Biological Activity of *Bacillus thuringiensis* and Associated Toxins against the Asian Longhorned Beetle (Coleoptera: Cerambycidae)[1]

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Abstract *Bacillus thuringiensis* Berliner var. *tenebrionis* and *B. thuringiensis* toxins were assayed against larval and adult Asian longhorned beetles, *Anoplophora glabripennis* (*A. glabripennis*). Preliminary *in vitro* assays showed some toxins to be active on whole midgut preparations in voltage clamp assays and in assays on brush border membrane vesicles formed from midgut epithelial cells. For *in vivo* tests, a commercially-available product (Novodor®) was incorporated into artificial diet, upon which larvae were allowed to feed *ad lib*. In other tests, droplets of solubilized *B. thuringiensis* toxins were fed to larval and adult beetles using a micropipette. None of the *in vivo* assays showed significant negative effects on either larvae or adults. We believe that some aspect of *A. glabripennis* midgut chemistry may be incompatible with toxin activation or mode of action.

The Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky), is a large wood-boring cerambycid native to parts of mainland China and Korea. *Anoplophora glabripennis* is a polyphagous feeder with a marked preference for poplar, maple and willow. Adult beetles feed and mate on the leaves, branches, and bole of trees, and female beetles chew slits in the bark in which to deposit a single egg. After hatching, larvae burrow and consume wood under the bark surface, eventually penetrating deeply into trees. The damage resulting from larval feeding can be sufficient to render large trees unsound, creating hazardous conditions in populated areas.

Anoplophora glabripennis was accidentally introduced into the United States in solid wood packing material from China, discarded in areas adjacent to airports. There were likely a number of such introductions in the 1980s and 1990s. Populations of *A. glabripennis* now exist in and near New York City, NY and Chicago, IL. These populations have been the targets of intense efforts aimed at eradication, usually in the form of removal and burning of infested trees. Less intrusive methods of control are currently being sought, especially biological control methods using parasites and microbial pesticides.

Bacillus thuringiensis Berliner, an aerobic soil-dwelling bacterium, has a long history of use in biopesticides. *Bacillus thuringiensis* and its subspecies produce insecticidal proteins during sporulation, the primary of which is δ-endotoxin. Because the δ-endotoxins are derived from bacterial Cry genes, they are also known as Cry toxins (e.g., CryI or Cry IIIa). Some Cry toxins, notably Cry1b, have been shown to have

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activity against Coleoptera (Krieg et al. 1983, Dai et al. 1989, Zehnder and Gelernter 1989, Bradley et al. 1995, James et al. 1999). Cry toxins and *B. thuringiensis* have been used to control insects in several orders and families, although successes in the control of coleopteran pests have been relatively few (Bauer 1990, Kaelin et al. 1999). Bauer and Pancratz (1992) reported on the efficacy of *B. thuringiensis* var. *san diego* against the cottonwood leaf beetle, *Chrysomela scripta* F., and described the effects of intoxication on midgut cells. *Anoplophora glabripennis* presents several difficulties as a target for applied *B. thuringiensis*. *Anoplophora glabripennis* larvae are generally inaccessible within the wood of the bole and branches; only the adult beetles feed on the outer surface of trees, therefore, only adult beetles could be easily targeted by surface applications. In other species it has been noted that adult beetles are typically more difficult to kill using *B. thuringiensis* and derived toxins. However, new genetic manipulation techniques raise the possibility of using the genes coding for effective toxins in transgenic trees (Bauer 1997) which would facilitate intoxication of larvae.

A quick method for testing possible *in vivo* efficacy of *B. thuringiensis* toxins is the voltage clamping assay, a method of using current flow across midgut tissue to detect pore formation. This is the first time, to our knowledge, that this technique has been applied to Coleoptera, probably because of the relatively small size of most beetles. Our *in vitro* work using voltage clamp assays of *A. glabripennis* midgut tissue, and brush border vesicle assays (D. Dean, pers. comm.) indicated that *B. thuringiensis* toxins were capable of causing pore formation in midgut cells. Because of the need for an effective control for *A. glabripennis*, we initiated tests of *B. thuringiensis* products and toxins *in vivo*.

We describe herein results of using diet incorporation and droplet feeding assays to test a commercial *B. thuringiensis* product and *B. thuringiensis* Cry toxins against *A. glabripennis* larvae and adults.

Materials and Methods

Preliminary voltage clamp assays. Using a limited number (8) of fifth-instar *A. glabripennis* larvae, voltage clamp assays of several toxins were conducted on midgut tissue using the technique of Wolfsberger et al. (1987), described here briefly. Larvae were cut open longitudinally, and an 2 cm length of midgut tissue was removed. This tissue was placed between two chambers containing an electrolytic solution of saline as per Harvey and Wolfersberger (1979) and Harvey et al. (1990). Current flow across the membrane was measured using a EVC-4000 Multi Channel Voltage/Current Clamp (World Precision Instruments, Inc., Sarasota, FL). Upon stabilization of current flow across the membrane, usually after approximately 10 min, a solubilized toxin was added to the solution in the lumen-side chamber, and changes in current flow noted. A rapid drop in current flow across the membrane is typically associated with toxin-mediated pore formation, which in turn signifies possible *in vivo* toxicity.

***Anoplophora glabripennis* on Novodor®-treated artificial diet.** Thirty *A. glabripennis* larvae of unknown age were obtained from poplar (*Populus* spp.) trees in China (by M. Smith) and returned to the United States for experimental use. These larvae were maintained on small twigs of maple (*Acer* spp.) until arrival at a quarantine facility in Ansonia, CT. Immediately before beginning the diet-incorporation bioassay, all larvae were weighed using an electronic balance accurate to 0.001 grams. Larvae were then divided into two groups. Initial weights of larvae ranged from 0.0595 to 0.5772 g.

Each larva was then placed in a cup containing a modified version of artificial red oak borer diet (Galford 1985). Those in the control group were placed in a cup containing diet alone, and those in the test group were placed in a cup containing diet into which was incorporated the *B. thuringiensis* var. *tenebrionis* product Novodor® (Valent Biosciences Corp., Libertyville, IL). Novodor® flowable concentrate was incorporated into the diet to a final concentration of approximately 170 Leptinotarsa Potency Units (LTUs) per ml diet. It was assumed that the high LTU concentration would evoke a response in larvae if they were at all susceptible to the product. Larvae were reared in an environmental growth chamber at 25°C, 60% ± 5% Rh, at a 16L:8D photoperiod and allowed to feed on diet freely for 2 wks. Larvae were examined and weighed twice during this period, and once at the conclusion of the test.

Morbidity and changes in larval weight were used to measure treatment efficacy. To compare mean weights through time the data were modeled as a two-way ANOVA with time as a repeated measure. Because subsequent growth measures are autocorrelated, the covariance structure of the repeated measures was fit with an AR(1) structure (SAS, Proc Mixed, Version 8.2). Residual analysis confirmed that model residuals met basic distributional assumptions. The type I error rate for pair-wise comparisons for each time step were Tukey adjusted.

***Anoplophora glabripennis* larvae droplet-fed Cry toxins.** Fifty-seven fourth-instar *A. glabripennis* larvae were obtained from a colony maintained at the USDA Forest Service Ansonia Quarantine facility in Ansonia, CT. These were randomly assigned to a control group (n = 28), and a group of larvae that were treated with *B. thuringiensis* toxin Cry 1B (n = 29). Larval weight was measured and recorded weekly for 3 wks. Morbidity and changes in larval weight were used to measure treatment efficacy as in the previous experiment.

***Anoplophora glabripennis* adults droplet-fed Cry toxins.** Adult *A. glabripennis* were collected from a farm in Yinchuan, China, 1 d before being used in the assay. All beetles were found on poplar (*Populus* spp.) trees, which had been planted as windbreaks on the perimeter of a corn field. Ages of beetles were not known. We returned to a laboratory in Beijing, divided these beetles at random into 6 groups of 15 to 17 adults each, and randomly assigned them to 1 of 5 solubilized Cry toxin treatments (Table 1) and a control.

Cry toxin treatments were administered as follows. Adult beetles were held by the thorax, and a 4 microliter droplet was placed directly on their mouthparts using an Eppendorf® Repeater® Plus Pipette (Brinkman Instruments, Inc., Westbury, NY)

Table 1. Description of solubilized Cry toxins droplet-fed to adult Asian longhorned beetles

Toxin name	Description
G4S	Cry1a
1B477	Contains a Cry8c gene producing a 70 kDa protein
1B164	Not identified, produces a 70 kDa protein as a square crystal
1B441	Contains a Cry3Bb gene producing a 70 kDa protein
1B348	Contains a Cry7A gene producing a 130 kDa protein

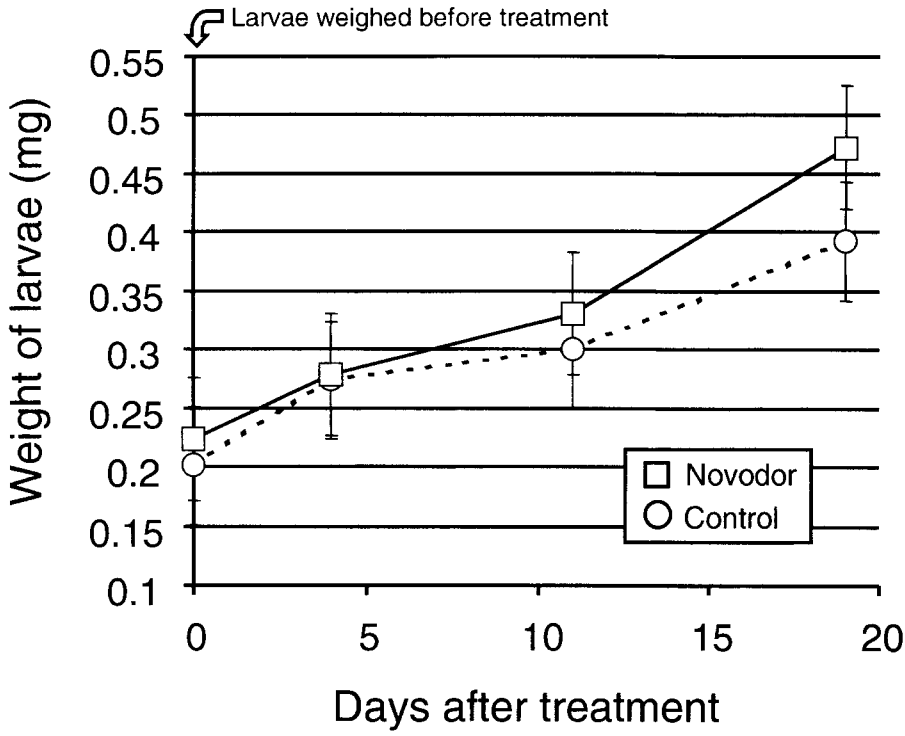

Fig. 1. Asian longhorned beetle larval weights (mg) fed on Novodor®-treated and untreated artificial diet.

fitted with a 0.1 ml Eppendorf® Combitip Plus. Only beetles that imbibed the whole droplet were included in the test. Control beetles were fed a 4-µl droplet of sterile distilled water. After treatment, adult beetles were kept individually in cylindrical steel screen cages 16 cm diam and 24 cm tall. The cages were held in a large environmentally-controlled room at 27°C, 65% ± 5% RH, and a 16L:8D photoperiod for 17 d. Fresh poplar foliage and small twigs were placed in the screen cages every other day.

The survival of adult beetles was determined daily for 17 d after treatments were applied. We used a simple logistic regression to evaluate if there was a relationship between survival rate and treatment.

Results and Discussion

Preliminary voltage clamp assays. Our preliminary trials using solubilized toxins indicated that certain *B. thuringiensis* toxins might be effective against *A. glabripennis* larvae. Addition of several of the Cry toxins resulted in a sudden and visually significant drop in conductivity across the tested membranes. It was these results that instigated the additional work described in this paper. Assays using brush border membrane vesicles indicated that Cry 1b was the toxin most likely to be effective *in vivo*.

***Anoplophora glabripennis* larvae on Novodor®-treated diet.** The results of our simple logistic regression indicated a non-significant time × treatment interaction as well as a non-significant treatment effect (Fig. 1). The means at each time were significantly different, i.e., the larvae grew significantly heavier between weighings; however, the two treatment groups grew at approximately the same rate. We attribute a trend toward heavier weights in the treatment larvae to the feeding stimulants added to this commercial preparation of *B. thuringiensis.*

***A. glabripennis* larvae droplet-fed Cry toxins.** The results of droplet-feeding Cry 1B toxins to larvae (Fig. 2) were similar to those in our Novodor® experiment. There was a non-significant time × treatment interaction as well as a non-significant treatment effect.

***Anoplophora glabripennis* adults droplet-fed Cry toxins.** There were no differences between treatments and the control group survival rates ($P = 0.25$) (Fig. 3) of adult *A. glabripennis* droplet-fed various toxins (Table 1). The overall mean survival rate was 7.3 ± 0.31 SE adult beetles.

Despite the activity of various *B. thuringiensis* toxins against *A. glabripennis* midgut tissue *in vitro* we did not see a significant effect of toxins assayed *in vivo.* There

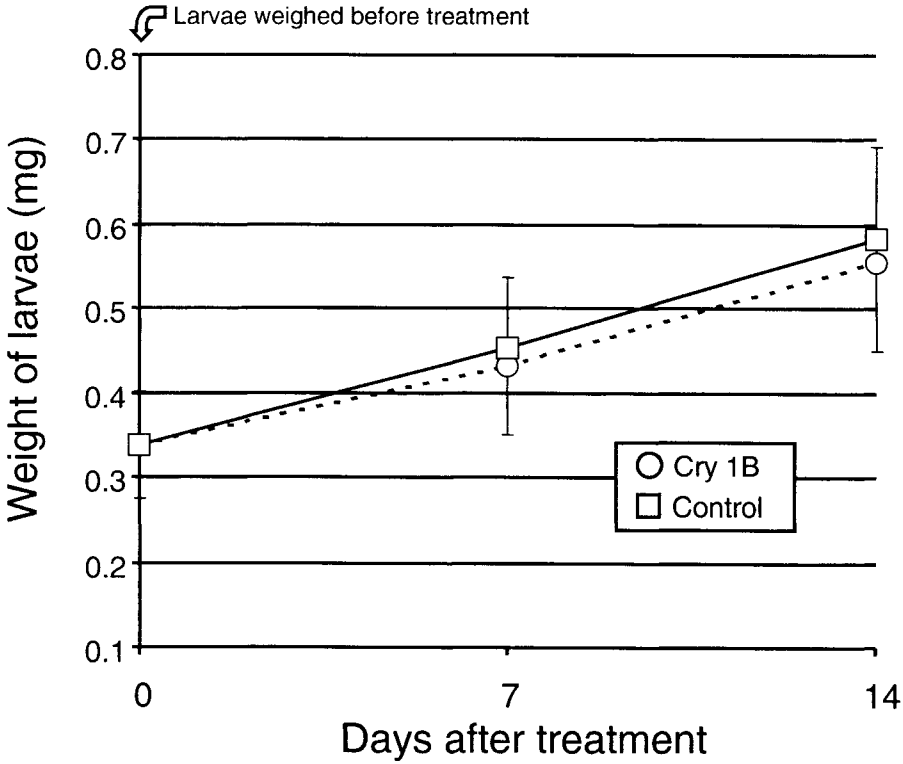

Fig. 2. Mean weights of Asian longhorned beetle larvae droplet-fed solubilized Cry1B toxin.

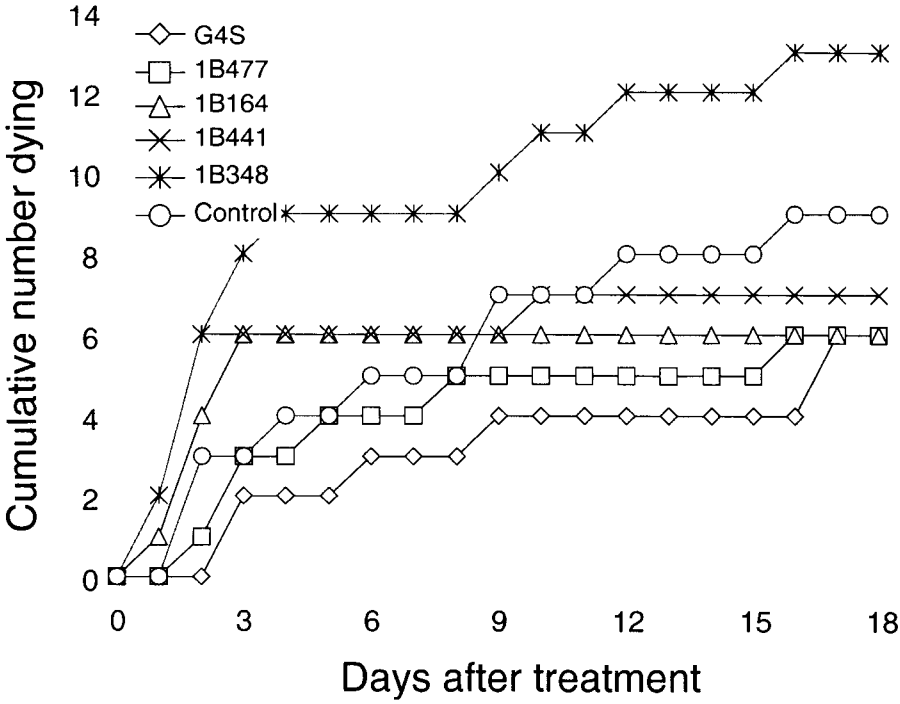

Fig. 3. Cumulative mortality of Asian longhorned beetle adults droplet-fed solubilized Cry toxins.

are several possible explanations for these findings. As noted by other researchers, the pH inside the active insect midgut varies according to insect species, chemical makeup of recently ingested food, and even insect health. Part of the activation sequence of *B. thuringiensis* endotoxins requires specific pH conditions that, while they may be met in the buffered solution used for our *in vitro* assays, may or may not be present inside living *A. glabripennis* larvae or adults. Alternatively, it is possible that some other unknown factors caused false positive *in vitro* results, when in fact no appropriate receptors exist on the surface of *A. glabripennis* midgut cells.

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