Short Communication

Biological Activity of *Bacillus thuringiensis* (Bacillales: Bacillaceae) in *Anastrepha fraterculus* (Diptera: Tephritidae)

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Abstract

*Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae) is considered to be one of the major pest insects in fruit orchards worldwide. *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) strains are widely used as biological control agents and show high biological activity against different insect species. The objective of this study was to evaluate the biological activity of different strains of *B. thuringiensis* against *A. fraterculus* larvae and adults. Bioassays were performed using suspensions of bacterial spores/crystals of *B. thuringiensis* var. israelensis (*Bti*), kurstaki (*Btk*), and oswaldocruzi (*Bto*) at three concentrations [2 × 10⁷, 2 × 10⁸, and 2 × 10⁹ colony-forming units per ml (CFU ml⁻¹)]. At a concentration of 2 × 10⁸ CFU ml⁻¹, a significant larval effect (mortality 60%) was observed when compared with the control treatment. Larvae that ingested spore/crystal suspensions of *Bti*, *Btk*, or *Bto* bacterial strains exhibited significant larval and pupal deformations, leading to a significant decrease (~50%) in the completion of the insects’ biological cycle (egg to adult). The *B. thuringiensis* strains (*Bti*, *Btk*, or *Bto*) at a concentration of 2 × 10⁸ CFU ml⁻¹ in combination with one food attractant (BioAnastrepha 3% or CeraTrap 1.5%) in formulations of toxic baits provided high mortality (mortality > 85%) of *A. fraterculus* adults 7 d after treatment. However, the *Btk* strain in combination with CeraTrap 1.5% caused mortality of 40%. On the basis of these results, the native bacterial strains *Bti*, *Btk*, and *Bto* were considered to be promising candidates as biological control agents against *A. fraterculus*.

Key words: South American fruit fly, Biological control, Diptera, *Bacillus thuringiensis*

The South American fruit fly *Anastrepha fraterculus* (Wiedemann, 1830) (Diptera: Tephritidae) is considered to be one of the main fruit pests worldwide (Zucchi 2008). The economical relevance of this association is the possibility of direct damage when introducing their ovipositor to deposit their eggs and the possibility of infection by other pathogens through these holes present in the fruits (Machota Junior et al. 2016). In Brazil, current management strategies against *A. fraterculus* include a spray application of phosphorus and pyrethroids insecticides (Harter et al. 2015). However, worldwide studies have shown that using toxic baits can be a management option for infested areas (Navarro-Llopis et al. 2012, Borges et al. 2015).

In an effort to develop rational pest management tools for *A. fraterculus*, the use of *Bacillus thuringiensis* Berliner (1915) (Bacillales: Bacillaceae) may be a feasible option for management (Frankenhuysen 2009). Currently, there are no reports on the pathogenicity of *B. thuringiensis* against *A. fraterculus* immature phase. However, several studies have demonstrated significant biological activity of *B. thuringiensis* against larvae and adults of species from the families Tephritidae, Drosophilidae, and Sepsidae (Harter et al. 2015, Shisheh et al. 2015, Matsunaga et al. 2015). Therefore, in this study, we aimed to evaluate the biological activity of *B. thuringiensis* against *A. fraterculus* larvae and adults and the effects on the biological cycle.
Materials and Methods

Insect Rearing

*A. fraterculus* adults were raised and maintained in the laboratory using plastic cages (50 liter) with ~300 couples per cage. Insects were fed with solid food composed of refined sugar, wheat germ, and brewer's yeast (3:1:1 ratio) (Gonçalves et al. 2013). After oviposition, eggs were collected in running water and transferred to Erlenmeyer glass flasks (500 ml) for the 24-h aeration process (Gonçalves et al. 2013). After this time, 0.5 ml of eggs was deposited (~9,200 eggs) on a filter paper and placed inside plastic containers (500 ml) containing artificial diet (300 ml). The *A. fraterculus* larvae were raised on the artificial diet for ~9 d until pupation occurred and adults emerged (Nunes et al. 2013).

*B. thuringiensis* Strains

Strains used in the bioassays were *B. thuringiensis* var. *israelensis* (Bti), *B. thuringiensis* var. *kurstaki* (Btk), and *B. thuringiensis* var. *osvaldocruzii* (Bto), which were obtained from Department of Microbiology and Parasitology at the Federal University of Pelotas, Brazil. Strains were recovered in brain heart infusion (BHI) agar plates (Acumedia), incubated at 28°C, and subcultured in 1000 ml Erlenmeyer flasks containing 200 ml of NYSM (Nutrient Broth, Yeast extract, MnCl₂, MgCl₂, and CaCl₂). Then, the flasks were placed in an orbital shaker at 150 rpm at 28°C for 72 h. Sporulation of each bacterial strain was confirmed using optical microscopy (microscope at 100× magnification, Olympus). When ~90% of each culture had developed spores, the cultures were heated in an oven at 80°C for 15 min to eliminate vegetative cells. A Gram stain was performed on each bacterial culture to determine the purity of the spores. The average concentration of spores was determined as 2 × 10^12 colony-forming units per ml (CFU ml⁻¹) in BHI agar plates for both bacterial strains. Later, the bacterial cultures for each strain were stored at 4°C until the bioassays were performed. To incorporate bacteria into the bioassays, 2 × 10^12 CFU ml⁻¹ of each bacterium was diluted in a 10-fold serial dilution, directly in the diet at different amounts, depending on the desired concentration (2 × 10^⁷, 2 × 10⁸, or 2 × 10⁹ CFU ml⁻¹).

Biological Activity Against *A. fraterculus* Immature

For characterization of the biological activity (treatments) of *Bti*, *Btk*, and *Bto* strains against *A. fraterculus* immature, *Bt* strains were incorporated into an artificial diet (Nunes et al. 2013); however, Nipagim was excluded. After preparation, 300 ml of the artificial diet was poured in plastic containers (500 ml). Before solidification, 2 ml of bacterial suspension was added to the artificial diet using a micropipette (Zimmer et al. 2013). Three concentrations (2 × 10⁷, 2 × 10⁸, and 2 × 10⁹ CFU ml⁻¹) of each bacterial strain were tested. The control treatment was performed using artificial diet without bacterial strains. After 24 h, 100 eggs of *A. fraterculus* were inoculated with the aid of LabMate micropipette. After inoculation, containers were closed and incubated (temperature at 25 ± 1°C, 75 ± 10% relative humidity and 12 h photophase). The experimental design was completely randomized, with five repetitions per concentration of *B. thuringiensis*, and each repetition had 100 *A. fraterculus* eggs. After 9 d of inoculation, the larvae were taken off the artificial diet using a fine sieve and running water, and they were transferred to Gerbox brand plastic containers with 3 cm of fine vermiculite to aid in pupation and adults to emerge. Biological parameters evaluated were as follows: percentage (%) of larval mortality; completion of the biological cycle (egg to adult, %); deformation of larvae, pupae, and adults (%); and sex ratio. Dead larvae were defined as those which did not respond to touch by a fine-tipped brush, and deformed larvae were defined as those with changes in their shape and color compared with larvae from the control treatment. Deformity in adults was determined after their emergence based on anomalies on their abdomens and wings.

Biological Activity Against *A. fraterculus* Adults

For the bioassay was used to provide a 2 × 10^⁹ CFU ml⁻¹ concentration of each *B. thuringiensis* strain due to provide the greatest biological activity and negative effects against immature *A. fraterculus*. Ten-d-old adults were kept without food for 12 h and were then incubated in transparent plastic cages (100 ml) and inversely placed on acrylic plates (12 cm in diameter). BioAnastrepha at 3% (hydrolyzed corn protein—Biocontrol LTDA, São Paulo, Brazil) and CeraTrap at 1.5% (enzymatic hydrolyzed animal protein—Bioiberica S.A., Barcelona, Spain) was used as the food attractant. The pH of the food attractants were adjusted to 7.0 using an acid and a base (1M HC and 1M NaOH, respectively) before they were mixed with *B. thuringiensis* strains. After the toxic baits were formulated (food attractant + Bti, Btk, or Bto strains), they were supplied via capillarity using Eppendorf tubes perforated at the bottom and placed at the tops of the cages; therefore, that the insects could feed without any other type of contact with the food attractants. In the negative control, insects were only fed with food attractants (BioAnastrepha 3% or CeraTrap 1.5%). The experimental design was completely randomized with 20 repetitions per treatment, and each repetition consisted of one pair of insects (*n* = 40) per treatment. Live and dead insects were recorded daily over 7 d after treatment (DAT). The mortality rate for each treatment was corrected with that for the corresponding negative control (food attractant only) formulation using Abbott’s formula (1925).

Statistical Analysis

Data on the completion of insects’ biological cycle (egg to adult) and larval, pupal, and adult deformations and biological activity and the toxic effect on *A. fraterculus* larvae and adults, were considered in the normality analysis using the Bartlett Shapiro–Wilk test. Next, analysis of variance (ANOVA) was applied to data, and average results were compared using Tukey’s test (*P* < 0.05) (PROC ANOVA, SAS Institute 2011). Possible deviations in the sex ratio (sr) were compared using the chi-squared test (*χ²*) (*P* ≤ 0.05) (PROC FREQ, SAS Institute 2011).

**Fig. 1.** Larval mortality (average ± SE) of *A. fraterculus* when fed an artificial diet containing different concentrations of *B. thuringiensis*. Same lowercase letters (at the same concentration) and capital letters (comparison between concentrations) in the bars do not differ statistically from each other according to Tukey’s test (*P* < 0.05).
Results

Biological Activity Against Immature

We observed a significant increase ($P < 0.05$) in the susceptibility of *A. fraterculus* larvae depending on the concentration of spores added to their artificial diet. Results were similar for all *B. thuringiensis* strains (*Bti*, *Btk*, and *Bto*) compared with the control treatment (Fig. 1). The lowest larval mortality rate was observed at a concentration of $2 \times 10^7$ CFU ml$^{-1}$ of *Bti*, *Btk*, and *Bto* (14.9%, 3.2%, or 14.2%) strains 9 DAT (Fig. 1), whereas the highest concentration ($2 \times 10^9$ CFU ml$^{-1}$) of *Bti*, *Btk*, and *Bto* strains led to a significant increase ($F_{3,16} = 33.52$; $P < 0.0001$) in *A. fraterculus* larval mortality (42.2, 49.9, and 58.1%, respectively) (Fig. 1). Consequently, a significant decrease ($P < 0.05$) in the completion of the biological cycle (egg to adult) of *A. fraterculus* was observed for each strain tested at all concentrations of spores (Fig. 2). Compared with the control treatment (87.8% completion), the highest concentration ($2 \times 10^9$ CFU ml$^{-1}$) of *Bti*, *Btk*, and *Bto* led to a significant decrease (~50%) ($P < 0.05$) in the ability of the insects to complete their biological cycle (egg to adult) (43.3% completion [$F_{1,16} = 5.62$; $P < 0.0189$]; 45.8% completion [$F_{1,16} = 8.05$; $P < 0.0061$]; and 26.5% [$F_{1,16} = 24.49$; $P < 0.0001$], respectively) (Fig. 2). Significant variations were also observed in the percentage of deformed insects relative to the control treatment during their larval phase ($F_{1,16} = 33.05$; $P < 0.0001$), pupal phase ($F_{1,16} = 9.90$; $P < 0.0006$), and adult phase ($F_{1,16} = 2.31$; $P < 0.0055$) when they were fed with an artificial diet containing one of the three *B. thuringiensis* strains (Table 1). However, the chi-squared test ($\chi^2$) did not show significant differences ($P < 0.05$) in the sr, regardless of which *B. thuringiensis* strain was used (Table 1).

Biological Activity Against Adults

*A. fraterculus* adults were found to exhibit greater susceptibility at 7 DAT when BioAnastrepha at 3% or Ceratrap at 1.5% was mixed with *Bti* (90.0% and 100% mortality, respectively) or *Bto* (85.0% and 95.0% mortality, respectively); however, not found to differ significantly ($P < 0.05$) (Fig. 3). When the *Btk* strain was combined with BioAnastrepha at 3%, a 46.7% mortality rate was observed among *A. fraterculus* adults, which is statistically lower ($P < 0.05$) than the *Btk* (87.8% completion), *Bto* (85.0% completion), and *Bti* (90.0% and 100% mortality, respectively) or *Bto* (85.0% and 95.0% mortality, respectively). The susceptibility of larval and adult tephritids to different strains of *B. thuringiensis* has been demonstrated in several studies (Aboussaid et al. 2010, Molina et al. 2010, Illias et al. 2013, BUentello-Wong et al. 2015, Shishir et al. 2015, Cossentine et al. 2016). In this study, we

Discussion

The susceptibility of larval and adult tephritid to different strains of *B. thuringiensis* has been demonstrated in several studies (Aboussaid et al. 2010, Molina et al. 2010, Illias et al. 2013, BUentello-Wong et al. 2015, Shishir et al. 2015, Cossentine et al. 2016). In this study, we

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**Table 1.** Number (average ± SE) of larval in the third instar, pupae, and adult deformations, and the sex ratio of *A. fraterculus* when fed an artificial diet containing different concentrations of *B. thuringiensis*

<table>
<thead>
<tr>
<th>Concentration (CFU ml$^{-1}$)</th>
<th>10$^7$</th>
<th>10$^8$</th>
<th>10$^9$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of deformed larvae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bti</em> strain</td>
<td>8.80 ± 1.56 a</td>
<td>21.00 ± 5.77 a</td>
<td>3.00 ± 0.84 c</td>
</tr>
<tr>
<td><em>Btk</em> strain</td>
<td>10.20 ± 0.73 a</td>
<td>19.25 ± 2.56 a</td>
<td>46.25 ± 3.77 a</td>
</tr>
<tr>
<td><em>Bto</em> strain</td>
<td>10.60 ± 1.43 a</td>
<td>19.20 ± 2.61 a</td>
<td>26.80 ± 3.02 b</td>
</tr>
<tr>
<td>Control</td>
<td>1.60 ± 0.51 b</td>
<td>0.00 ± 0.00 b</td>
<td>0.00 ± 0.00 c</td>
</tr>
<tr>
<td>Number of deformed pupae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bti</em> strain</td>
<td>2.00 ± 1.15 b</td>
<td>7.75 ± 1.65 b</td>
<td>8.67 ± 2.03 b</td>
</tr>
<tr>
<td><em>Btk</em> strain</td>
<td>11.00 ± 1.90 a</td>
<td>26.00 ± 3.49 a</td>
<td>4.25 ± 1.49 a</td>
</tr>
<tr>
<td><em>Bto</em> strain</td>
<td>4.60 ± 1.17 ab</td>
<td>33.80 ± 1.98 a</td>
<td>18.25 ± 0.85 a</td>
</tr>
<tr>
<td>Control</td>
<td>2.40 ± 0.9 b</td>
<td>1.80 ± 0.73 b</td>
<td>2.00 ± 0.55 c</td>
</tr>
<tr>
<td>Number of deformed adults</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bti</em> strain</td>
<td>7.00 ± 1.08 b</td>
<td>7.60 ± 0.51 a</td>
<td>8.25 ± 0.85 a</td>
</tr>
<tr>
<td><em>Btk</em> strain</td>
<td>9.25 ± 1.03 b</td>
<td>5.00 ± 1.41 a</td>
<td>7.20 ± 1.36 a</td>
</tr>
<tr>
<td><em>Bto</em> strain</td>
<td>15.75 ± 2.75 a</td>
<td>6.75 ± 2.17 a</td>
<td>3.00 ± 1.53 a</td>
</tr>
<tr>
<td>Control</td>
<td>0.80 ± 0.49 c</td>
<td>1.80 ± 0.49 b</td>
<td>2.00 ± 0.84 b</td>
</tr>
<tr>
<td>Sex ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bti</em> strain</td>
<td>0.46 ± 0.04 ns</td>
<td>0.49 ± 0.01 ns</td>
<td>0.48 ± 0.04 ns</td>
</tr>
<tr>
<td><em>Btk</em> strain</td>
<td>0.47 ± 0.03</td>
<td>0.38 ± 0.02</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td><em>Bto</em> strain</td>
<td>0.43 ± 0.02</td>
<td>0.42 ± 0.00</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>Control</td>
<td>0.43 ± 0.02</td>
<td>0.40 ± 0.06</td>
<td>0.36 ± 0.02</td>
</tr>
</tbody>
</table>

Same lowercase letters in the columns for each variable analyzed do not differ significantly from each other according to Tukey’s test ($P < 0.05$). ns, non significant in the column according to the chi-squared test ($\chi^2$) ($P < 0.05$).
tested three native strains of \( B. \text{ thuringiensis} \): \( Bti \), \( Btk \), and \( Bto \). These strains had a direct effect (mortality) on \( A. \text{ fraterculus} \) larvae. They also had indirect effects, such as \( A. \text{ fraterculus} \) larval, pupal, and adult deformations. These indirect effects during the insects’ immature phase led to a significant decrease (50%) in larval, pupal, and adult deformations. These indirect effects during larvae. They also had indirect effects, such as

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**References Cited**


