated with a vortex and distributed into several cryovials. The cryovials were kept at  $4^{\circ}\text{C}$  until shipment to the United States under APHIS PPQ permit. At the Texas laboratory, the density of colony-forming units (cfu) was determined by serially diluting aliquots of the bacterial suspensions onto Sabouraud dextrose yeast extract agar and counting colonies after 48 hours.

### *Fungal suspensions*

120 *B. bassiana* strain GHA (ARSEF 6444) conidia in the form of a dry, technical grade powder were obtained from the Northern Plains Agricultural Research Laboratory, USDA-ARS, Sidney, MT. This fungal strain is different from that used in Meikle et al. (2012a). Conidial concentration and viability were measured in that laboratory just prior to transfer as  $1.4 \times 10^{11}$  conidia/g and 85% viability. The conidia were sent by overnight express to the Honey Bee Research Unit, USDA-ARS, in Weslaco, TX, where the experiments were conducted and stored at  $4^{\circ}\text{C}$  until the day of the experiment. 'Low', 'medium' and 'high' suspensions were prepared by mixing by vortexing 2.2, 22 or 220 mg of conidia, respectively, with 10 ml of a solution of sterile aqueous 0.02% sterile SilWet L77 (Loveland Products, Inc.). The 'low' suspension was calculated to have about  $2.5 \times 10^7$  conidia/ml, the 'medium' suspension about  $2.5 \times 10^8$  conidia/ml and the 'high' suspension at  $2.5 \times 10^9$  conidia/ml.

### Experiment 1

135 Waxworms were obtained from Carolina Biological Supply, Co. (Burlington, NC, USA). A separate shipment of waxworms was obtained for each experiment; each shipment contained 75–115 waxworms of third instar or older, along with enough insect diet to maintain the waxworms until the experiments. Waxworms were placed individually in Petri dishes (5.5 cm diameter) with a piece of Whatman filter paper (3 cm diameter); no diet was included. Waxworms were divided into seven approximately equal groups with 11–17 waxworms in each group, depending on larval availability at the time of the experiment. Each group of waxworms was assigned to one of seven treatments:

1. 10  $\mu$ l of Silwet solution, followed by 10  $\mu$ l of 50 mM phosphate buffer;
2. 10  $\mu$ l of Silwet solution, followed by 10  $\mu$ l PSP1 suspension alone;
3. 10  $\mu$ l of Silwet solution, followed by 10  $\mu$ l PSP2 suspension alone;
4. 10  $\mu$ l of phosphate buffer, followed by 10  $\mu$ l GHA alone;
5. 10  $\mu$ l of PSP1 followed by 10  $\mu$ l GHA;
6. 10  $\mu$ l of PSP2 followed by 10  $\mu$ l GHA; and
7. 1.7. Untreated control.

150 The conidial dose per waxworm, about  $10^5$ – $10^7$ , was somewhat higher than that observed per bee in field experiments involving treated bee hives (Meikle et al. 2007, 2009); conidial dose per varroa mite has not been reported from field experiments. Waxworms were treated individually, and treatment suspensions were applied directly to the insect's cuticle using a 2–20- $\mu$ l micropipette (Pipetman Gilson, Inc., Middleton, WI, USA). If the insect was treated with only one microbial suspension, the blank was applied first. For the sake of consistency, if the insect was treated with both fungal and bacterial suspensions, the bacteria were applied first. No more than a few minutes elapsed between applications of different solutions on any given insect.

160 After application of the solutions and suspensions, the filter paper in each Petri dish was moistened with 0.1 ml sterilised water, the dish was sealed with Parafilm and placed in a controlled temperature and humidity (CTH) incubator at 30°C and about 50% r.h. Insects were monitored daily until they died, emerged as adults or the study was terminated. All dead insects were plated onto water agar with chloramphenicol and observed for at least 21 days for growth and sporulation of *B. bassiana*. The proportion of cadavers found infected with *B. bassiana* was used to calculate the infection rate for the treatment group. The experiment was conducted twice at each *B. bassiana* suspension for a total of six trials.

### Experiment 2

170 Experiment 2 was similar to experiment 1 except that a Potter Spray Tower (Burkard Manufacturing, Ltd., Herts., UK) was used to apply the conidial suspension, rather than a pipette. Experiment 2 was intended to simulate a spray treatment of *B. bassiana* conidia into an environment, e.g. the interior of a bee hive, which had been previously inoculated with *P. fluorescens*, e.g. from a prior treatment with contaminated biopesticide. The relationship between the spray tower load and the final conidial coverage of the sprayed surface was first estimated by applying 2 ml of



suspensions containing either  $2.8 \times 10^7$  or  $2.6 \times 10^6$  conidia/ml, onto a hemocytometer (Hausser Scientific, Horsham, PA, USA) on the treatment platform and spraying the suspension at a pressure of 0.75 bar. After spraying, a 0.1-mm thick cover slip was placed over the hemocytometer, and the number of spores per square millimeter was counted using a binocular microscope (Meijer, Inc., Tokyo, Japan) at  $400 \times$ . The conidial concentration was measured three times for each dose.

The seven treatment groups were as follows:

1. 10  $\mu$ l blank SilWet solution.
2. 10  $\mu$ l of PSP1, followed by 2 ml GHA  $2.5 \times 10^7$  conidia/ml in spray tower.
3. 10  $\mu$ l of PSP2, followed by 2 ml GHA  $2.5 \times 10^7$  conidia/ml in spray tower.
4. 2 ml GHA suspension of  $2.5 \times 10^7$  in spray tower only.
5. 10  $\mu$ l of PSP1, followed by 2 ml GHA  $2.5 \times 10^8$  conidia/ml in spray tower.
6. 10  $\mu$ l of PSP2, followed by 2 ml GHA  $2.5 \times 10^8$  conidia/ml in spray tower.
7. 2 ml GHA suspension of  $2.5 \times 10^8$  in spray tower only.

The 'high' conidial dose was not included because of the risk of plugging the spray nozzle and affecting the treatments. Each treatment group contained 12 waxworms, with three waxworms being treated at each spraying. After treatment with the bacteria, 2–3 hours elapsed before treatment with GHA. After treatment with GHA, waxworms were placed at 30°C in a CTH incubator as described for experiment 1 and observed until death or emergence as an adult. Dead larvae were plated on water agar as described above.

This experiment was repeated with two changes: (1) rather than treating waxworms with bacterial suspension using a micropipette, a small (5.5 cm diameter) Petri dish was filled with 1–2 ml of bacterial suspension and waxworms were immersed for 10–15 sec in the suspension to ensure complete coverage, with an additional 50  $\mu$ l of bacterial suspension added to the filter paper in the final Petri dish; and (2) two treatment groups of waxworms exposed to bacteria (one group for each strain) using the immersion method were added as a control for the application method. Each treatment group contained 15 waxworms.

### Statistical analysis

Comparisons of adult emergence and infection rates for the different treatment groups were made using a mixed-model ANOVA (version 9.2; SAS, Inc.). Proportion data were transformed as arcsine of the square root prior to analysis.

For survivorship analyses, insect data were censored if the insect died due to injury from handling during the study or the insect was still alive at the end of the study as either a larva or a pupa. All insects that either died or emerged as adults during the study were not censored. Emerged adults were considered to have escaped the treatment with GHA they had as larvae. Thus, the question addressed by the survivorship analysis was: Do the treatments significantly affect the length of time between application and either death or emergence as an adult? Data on the incidence of *B. bassiana* infection and the proportion of insects successfully pupating were examined separately from the survivorship data.

Insect survivorship was analysed using one of two procedures: the Kaplan–Meier product limit method (SigmaPlot, version 11.0; Systat Software, Inc.) and the

220 proportional hazards, or Cox, regression method (version 9.2; SAS Inc.). The  
Kaplan–Meier method, in which survivorship was estimated using continuous time  
data (Singer and Willett 2003) and pairwise comparisons of treatment groups made  
using the Holm-Sidak method, is in many ways preferable because the survivorship  
225 curves generated are easy to interpret and they have a value for all observed event  
times, unlike Cox regression (Singer and Willett 2003). However, the Kaplan–Meier  
method does not take into account the effects of random factors as does the Cox  
regression and so is unsuitable for analyses across two or more replicate trials unless  
data are pooled. Cox regression shows the risk for an event (death or emergence) for  
an individual relative to a given reference group (Singer and Willett 2003). Thus, for  
230 analyses across two trials at each conidial concentration in experiment 1, Cox  
regression was used, and for analyses of each of the two trials in experiment 2, the  
Kaplan–Meier product limit method was used. Cox regression analyses were  
conducted with treatment as a fixed factor and experiment number as a random  
factor. Analyses consisted of three successive parts: the first analysis, in which all  
235 treatment groups included with GHA alone as the reference group; followed by the  
second analysis in which GHA alone and any groups not significantly different from  
that group were excluded; followed by the third analysis in which the reference group  
from the second analysis and all not significantly different groups were excluded.  
Kaplan–Meier analyses of the two trials in experiment 2 were conducted within  
240 GHA conidial concentration, such that the first analysis included comparisons  
among treatment groups 2.1, 2.2, 2.3 and 2.4, and the 2nd analysis included groups  
2.1, 2.5, 2.6 and 2.7.

## Results

### *Microbial concentrations*

245 Bacterial concentrations were checked five times for each strain, just before the  
experiments. No time trend was observed, that is, concentrations were relatively  
stable over time. Average concentrations of PSP1 were  $6.8 \pm 1.7 \times 10^7$  cfu/ml, and  
those of PSP2 were  $7.0 \pm 1.7 \times 10^7$  cfu/ml. Germination rate of the *B. bassiana* GHA  
conidia as determined immediately before the experiment was  $84.5 \pm 1.7\%$  with  $1.4 \times$   
250  $10^{11}$  conidia/g, which were in agreement with the original determinations.

### *Experiment 1*

Survival to emergence as an adult was significantly lower for waxworms treated with  
low ( $F = 14.06$ ;  $df = 6,7$ ;  $P = 0.0014$ ), medium ( $F = 7.73$ ;  $df = 6,6$ ;  $P = 0.0126$ ) and  
255 high ( $F = 193.87$ ;  $df = 6,7$ ;  $P < 0.0001$ ) GHA conidial suspensions compared to  
waxworms treated with bacterial suspensions alone, control solutions or left  
untreated (Figure 1A). Infection was, as expected, strongly associated with GHA  
conidial concentration at the low ( $F = 11.24$ ;  $df = 6,6$ ;  $P = 0.0048$ ) and medium  
rates ( $F = 42.08$ ;  $df = 6,7$ ;  $P < 0.0001$ ); at high conidial concentrations, cadavers of  
260 all waxworms treated with GHA sporulated, while no waxworms from other groups  
did (Figure 1B). It should be noted that, because the cadavers were not surface  
sterilised, confirmed infection by *B. bassiana* here does not necessarily mean the  
waxworm died from infection, as *B. bassiana* can act as both a pathogen and a

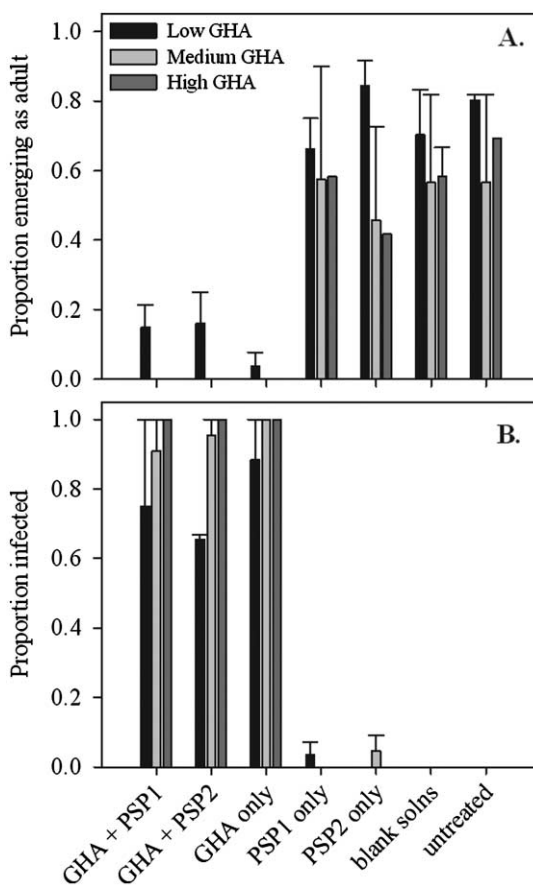


Figure 1. Mean ( $\pm$ SE): (A) survivorship to adult and (B) infection rates for *G. mellonella* larvae subjected to one of the seven treatments in experiment 1: (1) PSP1 with and (2) without GHA, (3) PSP2 with and without GHA, (4) GHA alone, (5) larvae treated with blank phosphate buffer and (6) dilute detergent and (7) untreated larvae. Three concentrations of GHA suspensions were used:  $2.5 \times 10^7$ ,  $2.5 \times 10^8$  and  $2.5 \times 10^9$  conidia/ml. Error bars show experiment-wide standard error.

saprophyte (Tanada and Kaya 1993). However, given the conducive conditions for fungal growth, a lack of infection strongly suggests that the waxworm did not die of *B. bassiana* infection. At the low conidial concentration, some waxworms treated only with GHA escaped infection but not at higher conidial concentrations. Two waxworms from groups not treated with GHA did show signs of infection; however, the growth of the *B. bassiana* was slow and appeared only 2–3 weeks after treatment, suggesting that infection likely occurred during handling the waxworms after they had died of other causes.

Cox regression results for larval survivorship were similar between the low and medium GHA conidial concentration trials (Table 1). The same reference group, GHA alone, was used for initial analyses at all conidial concentrations. The PSP2 + GHA group had a significantly lower proportional hazard than either PSP1 + GHA or GHA alone at both conidial concentrations. In the second analysis, in which

Table 1. Cox proportional regression results for experiments in which waxworms were exposed to various combinations of GHA conidia suspensions, PSP1 suspensions, PSP2 suspensions and blank solutions (see text for details).

[GHA] ml <sup>-1</sup>		Treatment	Hazard	Wald $\chi^2$	<i>P</i>	
2.6 × 10 <sup>7</sup>	First analysis	Blank solutions	0.133	35.038	<0.001	
		Ref. group	GHA + PSP1	0.974	0.008	0.002
	GHA only	GHA + PSP2	0.378	9.907	0.928	
		PSP1 only	0.147	33.750	<0.001	
		PSP2 only	0.098	43.141	<0.001	
		Untreated	0.117	38.232	<0.001	
		Second analysis	Blank solutions	0.364	9.839	0.002
			Ref. group	PSP1 only	0.398	8.648
	2.6 × 10 <sup>8</sup>	First analysis	Blank solutions	0.060	63.914	<0.001
			Ref. group	GHA + PSP1	0.628	2.748
GHA only		GHA + PSP2	0.412	9.283	0.002	
		PSP1 only	0.038	79.296	<0.001	
		PSP2 only	0.033	85.270	<0.001	
		Untreated	0.046	74.137	<0.001	
		Second analysis	Blank solutions	0.095	45.210	<0.001
			Ref. group	PSP1 only	0.061	59.352
GHA + PSP2	PSP2 only	0.053	64.635	<0.001		
	Untreated	0.074	54.213	<0.001		

Note: The response variable was the longevity of the waxworms in days after exposure. ‘Ref. group’ refers to the reference group for the particular analysis (the group for which longevity was shortest) and ‘Hazard’ shows proportional hazards relative to that group.

GHA alone and GHA + PSP1 groups had been removed, the PSP2 + GHA group was significantly different from the remaining groups. In the third analysis, the PSP2 + GHA group was removed, and a Cox regression conducted with the remaining four treatment groups; none of the results from the third analyses at either conidial concentration was significant ( $P = 0.65$  for the low dose and  $P = 0.24$  for the medium dose). To illustrate the different effects of the two bacterial strains on larval survivorship, Kaplan–Meier analyses were conducted for the trials with low and medium conidial suspensions by pooling the data across the experiments (Figure 2). Regression results for the high conidial concentration trials showed no significant interaction between bacteria and GHA; the proportional hazard coefficients for waxworms treated with PSP1 and GHA or PSP2 and GHA were not significantly different from those treated with GHA alone ( $P = 0.69$  and  $P = 0.64$ , respectively). When those treatment groups were removed from the analysis, the regression was not significant ( $P = 0.65$ ).

### Experiment 2

The spray tower provided  $385.0 \pm 22.7$  conidia/mm<sup>2</sup> using 2 ml of a  $2.8 \times 10^7$  conidia/ml (corresponding to low dose) suspension and  $39.7 \pm 3.9$  conidia/mm<sup>2</sup> with 2 ml of a  $2.6 \times 10^6$  conidia/ml suspension. Conidia dispersal values are within 5% of those

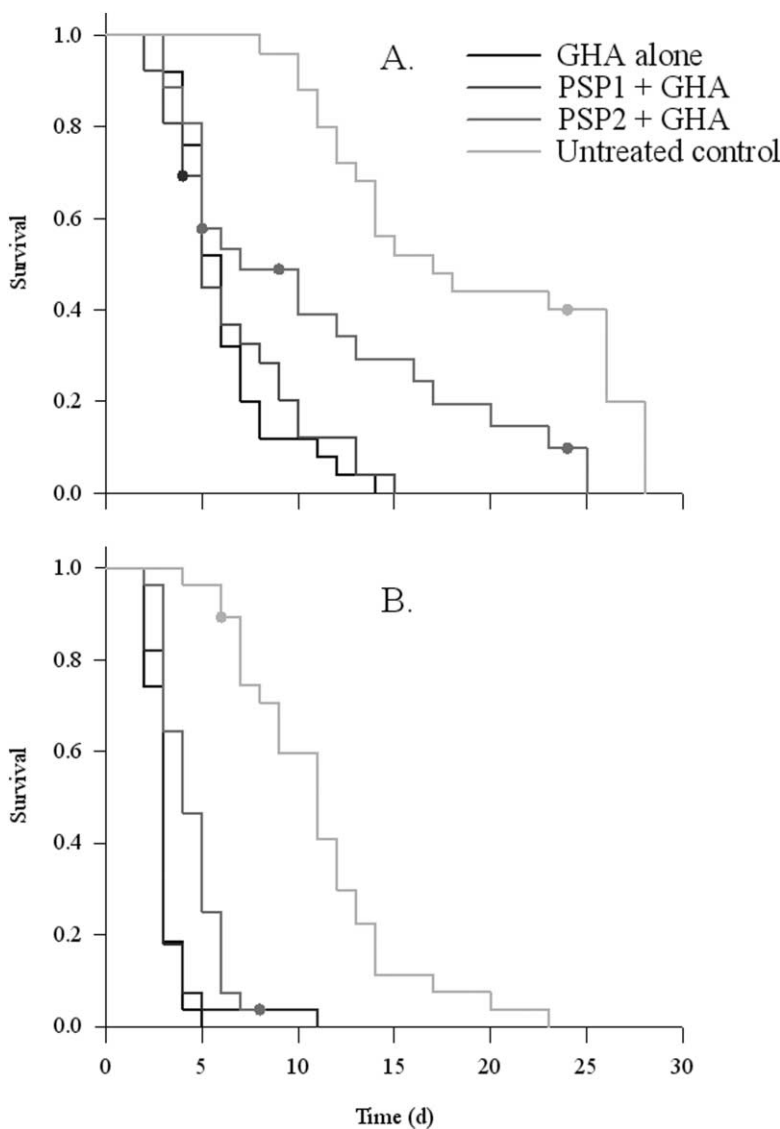


Figure 2. Kaplan–Meier estimates of the continuous-time survivorship functions for waxworms in one of the four treatments of experiment 1: treated with (1) 10  $\mu$ l buffer followed by 10  $\mu$ l GHA conidial suspension (GHA alone) (■); (2) 10  $\mu$ l PSP1 suspension followed by 10  $\mu$ l GHA (PSP1 + GHA) (■); (3) 10  $\mu$ l PSP2 followed by 10  $\mu$ l GHA (PSP2 + GHA) (■) and (4) untreated control (■). (A) Curves for two trials in which the GHA suspension was  $2.5 \times 10^7$  conidia/ml; (B) curves for two trials in which the GHA suspension was  $2.5 \times 10^8$  conidia/ml. Because data were pooled across trials, these Kaplan–Meier analyses were used for graphical purposes only.

AQ3 reported by Meikle, Mercadier, Girod, Derouané and Jones (2006) for similar conidia concentrations using the same model of spray tower.

Kaplan–Meier analyses were similar between the two trials (Figure 3). For treatment groups in both trials in the low GHA dose analysis, waxworms in all

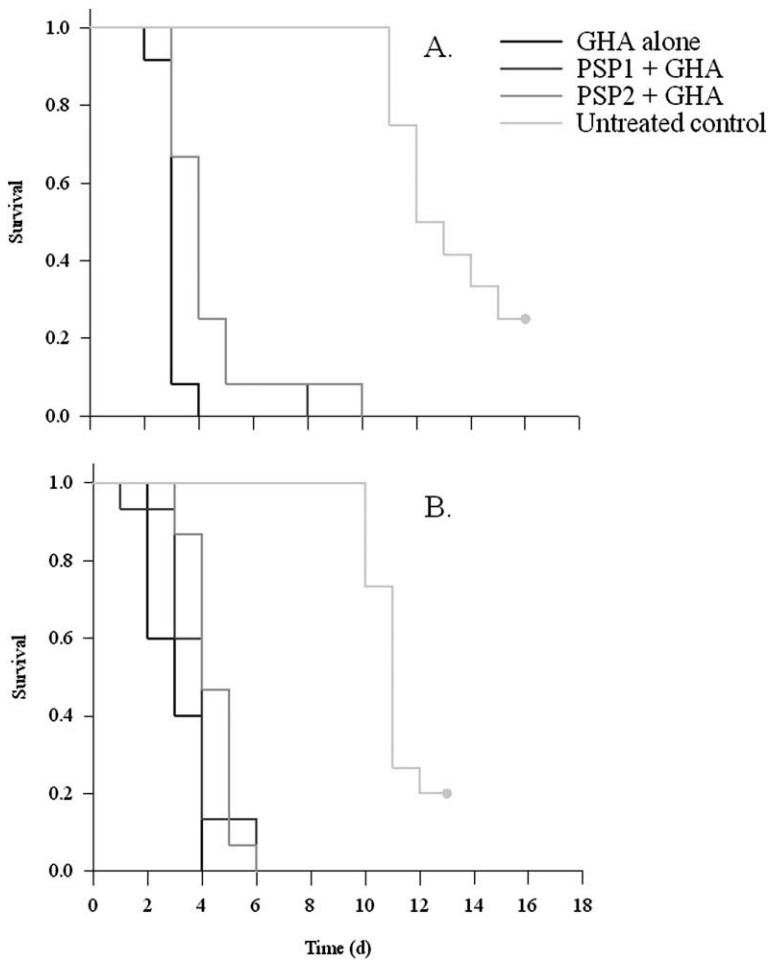


Figure 3. Kaplan–Meier estimates of the continuous-time survivorship functions for waxworms in one of the four treatments of experiment 2: treated with (1) blank phosphate buffer followed by GHA conidial spray was estimated at about 4000 conidia/mm<sup>2</sup> (GHA alone) (■); (2) 10 μl PSP1 suspension followed by the GHA conidial spray (PSP1 + GHA) (■); (3) 10 μl PSP2 followed by the GHA conidial spray (PSP2 + GHA) (■) and (4) untreated control (■). (A) Curves for a trial in which 10 μl of the bacterial suspension or blank phosphate buffer was applied using a micropipette; (B) curves for a trial in which waxworms were immersed in the bacterial suspension or buffer.

groups treated with GHA died significantly faster than those in the control groups (unadj.  $P < 0.0001$  for all comparisons in both trials), but there were no differences among the GHA-treated groups themselves. For treatment groups in the medium GHA dose analysis, waxworms treated with PSP2 + GHA survived  $4.3 \pm 0.6$  d longer in the first trial and  $4.4 \pm 0.2$  d longer in the second trial than those treated with only GHA, which survived  $3.0 \pm 0.1$  d and  $3.0 \pm 0.2$  d, respectively (unadj.  $P = 0.0027$  and  $0.0004$ , respectively) (Figure 2). In the first trial, waxworms treated with PSP1 + GHA also survived longer than those only treated with GHA ( $P = 0.0027$ ) but

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not in the second trial. In the second trial, one larva treated with a medium dose of GHA survived to emerge as an adult. There were no significant differences in survivorship between insects treated with either bacterial strain alone or the control ( $P = 0.53$ ).

## Discussion

Varroa mites are often managed with chemical pesticides (Rosenkranz, Aumeier, and Ziegelmann 2010). An effective biopesticide against Varroa would offer, as in other agricultural systems, several advantages over chemical control for producers and consumers by reducing chemical contamination of products; negative impacts on bees or other desirable arthropods; and the development of pesticide resistance by the target pest (Rosenkranz et al. 2010). However, these advantages may be undermined when the biopesticide is contaminated by other microbes. Such contaminations may affect agricultural endproducts, desirable arthropods may be negatively impacted and the efficacy of the biopesticide may be compromised. In this particular instance, a *B. bassiana*-based biopesticide intended to be used in bee hives against Varroa mites was contaminated with two strains of *P. fluorescens*, PSP1 and PSP2 (Meikle et al. 2012a).

The goal of this study was to investigate whether a *P. fluorescens* strain could affect *B. bassiana* virulence. Clearly, there are major differences between the conditions in this study and those in the field experiment, described in Meikle et al. (2012a), which provided the original impetus for this research. This study involved *B. bassiana*, an arthropod host and the two strains of bacteria, using controlled dosages in a controlled environment, while the field experiment involved *B. bassiana*, the same bacteria, two arthropod species (either of which could act as pathogen hosts), probably variable dosages and largely uncontrolled conditions. In addition, the particular varroa subpopulation, i.e. phoretic, capped-cell or frame, affected by the biopesticide in the field experiment was not known with certainty (Meikle, Sammataro, Neumann, and Pflugfelder 2012b), and that would have implications in designing laboratory experiments to explicitly simulate field conditions. Thus, this study used a simpler system to examine infection interactions, which, if detected, may justify further examination in more complex systems.

Survivorship of waxworms treated with only a suspension of either bacterial strain was not different from that of either untreated waxworms or waxworms treated with blank solutions, either for direct topical application with 10  $\mu$ l of a phosphate buffer-based suspension or immersion in the suspension for a much higher dose. While exposure to certain *P. fluorescens* strains has had negative effects on *Drosophila melanogaster* (Olcott et al. 2010), fourth instar *Hippodamia convergens* larvae (James and Lighthart 1992) and *Odontotermes obesus* (Devi and Kothamasi 2009), the *P. fluorescens* strains in this study had no measurable effect on waxworm longevity.

Waxworms treated directly on their cuticle with both a PSP2 suspension and a GHA suspension of either  $2.5 \times 10^7$  (low) or  $2.5 \times 10^8$  (medium) conidia/ml lived significantly longer than waxworms treated with GHA alone. The bacteria had no effect when waxworms were treated with high dose of  $2.5 \times 10^9$  conidia/ml; all waxworms died rapidly in those treatment groups. Treatment with PSP1 did not have this effect; in all experiments, longevity of waxworms treated with PSP1 + GHA was

indistinguishable from those treated with GHA alone. When GHA was applied with a spray tower using the low and medium dose, no effects of bacterial exposure were observed with the low dose, and effects at the medium dose were significant but weaker.

The effects noted here were consistent. Waxworms treated with PSP2 + GHA tended to live longer, on average, than those treated only with GHA, but they still did not live as long as those in the control group. Treatment with PSP2 increased the variance in the survivorship; while many waxworms treated with PSP2 + GHA died at about the same rate as those treated with GHA alone, some individuals survived much longer. On two occasions, waxworms treated with PSP2 + GHA escaped infection entirely and emerged as adults while no waxworms in the corresponding PSP1 + GHA and GHA-only groups did so. In both spray tower trials, the maximum longevity of waxworms treated with the medium dose of GHA was 4 d, but for waxworms also treated with PSP2, it was 10 d in the first trial and 6 d in the second.

By inoculating waxworms with bacteria first and waiting 2–3 h before treatment with GHA, the spray tower experiments were expected to increase larval survivorship and reduce the infection rate. When the GHA conidia were applied with a spray tower, the coverage of the waxworms by conidia was probably much better than when applied with a micropipette, even if the number of conidia expected to contact the waxworms was lower. Using the micropipette, insects were treated mainly along one side, usually the dorsal surface, while the immersion method and the spray tower treated a much greater proportion of the larval surface. However, despite the higher exposure of larval surface to bacterial cells, the effects of the bacteria on larval longevity were in some respects weaker in the spray tower trials than the direct topical application. Either the immersion technique did not help the bacteria ‘colonize’ the larval cuticle in spite of the higher exposure (perhaps due to insufficient time or nutrients) or application of conidia using the spray tower improved fungal distribution or activity to more than compensate for the higher bacterial exposure.

The mechanism by which some *P. fluorescens* strains might affect *B. bassiana* virulence is not known. Strains of *P. fluorescens* produce surfactants, such as lipopeptides, which lower the surface tension of water, causing rapid lysis of zoospores among zoosporic fungi, and reducing spore germination and hyphal growth among other species of plant pathogenic fungi (Nielsen, Thrane, Christophersen, Anthoni, and Sørensen 2000; de Souza et al. 2003), resulting in lower plant infection rates *in vivo*. The ability to produce these surfactants varies greatly among strains of *P. fluorescens* (de Souza et al. 2003), which may explain why one strain affected *B. bassiana* infection and the other did not. There may be other mechanisms of bacterial inference as well.

These results underscore the importance of checking mass-produced arthropod pathogens for bacterial as well as fungal contaminants. The role of bacterial interference of *B. bassiana* infection in the original field trial described by Meikle et al. (2012a) is still not entirely clear but several aspects remain to be examined. For example, temperature may have affected the bacteria, the fungi or both. In the caged bee study described in Meikle et al. (2012a), raising the temperature from 30°C to 33°C, which is closer to brood nest temperature (Human, Nicolson, and Dietemann 2006), changed the longevity of bees fed PSP1-inoculated food from significantly greater than bees fed *B. bassiana*-inoculated food to significantly less. Temperatures in this range can have strong effects on the growth of entomopathogenic fungi



(Fargues, Maniania, Delmas, and Smits 1992) and may also affect the activity of PSP2.

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