Toxicity of *Bacillus thuringiensis* β-exotoxin to Three Species of Fruit Flies (Diptera: Tephritidae)

JORGE TOLEDO,[1] PABLO LIEDO,[1] TREVOR WILLIAMS,[1] AND JORGE IBARRA[2]

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ABSTRACT The current study describes toxic effects of the *Bacillus thuringiensis* β-exotoxin toward 3rd instars of 3 fruit fly species: *Anastrepha ludens* (Loew), *A. obliqua* (Macquart), and *A. serpentina* (Wiedemann). The β-exotoxin was highly toxic to all 3 species tested, with LC_{50} values calculated as 0.641, 0.512, and 0.408 μg/cm^2 of filter paper used to expose the larvae, for *A. ludens, A. obliqua,* and *A. serpentina*, respectively. Exposure to β-exotoxin was associated with an increase in the incidence of deformed pupae. The adult survivors from β-exotoxin treatments showed no negative effects in terms of their longevity, fecundity, or egg eclosion (fertility). We conclude that the β-exotoxin may have potential as a control agent for fruit fly pests.

KEY WORDS *Anastrepha* spp., bioassay, β-exotoxin, sublethal effects, Mexico, larvalcide

FRUIT FLIES OF the genus *Anastrepha* (Schiner) (Diptera: Tephritidae) represent a major problem for fruit and vegetable production from the southern United States to the North of Argentina (Aluja 1994). A diversity of methods are currently employed to combat these pests, including insecticidal baits for control of adult flies, male sterile fly releases, or soil applications of insecticides to kill larvae and emerging adults.

Anastrepha larvae leave the fruit and are in contact with the soil for ≈24 h before pupation. The pupal stage lasts some 15 d, depending on environmental conditions (Aluja 1984). Soil applications of insecticides such as diazinon have been considered a useful contribution to fly control in systems based on integrated pest management (Penrose 1993). This practice does, however, have a major impact on soil fauna, including numerous beneficial arthropods (e.g., ants, predatory beetles) because of the broad spectrum activity of such insecticides. This has stimulated the search for alternative agents for fruit fly control that offer a reduced environmental impact.

The β-exotoxin of *Bacillus thuringiensis* Berliner is produced and secreted into the growth medium during the phase of active vegetative growth of this bacterium (Faust and Bulla 1982). The exotoxin is not produced by all strains of *B. thuringiensis,* but exotoxin production has been reported in association with the presence of plasmids coding for the δ-endotoxin (Levinson et al. 1990). The β-exotoxin is thermostable, water soluble, dialyzable, and is structurally similar to a nucleotide (Faust and Bulla 1982). It is not readily degraded by exposure to UV radiation (Hitchings 1967).

The β-exotoxin has been shown to be highly toxic to a number of insect species from diverse orders (Burgerjon 1974). Dipteran larvae appear to be particularly susceptible and deleterious effects have been observed after topical application, ingestion, or injection of the exotoxin (Sebesta et al. 1981). Apart from mortality, other observed effects include inhibition of larval development or pupal malformations (Gingrich and Eschle 1971, Wasti et al. 1973, Haufler and Kunz 1985).

There have been very few studies of the effect of *B. thuringiensis* toxins toward fruit flies. Yamvrias and Anagnou (1989) observed high levels of mortality of *Bactrocera* (*Dacus*) *oleae* (Gmelin) larvae after application of an aqueous suspension of *B. thuringiensis,* although it is not known if β-exotoxin was present in their experimental preparation. In a different study, 55 strains of *B. thuringiensis* were tested for activity against larvae of *Anastrepha ludens* (Loew) with a resulting mortality of 4–63%, although the β-exotoxin content of these preparations was not specified (Robacker et al. 1996).

The objetive of the current study was to determine the potential of *B. thuringiensis* β-exotoxin as an agent for control of *Anastrepha* species. This was achieved by bioassay of the direct effects of a pure β-exotoxin product on insect mortality and sublethal effects of β-exotoxin treatment in survivors of the bioassays.

Materials and Methods

Third-instar *Anastrepha ludens, A. obliqua* (Macquart), and *A. serpentina* (Wiedemann) were obtained from the Moscafrut production plant at Metapa, Chiapos in which cultures are maintained on a semisynthetic diet as described by Domínguez et al. (1996), Moreno (1996), and Pinson et al. (1993), respectively. The β-exotoxin was obtained as experi-

[1] El Colegio de la Frontera Sur (ECOSUR), A. P. 36, Tapachula 30700, Chiapas, Mexico.
[2] CINVESTAV-IPN, A. P. 629, Irapuato 36500, Gto., Mexico.

Table 1. Toxicity of *B. thuringiensis* β-exotoxin to 3 species of *Anastrepha* fruit flies

Species	*n*	$LC_{50} \pm$ SE (μg/cm^2) (range of 95% CI)	χ^2	LC_{95}	Equation of regression line
A. ludens	1,166	0.641 ± 0.064a (0.471–0.878)	3.392	4.694	$Y = 5.424 + 2.157X$
A. obliqua	840	0.512 ± 0.033ab (0.387–0.678)	3.366	2.560	$Y = 5.701 + 2.433X$
A. serpentina	1,110	0.408 ± 0.019b (0.289–0.466)	5.967	3.652	$Y = 5.688 + 1.767X$

LC_{50} values followed by the same letter are not significantly different (LSD test at 5% level).

mental product from Abbott (ABG-6277A, batch number 28–037-BR, Chicago, IL) in the form of a 25% (WP) wettable powder. The product was <5 mo old. Experiments were performed in the laboratories of ECOSUR, Tapachula, Mexico, at a temperature of 26 ± 1°C, 70 ± 5% RH, and a photoperiod of 12:12 (L:D) h, except where otherwise stated.

Bioassay Procedures. Bioassays were performed in 9-cm-diameter petri dishes containing a filter paper disk impregnated with 1 ml of the toxin solution or distilled water in the case of the controls. Third-instar *Anastrepha* larvae were selected randomly and placed in groups of 30 in each petri dish. The concentrations tested were 0.091, 0.152, 0.253, 0.421, 0.702, 1.169, 1.949, and 3.248 μg of β-exotoxin per square centimeter of filter paper. After 24 h exposure, larvae were transferred to small containers, containing damp vermiculite, where they were left to pupate for 12 d. Pupae were then separated from vermiculite by gentle sieving, and the number of deformed pupae was recorded. Normal pupae from each treatment were placed in glass cages (30 by 30 by 30 cm) to permit adult emergence. Upon emergence, adult flies were examined for obvious deformations and were classified as normal or deformed.

The concentration-mortality response was subjected to Probit analysis (PROC Probit, SAS Institute 1992). The number of bioassays performed was 4 for *A. ludens* and *A. serpentina,* and 3 for *A. obliqua* to ensure statistical validity of the results (Ibarra and Federici 1987). The calculated LC_{50} values from each replicate were subjected to analysis of variance (ANOVA) in which fly species were considered as treatments. Means separation was achieved using the least significant difference (LSD) procedure (SAS Institute 1992).

Sublethal Effects in Adults. The effect of the β-exotoxin on adult flies that survived exposure in the larval stage was studied in 2 experiments. The 1st experiment used *A. ludens,* which was the species that showed least susceptibility to the β-exotoxin. Ten female and 10 male adult *A. ludens* that emerged from each concentration of the bioassay over a period of 24 h were placed in a cage and given water and a food source of sugar and hydrolyzed protein (3:1). After 10 d, when sexually mature, 3 Parafilm-wrapped green colored agar spheres were placed in the cage as oviposition substrates (Freeman and Carey 1990).

Oviposition targets were replaced every 24 h and exposed targets were dissected and the number of eggs was recorded. A randomly chosen subsample of 100 eggs was placed on moist filter paper and held in a petri dish at 28 ± 0.5°C for 5 d, after which the percentage eclosion was checked and recorded.

Longevity in the cages was registered by noting the daily mortality of adult flies. Results were subjected to ANOVA followed by means separation using the LSD procedure ($P \leq 0.05$) (SAS Institute 1992).

In the 2nd experiment, 3rd instars of each species were exposed to an LC_{50} concentration of β-exotoxin for 24 h and then transferred to damp vermiculite as previously described. Control larvae were treated with distilled water. When adults emerged, 10 females and 10 males of each species were confined in a cage with water and sugar-protein diet. Longevity, fecundity and egg viability of these flies was determined as described above. Data were analyzed by Student *t*-test of the means for each species separately (Steel and Torrie 1988).

Results

β-exotoxin Toxicity in Juvenile Stages. High levels of toxicity were detected in the bioassays with LC_{50} values of 0.641, 0.512, and 0.408 μg of β-exotoxin per square centimeter of filter paper for *A. ludens, A. obliqua,* and *A. serpentina,* respectively. These values were significantly different between *A. ludens* and *A. serpentina* ($F = 7.18$; df = 2, 8; $P < 0.001$) (Table 1). Overall mortality in nontreated control insects (larvae + pupae) was low in all bioassays (never >11.94%). The results given in Table 1 were subject to Abbott's correction (Abbott 1925). There was no evidence of β-exotoxin contamination among different treatments at any stage.

The greatest effect of β-exotoxin treatment was evident in the pupal stage; the β-exotoxin appeared to interfere with pupation and adult emergence. The incidence of malformed pupae ranged from 0.83 to 5.0% but was not correlated with β-exotoxin concentration. In contrast, mortality at the larval stage was low (2.5–7.5%, depending on β-exotoxin concentration) and larval mortality in the controls was very low (0.55%).

Sublethal Effects of β-exotoxin in Adult Flies. There were no negative effects apparent in the longevity of *A. ludens* adults that had been exposed as larvae to β-exotoxin when compared with controls or among treatments [concentrations of β-exotoxin] ($F = 1.25$; df = 7, 139; not significant) (Table 2).

Table 2. Longevity, fecundity, and fertility of *A. ludens* adults obtained from larvae exposed to a range of concentrations of β-exotoxin

Concn of β-exotoxin, μg/cm^2	Mean longevity ± SE, days	Mean daily fecundity ± SE (eggs/female/day)	Mean % eclosion of eggs ± SE
Control	66.6 ± 10.9a	14.95 ± 1.36e	34.36 ± 2.85f
0.091	56.2 ± 5.5a	43.40 ± 1.74a	66.00 ± 3.40a
0.152	79.5 ± 8.6a	28.06 ± 1.52c	58.08 ± 2.79ab
0.253	69.7 ± 8.6a	27.42 ± 1.94cd	43.94 ± 3.22de
0.421	57.7 ± 7.2a	33.70 ± 1.67b	52.04 ± 3.55bcd
0.702	65.7 ± 6.3a	34.17 ± 2.39b	54.63 ± 4.19bc
1.169	56.5 ± 6.3a	24.63 ± 1.65cd	41.21 ± 3.61ef
1.949	68.9 ± 8.1a	23.34 ± 1.98d	41.03 ± 3.62ef

Values in the same column followed by the same letter are not significantly different (LSD test at 5% level).

Unexpectedly, the fecundity of female survivors of the β-exotoxin exposure was significantly greater than that observed in nontreated flies, for all concentrations tested ($F = 22.29$; df = 7. 581; $P < 0.001$). The magnitude of this effect did not appear to be related to β-exotoxin concentration. Egg eclosion (fertility) was also significantly greater in the surviving adults of the β-exotoxin treated larvae compared with the adults emerging from nontreated larvae ($F = 9.75$; df = 7, 575; $P < 0.001$). Eclosion of eggs was generally highest for the survivors of the low and moderate concentrations of β-exotoxin, being 11–32% higher than controls. Survivors of the 2 highest concentrations of β-exotoxin also had fertilities significantly higher than controls, although the difference was not as evident as in the other concentrations (Table 2).

After exposure of each species to an LC_{50} concentration of β-exotoxin, the longevity of adult flies was significantly greater in the survivors of toxin exposure than in controls for *A. ludens* and *A. obliqua* but not for *A. serpentina* (Table 3). Fecundity did not differ between control and β-exotoxin exposed survivors for any species. Egg eclosion was significantly higher in the survivors of β-exotoxin treatment for *A. ludens* (86.4%) compared with controls (80.4%), but this difference was not seen in the other species (Table 3).

Table 3. Adult longevity, fecundity, and fertility of 3 species of *Anastrepha* obtained from larvae exposed to their respective LC_{50} concentrations of β-exotoxin compared with control flies

Treatment	Mean longevity ± SE, days	Mean daily fecundity ± SE (eggs/female/day)	Mean % eclosion of eggs ± SE
	A. ludens		
Treated	74.9 ± 7.6a	46.6 ± 2.7a	86.4 ± 2.1a
Control	62.3 ± 7.2b	42.6 ± 2.1a	80.4 ± 1.9b
	A. serpentina		
Treated	46.2 ± 6.1a	15.9 ± 0.8a	55.0 ± 4.6a
Control	38.5 ± 6.0a	16.1 ± 1.1a	57.1 ± 4.3a
	A. obliqua		
Treated	30.5 ± 3.7a	24.2 ± 4.4a	91.2 ± 3.3a
Control	19.9 ± 3.7b	19.2 ± 2.9a	87.7 ± 1.7a

Pairwise comparison of means in the same column and for each species separately were performed by Student *t*-test. Values followed by the same letter are not significantly different at 5% level.

Discussion

The β-exotoxin of *B. thuringiensis* was shown to be highly toxic to larvae of *Anastrepha* fruit flies, and this effect was consistent among the 3 species tested: LC_{50} values differed by a factor of not >1.60. The differences, such as they were, may be related to the thickness of the epidermis of each species or to intrinsic physiological factors (Rabossi et al. 1991, Hopkins and Kramer 1992). In fact, the abnormalities observed in insects that died during the larval stage were highly variable, which may reflect differences in the physiological state, titers of ecdysone, and other important hormones, at the moment of exposure to the β-exotoxin.

As the experimental larvae were exposed as 3rd instars, close to pupation, it is possible that more drastic symptoms of poisoning would have been seen had early instar larvae been used. Tanigoshi et al. (1990) reported that young nymphs of the bug *Lygus hesperus* Knight (Heteroptera: Miridae) were markedly more susceptible to topical applications of β-exotoxin than were late instar nymphs or adults.

The degree of toxicity observed in *Anastrepha* spp. larvae is comparable with that of other Diptera, Lepidoptera, and other pests insects. The LC_{50} of β-exotoxin in larvae of the fly *Hematobia irritans* (L.) was calculated at 2.79 μg/g of diet and symptoms of toxicity observed were similar to those seen in the current study (Haufler and Kunz 1985). In larvae of *Helicoverpa zea* (Boddie), *H. virescens* (F.), *Trichoplusia ni* (Hübner), *Pectinophora gossypiella* (Saunders), and *Spodoptera exigua* (Hübner), adult emergence was totally prevented by a concentration of 0.540 μg/cm2 of larval diet although the stage at which most mortality occurred was not mentioned (Ignoffo and Gregory 1972).

The composition, origin and mode of action of the β-exotoxin of *B. thuringiensis* is totally different from the δ-endotoxins commonly employed as bioinsecticides. Previous studies of the use of *B. thuringiensis* against fruit flies have focused on the search for effective δ-endotoxin activity and to his end, the mosquitocidal subspecies *B. thuringiensis* ssp. *israelensis* has been shown to induce important levels of mortality in larvae and adult fruit flies, but only at very high doses (Yamvrias and Anagnou 1989, Robacker et al. 1996). Programs of screening of isolates for toxicity to

A. ludens and *B. oleae* have also been carried out with varying success (Karamanlidou et al. 1991, Robacker et al. 1996), but in no case has the β-exotoxin content of these strains been tested; instead, attempts have been made to eliminate this substance from experimental formulations.

The results from the survivors of *A. ludens* from the bioassays and the tests of LC_{50} exposure to all 3 *Anastrepha* species were for the most part consistent, with greater longevity, higher fecundity, and improved egg eclosion in the survivors of β-exotoxin exposure compared with control flies. This trend was not expected and contradicts the observations of others (Ignoffo and Gregory 1972, Sebesta et al. 1981). We suggest that exposure to the β-exotoxin eliminated the weakest individuals in the experimental population, leaving survivors that were, on average, more vigorous and reproductively superior to the control population. However, this argument suggests that the survivors of the highest concentrations of β-exotoxin should have been the most vigorous and reproductively prolific, which was not, in fact, observed. Another possible explanation relates to the known inhibition of metamorphosis and new tissue formation by the β-exotoxin. The insect may respond to this inhibition by homeostasis of tissue formation, thus leaving proportionately more resources available for reproduction. Such ideas must, for the time being, remain speculative.

The adult survivors of β-exotoxin exposure did not show obvious signs of malformations, and there appeared to be no handicap to their successful mating and reproduction. This contrasts with 1 report in which lepidopteran survivors of β-exotoxin exposure showed antennal and bucal deformations that are likely to have direct effects on fecundity and survival (Ignoffo and Gregory 1972).

The β-exotoxin would appear to have clear potential for control of fruit flies, especially if applied to soil beneath the canopy of infested fruit trees. Nevertheless, the toxic effect of this substance extends to certain vertebrates, and many occidental countries have prohibited its use as an insecticide in its own right or as a contaminant in bioinsecticidal products based on the δ-endotoxins. Certain products, however, purposefully include the β-exotoxin, which allows a greater range of pest species to be effectively controlled than using the δ-endotoxin alone (e.g., Bitoxibacilin, available in Russia). The β-exotoxin has also been used in certain Nordic countries as a larvalcide for control of flies in pig farms.

The 2 types of toxin may also interact synergistically resulting in significant improvement of the effectiveness of *B. thuringiensis* as a bioinsecticide against several important insect pests, including *S. exigua* (Moar et al. 1986). Moreover, it is clear that chemical products such as diazinon used for fruit fly control are highly persistent and have toxicity to a broad range of soil arthropods above and beyond that of the β-exotoxin of *B. thuringiensis.* Field studies now in progress aim to quantify the impact of β-exotoxin applied to the soil for *Anastrepha* control, in terms of direct mortality of the pest and the impact on beneficial soil fauna.

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