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Susceptibility of adult *Exorista larvarum* to conventional and transgenic *Bacillus thuringiensis galleriae* toxin

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Abstract

Exorista larvarum (L.) (Diptera Tachinidae) is a polyphagous larval parasitoid of lepidopterans, including forest defoliators. Laboratory studies were conducted to investigate the side effects on adult parasitoid longevity and parasitization capacity of conventional and transgenic *Bacillus thuringiensis galleriae* (*Btg*) toxins, active against the pine processionary moth *Thaumetopoea pityocampa* (Denis et Schiffermuller) and the wax moth *Galleria mellonella* (L.). The flies were fed on lump sugar soaked with the bacterial suspensions and were thus treated by direct ingestion. In a first experiment, the Cry9Aa entomocidal toxin from *Btg* was administered at 3-times the dose to which the target lepidopterous species previously proved to be highly susceptible. *E. larvarum* male and female longevity from emergence and parasitization capacity (expressed as eggs/female laid on *G. mellonella* larvae and percentages of eggs which gave puparia) were not significantly affected by the treatment with the Cry9Aa toxin compared to the commercial *Bt* preparation Foray 48B or to distilled water (control). No significant differences were also found between the two controls. In a second experiment, adult longevity and parasitization capacity were not significantly affected by the treatment with a suspension of the epiphytic bacterium *Pseudomonas* Clb01 carrying the *cry9Aa Btg* gene compared to wild type *Pseudomonas* or distilled water. These results indicate that *E. larvarum* adults were not affected either by the conventional or transgenic *Btg* Cry9Aa toxin according to the parameters and under the conditions tested. To complement this study, future investigations will have to be performed in a more realistic scenario than in a laboratory situation.

Key words: entomocidal toxins; engineered epiphytic bacteria; parasitoids.

Introduction

Preparations based on strains of *Bacillus thuringiensis* (*Bt*) are widely used to control foliage-feeding Lepidoptera larvae in agricultural and forest environments (Glare and O'Callaghan, 2000), where tachinids (Diptera Tachinidae) are important biocontrol agents of the target insects and major components of biodiversity (Stireman *et al*., 2006; Dindo, 2011). Although concerns were raised about the potential negative impact of *Bt* on natural enemies (Cannon, 1996), there is no evidence of a clear direct effect (Federici, 2003; Ravensberg, 2011).

The use of epiphyitic bacteria modified with *Bt* genes has been considered as an interesting tool to overcome the problems related to the limited field stability of commercial *Bt*-products (Bora *et al*., 1994). An engineered epiphytic bacterium was constructed by using a pine epiphyte *Pseudomonas* sp. strain Clb01 (16S rDNA sequence 98% homologous to *P. graminis* and *P. lutea*) as a host for the gene encoding the Cry9Aa entomocidal toxin from *Bacillus thuringiensis galleriae* (*Btg*) (Alberghini *et al*., 2005). The *cry9Aa* gene used was selected due to the high efficacy shown by the *Btg* Cry9Aa toxin against first instar larvae of the pine processionary moth *Thaumetopoea pityocampa* (Denis et Schiffermuller) (Shevelev *et al*., 2001). In laboratory studies the bacterial construct showed effective toxicity toward larvae of both the model species *Galleria mellonella* (L.) (Alberghini *et al*., 2005) and *T. pityocampa* (Alberghini *et al*., 2006). Moreover, in a long-term greenhouse experiment the construct effectiveness against the pine processionary moth was more extended (over 100 days) compared to that of a commercial *Bt*-preparation (Foray 48B) (Alberghini *et al*., 2006). These results suggest the potential of *Bt* gene-carrying epiphytic bacteria for more efficient and persistent toxin delivery to the target species. To date, however, the construct has not yet been registered for use in the field, where it could be expected to be applied against the pine processionary moth as well as other lepidopterous defoliators. In this framework, studies must be undertaken in order to evaluate the possible adverse effects of both *Btg* and the engineered bacterium. First, its impact on resident phyllospheric bacteria was assessed on *Pinus mugo* under greenhouse conditions with reassuring results (Alberghini *et al*., 2008). Furthermore, in a laboratory study, Marchetti *et al.* (2009) evaluated the effects of *cry9Aa Pseudomonas* on the post-embryonic development of *Exorista larvarum* (L.) (Diptera Tachinidae), a larval parasitoid of forest defoliating lepidopterans, cultured in the factitious host *G. mellonella*. *E. larvarum* was selected as a model non-target species, because it is likely to be exposed to the epiphytic engineered bacterium in case of its prospective environmental applications, similarly to other tachinid parasitoids of forest lepidopterous defoliators. The post-embryonic development of *E. larvarum* was not found to be altered by host treatment with the bacterial construct under the conditions tested. The purpose of this paper was to complement that study by investigating the effects of both *Bt* and the engineered *Pseudomonas* Clb01 carrying the *cry9Aa Btg* gene on the longevity and parasitization capacity of *E. larvarum* adults. Actually *E. larvarum* adults are active, as a few other species of the same family, during the application time of commercial bacterial preparations against the most important forest pests

such as the gypsy moth *Lymantria dispar* (L.) (Luciano *et al*., 2003) and the pine processionary moth (Battisti *et al*., 1998). In addition, they could ingest or contact the bacterial construct which is very persistent on the leaf surface. Similarly to Marchetti *et al*. (2009), the effects of the transformed vs. the untransformed *Pseudomonas* sp. Clb01 were evaluated and the test was preceded by a comparison between the effects of the *Btg* Cry toxin alone and those of Foray 48B, a commercial preparation of *Bacillus thuringiensis kurstaki* (*Btk*).

Materials and methods

Insect culture

A laboratory colony of *E. larvarum* was maintained using *G. mellonella* as a factitious host as described by Michalkova *et al*. (2009). The colony was established in 1992 and renewed in 2004 from puparia obtained from *L. dispar* and *Hyphantria cunea* (Drury) larvae, both collected in the field in the province of Modena (Emilia Romagna, Northern Italy). *G. mellonella* larvae were reared on a wax-based artificial diet developed by Campadelli (1987), at 30 ± 1 °C, 65 ± 5% relative humidity (RH) and in complete darkness. *E. larvarum* adults were kept in plexiglas cages (40x30x30 cm) in a rearing chamber at 26 ± 1 °C, 65 ± 5% RH and at 16:8 L:D photoperiod. The flies were fed on lump sugar and cotton balls soaked in a honey and water solution (20% honey) (Depalo *et al*., 2010). For the tests, only lump sugar was used.

Test A. Effects of purified Cry9Aa entomocidal toxin and commercial *Btk*

Cry9Aa entomocidal toxin was purified from crystals of *Bacillus thuringiensis* subsp. *galleriae* 11-67 (*Btg*) as described by Chestukhina *et al*. (1994). A commercial preparation of *Bacillus thuringiensis kurstaki* (*Btk*) [Foray 48B® minimum concentration of 2.1% of active toxin (Cry1Aa, Cry1Ab, Cry1Ac, Cry2A), Valent Biosciences, Sumitomo Chemical Agro Europe, St. Didier au Mont d'Or, France] was also tested (Alberghini *et al*., 2006; Marchetti *et al*., 2009).

The pure toxin (1st treatment) was diluted to a concentration of Cry9Aa toxin per g of parasitoid adult diet (= lump sugar) equal to 22 µg, which corresponded to about 3 times the dosage applied per gram of natural or artificial food by Alberghini *et al*. (2006) and Marchetti *et al*. (2009) against target lepidopterous larvae. The commercial *Btk* preparation (2nd treatment) was also diluted to reach the same concentration. Distilled water (3rd treatment) was used as control.

Each treatment consisted of 10 *E. larvarum* adults no more than 12 h old (5 males and 5 females), which were placed together in a plexiglas cage (20x20x20 cm). Flies were supplied with 3 g lump sugar, which was previously soaked either with the suspensions or distilled water (1 ml/g sugar). A drinking trough with distilled water was also placed in each cage. Flies were provided with fresh food every 48 h until death.

To test fecundity, the females were supplied with *G. mellonella* larvae (three per female) daily until death. As the preoviposition period lasts 2 days at least, the larvae were exposed from the 3rd day (Dindo *et al*., 2007). The larvae were left in the cage for 30 min and, after counting the eggs laid on their bodies, they were placed in plastic boxes until puparium formation. Dead females were removed daily.

The treatments were replicated six times. Male and female longevity (in days) was recorded. To evaluate parasitization capacity, the eggs/female laid on host larvae (*e*) (= number of eggs/number of alive females) was calculated daily. The single *e* values were then added to determine the mean number of eggs laid on the larvae throughout female lifespan (*E*). The number of eggs which produced puparia and puparial yield (= percentage of eggs which produced puparia) were also calculated.

Test B. Effects of the engineered bacterium *Pseudomonas* sp. Clb01pDBCRY9 and *Pseudomonas* sp. Clb01

Pseudomonas sp. Clb01pDBCRY9 ("*cry9Aa-Pseudomonas*", Alberghini *et al*. 2005) and its parental plasmidless wild type were used. Both strains were grown at 30 °C in 500 ml cultures of modified King's medium by the methods described by Marchetti *et al*. (2009). After 48 h, the OD_{600} of both cultures were measured (with a Jasco 7800® spectrophotometer). Bacterial suspensions having reached $OD_{600} = 0.9$ were centrifuged at 5000 rpm for 5' in a Hettich MIKRO 22R centrifuge. The resulting cell pellets were resuspended in 15 ml sterile physiological solution (0.9% NaCl). The suspensions contained (mean values for 3 replicates) 2.32×10^7 CFU/ml (*cry9Aa-Pseudomonas*) and 2.36×10^7 CFU/ml (*Pseudomonas* wild type). The flies were fed on a bacterial suspension over a 7-day period. Throughout each replicate of the main experiment described below, the suspensions were stored in the fridge at +4 °C. On the 1st and on the 7th (last) day, a side test was conducted on third instar *G. mellonella* larvae, in order to ascertain the efficacy of the *cry9Aa-Pseudomonas* suspension throughout the experiment. Distilled water was used as control. The test was conducted as described by Marchetti *et al*. (2009) and consisted of 30 larvae per treatment.

For the main experiment three treatments were compared, namely distilled water, *cry9Aa-Pseudomonas* or wild type *Pseudomonas* suspensions. For each treatment 5 newly-emerged *E. larvarum* couples were placed in plexiglas cages, as described for test A. Each cage corresponded to a treatment and was provided with lump sugar, which was previously soaked either with the bacterial suspensions or distilled water in the same amount and proportions as in test A (= 1 ml/g). A drinking trough with distilled water was also placed in each cage. The treated lump sugar was changed daily for 7 days. Subsequently, in all treatments the adults were fed as in the standard rearing conditions, described above, until death.

Three replicates were carried out. Fly longevity, eggs/female, and number of eggs which produced puparia and puparial yields were evaluated as described in Test A.

Statistical analysis

In Test A the data were analyzed by one-way analysis of variance (ANOVA). In Test B the data for *G. mellonella* larval mortality (side test) were analysed by Kruskal-Wallis non parametric procedure, due to heteroscedasticity. The data for *E. larvarum* longevity and eggs/female laid on host larvae (*E*) were analysed by one-way ANOVA. In both tests, an arcsine transformation was used to transform percent values for analysis (Zar, 1984). In Test B, separate 2 by 2 contingency tables were used for testing the independence of treatment and number of eggs which produced puparia. Statistical tests were done with STATISTICA 6.0 (StatSoft, 2001).

Results

Test A. Effects of purified Cry9Aa entomocidal toxin and commercial *Btk*

Male and female longevity from emergence and eggs/female were not significantly affected by the treatment with purified toxin either compared to commercial *Btk* or to distilled water (control). For these parameters no significant differences were also found between commercial *Btk* and distilled water (table 1).

Test B. Effects of *Pseudomonas* sp. Clb01 and the engineered bacterium *Pseudomonas* sp. Clb01pDBCRY9

In the side test conducted on *G. mellonella* larvae, both on the 1st and on the 7th (last) day of the main experiment, the *cry9Aa-Pseudomonas* suspension produced 100% mortality, whereas in the control the mortality was (mean ± SE) 4.4 ± 2.9% (1st day) and 7.8 ± 2.8% (last day). The difference was significant (1st day: $H = 4.35$, $N = 6$, $P < 0.05$; last day: $H = 4.36$, $N = 6$, $P < 0.05$). The efficacy of the engineered bacterium suspension throughout the main experiment was thus shown. In this experiment, male and female *E. larvarum* longevity from emergence and eggs/female were not significantly affected by the treatment with *cry9Aa-Pseudomonas* or wild type *Pseudomonas* suspensions compared to distilled water (table 2). The number of eggs which produced puparia (and relevant puparial yield) was significantly higher for the flies treated with *cry9Aa-Pseudomonas* compared to wild type *Pseudomonas* ($\chi^2 = 10.02$; $P < 0.01$). For this parameter, no significant difference was found either between *cry9Aa-Pseudomonas* and distilled water ($\chi^2 = 3.48$; $P > 0.05$), or between distilled water and wild type *Pseudomonas* ($\chi^2 = 1.79$; $P > 0.05$) (figure 1).

Discussion

A number of laboratory studies have shown that *Bt*-preparations have no detectable effects on adult hymenopterous or tachinid parasitoids, either treated by direct ingestion or contact (Flexner *et al*., 1986; Chilcutt and Tabashnik, 1999; Xu *et al*., 2004; Liu *et al*., 2005; Ruiu *et al*., 2007; Ksentini *et al*., 2010). Blumberg *et al*. (1997) even reported a significant increase in the longevity of the adults of *Microplitis croceipes* (Cresson) fed on a *Bt*-product mixed in a honey solution. A few detrimental effects have however been shown, when high doses of the bacterium were applied (something

Table 1. Effects of purified Cry9Aa entomocidal toxin and commercial *Btk* (Foray 48B) on *E. larvarum* longevity from emergence (days), fecundity (eggs/female laid on host larvae throughout female lifespan) and puparial yield (% eggs which produced puparia).

Treatment	Male longevity (days)	Female longevity (days)	Eggs/female	Puparial yield (%)
Cry9Aa	13.5 ± 1.3	12.8 ± 2.2	59.3 ± 9.9	29.7 ± 2.7
Commercial *Btk*	14.3 ± 1.5	15.1 ± 1.9	49.8 ± 7.5	34.2 ± 4.4
Distilled water (control)	17.2 ± 0.7	18.5 ± 1.4	63.8 ± 6.2	32.9 ± 3.6
F (df)	2.61(2,15)	2.37 (2,15)	0.79 (2,15)	0.36 (2,15)
P	0.11	0.13	0.47	0.71

Within the means (± SE) of each column no significant difference was found ($P < 0.05$, one-way ANOVA). Number of replicates = 6, each consisting of 5 males and 5 females.

Table 2. Effects of the engineered bacterium *cry9Aa-Pseudomonas* and *Pseudomonas* sp. on *E. larvarum* longevity from emergence (days) and fecundity (eggs/female laid on host larvae throughout female lifespan).

Treatment	Male longevity (days)	Female longevity (days)	Eggs/female
Distilled water (control)	13.1 ± 3.9	13.9 ± 1.7	35.5 ± 14.7
cry9Aa-Pseudomonas	14.4 ± 1.2	12.5 ± 4.3	28.3 ± 12
Pseudomonas sp.	9.7 ± 2.9	13.6 ± 3.3	35.1 ± 9.9
F (df)	2.15 (2,6)	0.15 (2,6)	0.33 (2,6)
P	0.198	0.865	0.73

Means (± SE) in a column followed by the same letter are not significantly different ($P < 0.05$, one-way ANOVA). Number of replicates = 3, each consisting of 5 males and 5 females.

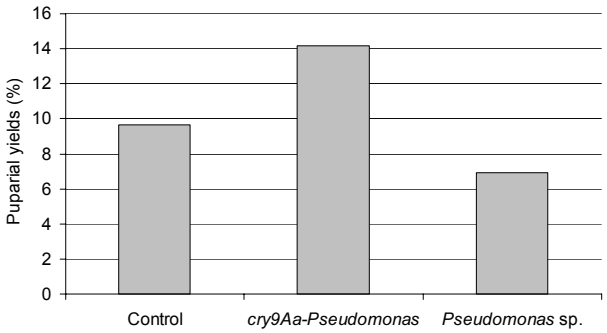

Figure 1. Puparial yield (% of eggs which produced puparia) obtained from eggs laid by *E. larvarum* treated with distilled water, *cry9Aa-Pseudomonas*, *Pseudomonas* sp. Number of eggs: 362 (distilled water), 360 (*cry9Aa-Pseudomonas*), 361 (*Pseudomonas* sp.). See text for statistics.

unlikely to occur under field or forest conditions) (Thoms and Watson, 1986; Salama *et al*., 1991). In the present study, *E. larvarum* adults were fed on lump sugar soaked with the bacterial suspensions and were thus treated by direct ingestion, but they also underwent contact as they were often observed while walking on the treated sugar. No negative effects on *E. larvarum* adults were found. In detail, both the Cry9Aa toxin and the *Btk* preparation did not affect the adult longevity when administered with food at 3-times the dose to which target lepidopterous species proved to be highly susceptible (Marchetti *et al*., 2009). Mating was also not affected as the females normally laid eggs on host larvae. Similarly, parasitization capacity (measured as eggs/female laid on host larvae and percentage of eggs which gave puparia) were not negatively influenced.

Compared to conventional *Bt*-preparations, the epiphytic bacteria modified with *Bt*-genes are more persistent on the leaf surface and are thus more likely to be ingested or contacted by parasitoid adults walking over the treated areas. Therefore, for these engineered bacteria it is even more important to assess the side effects on parasitoid adults when evaluating their environmental impact. In our study, the undiluted suspension of the bacterial construct *cry9Aa-Pseudomonas* from one side confirmed to be highly effective against the model target species *G. mellonella*, as in Alberghini *et al*. (2005) and Marchetti *et al*. (2009), and on the other side had no apparent detrimental effects on the adult longevity, mating and parasitization capacity of the parasitoid *E. larvarum*. These results complement the findings of Marchetti *et al*. (2009), who showed that the development of *E. larvarum* was not affected by host treatment either with Cry9Aa toxin or *cry9Aa-Pseudomonas.* It is also worth noting that in the side test conducted on *G. mellonella*, the bacterial construct was effective against the young larvae of this model species even after 7 days of storage at +4 °C, thus confirming its potential as a control agent of target lepidopterous species.

This paper represents a further step towards the evaluation of the side effects of *Bt* preparations and, in particular, *cry9Aa-Pseudomonas* on non-target organisms. Based on the parameters considered in this study as well as in the previous one (Marchetti *et al*., 2009), it can be concluded that the model parasitoid *E. larvarum* was not affected either by the commercial strain of *Btk*, a conventional preparation of *Btg*, or transgenic *Bacillus thuringiensis galleriae* toxin, under the conditions tested. Additional research is now required to assess the susceptibility of other non target species (including beneficial insects) to these different delivery forms of the Cry9Aa toxin. Studies will have also to be carried out in a more realistic environment than in a laboratory situation. It has to be stressed that not very many studies concerning the effects of *Bt* preparations on non target insects have so far been carried out under field or forest conditions. Most of them did not show negative side effects on beneficial insects (Lesko *et al.*, 1982; Flexner *et al*., 1986; Kuhne, 2010). A few adverse effects on some parasitoids have been reported, but they were not explained by the direct action of *Bt,* but rather to indirect effects, such as a high reduction of the host population in the treated area (Reardon *et al*., 1979), or, in the case of the tachinid fly *Blepharipa pratensis* (Meigen), limited ingestion of parasitoid microtype eggs by host larvae (Ticehurst *et al*., 1982). In general, it this desirable that more tests are conducted in agro-forestry ecosystems.

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