



## Short Communication

## *Bacillus thuringiensis* Cry3Aa toxin increases the susceptibility of *Crioceris quatuordecimpunctata* to *Beauveria bassiana* infection

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## ABSTRACT

The spotted asparagus beetle, *Crioceris quatuordecimpunctata* (Coleoptera: Chrysomelidae), is one of the most devastating pests of asparagus in China. Sprayed synthetic pesticides have been used to control *C. quatuordecimpunctata* damage, but they pose problems because of residues and harm to natural enemies. Neither the microbial coleopteran-specific toxin from *Bacillus thuringiensis tenebrionis*, Cry3Aa, nor the fungal pathogen *Beauveria bassiana* have sufficient activity to effectively control *C. quatuordecimpunctata* damage to asparagus. However, second instar *C. quatuordecimpunctata* larvae exposed to a sublethal dose of Cry3Aa toxin demonstrated significantly higher larval mortality when exposed to *B. bassiana*. Our results suggest that a combination of Cry3Aa and *B. bassiana* may be effective in reducing damage by *C. quatuordecimpunctata* larvae to asparagus.

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## 1. Introduction

The spotted asparagus beetle (SAB), *Crioceris quatuordecimpunctata* (Coleoptera: Chrysomelidae), is one of the most devastating pests of asparagus crops in China (Zhang et al., 2006; Gao et al., 2011). SAB is present throughout the crop growing cycle. Adults and larvae feed on the tips and spears, giving them a scarred appearance. If injury to the fern is severe, the crown is weakened, particularly if the asparagus stand is young, causing economic yield losses.

Tactics to control SAB damage are based on synthetic insecticides (Zhang et al., 2006) that may be incompatible with existing integrated pest management programs since they often affect natural enemies. Furthermore, intensive insecticide use may lead to pesticide residues, posing a serious food safety threat. Therefore, research is needed to implement environmentally-safe measures to manage SAB that result in the reduction of synthetic insecticides.

The entomopathogenic fungus *Beauveria bassiana* has been successfully applied in agricultural, horticultural, and forest systems as an alternative to harmful synthetic insecticides (Wraight and Ramos, 2005; Lord, 2007; Wraight et al., 2010). Biopesticide products containing *B. bassiana* as active ingredient are being developed, and some are commercially available for the control of various beetles (Wraight and Ramos, 2005; Faria and Wraight, 2007). However, *B. bassiana* alone cannot provide sufficiently high

levels of control within the brief period of time necessary to protect crops from economic damage (Wraight and Ramos, 2005).

Biopesticides based on *Bacillus thuringiensis* (Bt) represent another environmentally safe alternative to synthetics and have been used worldwide for more than 60 years for agricultural and forestry pests, accounting for more than 90% of all biopesticides (Brar et al., 2006). Bt toxins are expressed in transgenic crops to provide pest control with a reduced need for synthetic pesticides (Huang et al., 2007). Coleopteran-specific Cry3A toxins produced by Bt strains are toxic to some beetle pests (Van Frankenhuyzn, 2009). However, Cry3Aa displays low toxicity to SAB larvae, although Cry3Aa potency can be greatly increased by peptides derived from midgut receptors for this toxin (Gao et al., 2011).

Combinations of Bt and *B. bassiana* have been successful in increasing mortality in some insects (Wraight and Ramos, 2005). We hypothesized that sublethal effects of this toxin on SAB larvae would increase susceptibility to infection by entomopathogenic fungi. To test this hypothesis, we exposed selected stages of SAB larvae to Cry3Aa toxin and determined their subsequent susceptibility to *B. bassiana* infection using laboratory bioassays.

## 2. Materials and methods

## 2.1. Insects

Adult SAB were obtained from the Changping Asparagus Experimental Station (Beijing). The SAB colony was maintained on asparagus plants within an environmental chamber, 26 ± 2 °C and 70%

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relative humidity with a 14L:10D photoperiod. Newly hatched and second instar larvae were collected for bioassays.

## 2.2. Preparation of *Bt* Cry3Aa toxin

Cry3Aa was obtained from laboratory stocks of *B. thuringiensis*, as described in Gao et al. (2011). Cry3Aa protoxin was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and quantified by densitometry using a GS-800 Calibrated Densitometer and Quantity One image analysis software (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin standards ranging from 0.1 to 2.0 µg (data not shown).

## 2.3. *Beauveria* source

*B. bassiana* strain BbRSB was isolated from the striped stem borer, *Chilo suppressalis* (Lepidoptera: Pyralidae). The isolate was maintained on Sabouraud dextrose agar at  $26 \pm 1$  °C under continuous light. Conidia were harvested from 1- to 3-wk old cultures by flooding the aerial mycelia on the agar with sterile 0.05% Tween 80. The conidial concentration was determined with a hemocytometer and adjusted to  $1 \times 10^7$  conidia/ml with sterile 0.05% Tween-80. Viability of conidia of >90% was confirmed in counts of germinated and ungerminated conidia on Sabouraud dextrose agar.

## 2.4. Bioassays

### 2.4.1. *Bt*-Cry3Aa toxin bioassay

Assays with SAB larvae were conducted on fresh 5- to 8-cm-long sections of asparagus spears, using a dipping method. Briefly, the plant section was dipped for 10 s in the solution, held vertically to allow excess solution to drip, placed in a drying rack in a fume exhaust hood to air dry for 1 h, and then placed in a 9-cm Petri dish containing a moistened cotton ball.

Each bioassay was conducted with 20 SAB neonate or second instar larvae with different concentrations of Cry3Aa (400, 200, 100, 50, 25, and 0 µg toxin/ml PBS buffer) in triplicate with different SAB generations. Bioassays were incubated at  $26 \pm 2$  °C, 70% relative humidity and a photoperiod of 14L:10D. Larval mortality was determined after 96 h of exposure. Larvae were considered dead if they did not respond to contact.

Based on the results from the previous bioassays, we selected a dose of Cry3Aa of 10 µg/ml, which was not significantly different from the control ( $P > 0.05$ ), to evaluate sublethal effects on selected SAB growth parameters and subsequent Bb bioassays. To evaluate SAB larval weight and development time, bioassays were conducted with 20 s instar larvae in triplicate. Controls were treated with Tween 80 at 0.05% v/v. Larvae were pre-exposed to Cry3Aa- or PBS-treated asparagus spears for 96 h. Larval weight was measured before and after 8 d incubation, and developmental times from second-instar larvae to prepupae were recorded.

### 2.4.2. Effects of Cry3Aa toxin on infection of SAB larvae by *B. bassiana*

The effect of *B. bassiana* on SAB with or without toxin pretreatment was evaluated with second instar larvae. *B. bassiana* was tested at one dose,  $1 \times 10^7$  conidia/ml, alone or in combination with Cry3Aa at 10 µg toxin/ml. Controls were treated with Tween 80 at 0.05% v/v. Larvae were pre-exposed to Cry3Aa- or PBS-treated asparagus spears for 96 h, and each larva was then dipped for 10 s in the BbRSB conidial suspension or Tween 80 solution. Each replicate consisted of 20 larvae and was performed in triplicate with insects from different generations. Treated larvae were allowed to dry on filter paper, transferred to Petri dishes (3.5 cm) with untreated asparagus stems, and stored in a plastic box with 75% RH. The presence or absence of fungal mycelia on treated larvae

was recorded every other day until the larvae reached the prepupal stage (about 12–15 d after treated with *B. bassiana*).

## 2.5. Data analysis

Mortality values were corrected for background mortality using Abbott's formula for probit analysis, and LC<sub>50</sub> values were calculated by probit analysis using POLO-PC (LeOra Software, Berkeley, CA). The data for larval mortality, weight, and development time were assessed by analysis of variance. The data were normally distributed and not transformed. Means were separated using the protected LSD test (SAS Institute, 1988).

## 3. Results

### 3.1. *Bt*-Cry3Aa toxin bioassay

To determine the most effective dose of Cry3Aa and the more appropriate developmental stage of SAB larvae to use in bioassays, we evaluated the effect of increasing doses of Cry3Aa on neonate and second instar larvae (Fig. 1). The LC<sub>50</sub> for neonates was 3.9 µg/ml (95% CI 3.3–4.6,  $\chi^2 = 54.4$ , d.f. 13), and was 98.9 µg/ml (95% CI 82.9–118.0,  $\chi^2 = 48.1$ , d.f.13) for second instar. The LC<sub>50</sub> ratio is 25.6 (95% CI 22.7–28.6). Therefore, we chose second instar larvae and a dose of 10 µg/ml Cry3Aa that resulted mortality similar to control mortality to further investigate the effect of Cry3Aa protoxin on several life parameters. We also used this Cry3Aa dose in combination assays with Bb.

Second instar SAB larvae were exposed to 10 µg/ml Cry3Aa, and larvae were weighed and monitored for development time (Fig. 2). After 8 d, Cry3Aa toxin-treated larvae weighed significantly less ( $F = 108.73$ ;  $df = 1,5$ ;  $P = 0.0001$ , Fig. 2A) and had a significantly longer developmental period ( $F = 45.76$ ;  $df = 1,5$ ;  $P = 0.001$ , Fig. 2B) than control larvae. Therefore, while this dose was not lethal to SAB, fitness costs were incurred.

### 3.2. Effects of Cry3Aa toxin pre-treatment on larval infection by *B. bassiana*

Mortality was significantly different among the four treatments ( $F = 43.78$ ;  $df = 3,11$ ;  $P < 0.001$ ; Fig. 3). The activity of *B. bassiana* strain BbRSB was tested with a single dose of conidia ( $1 \times 10^7$  conidia/ml) against second instar SAB larvae. This concentration resulted in  $41.67 \pm 5.09\%$  mortality in bioassays with SAB larvae. However, larvae that were pre-treated with a sublethal dose of Cry3Aa (10 µg/ml) suffered significantly greater mortality of

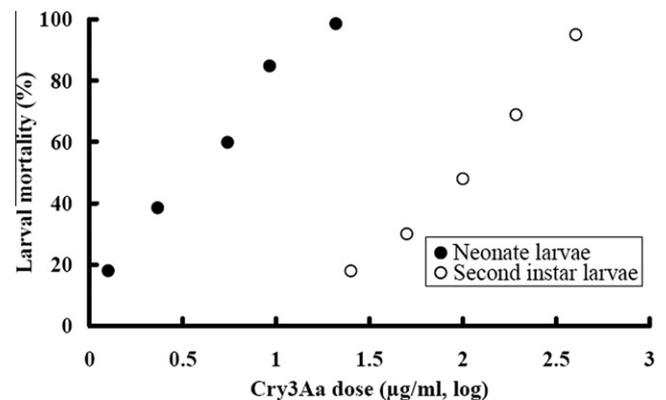
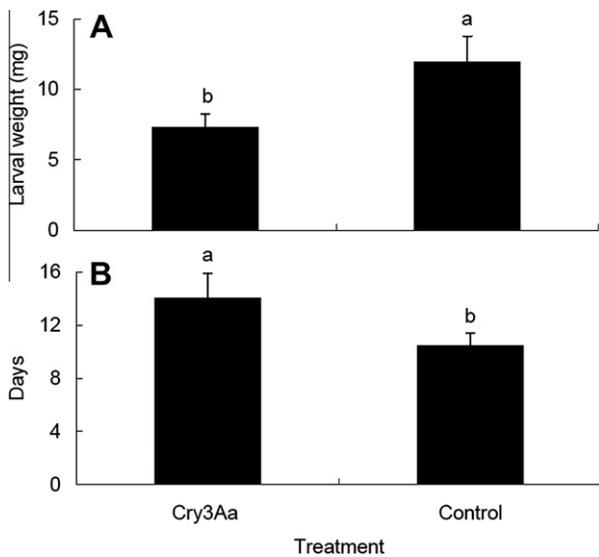
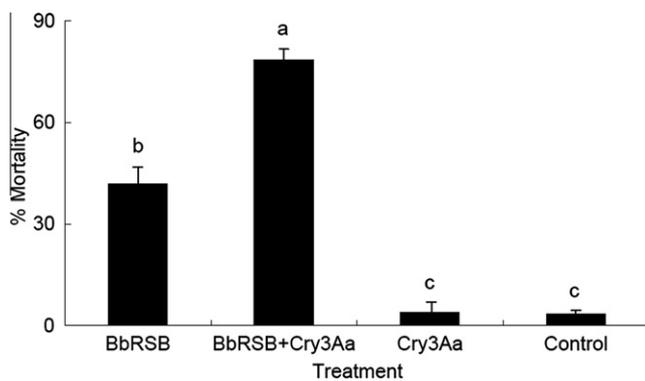


Fig. 1. Effect of increasing doses of Cry3Aa protoxin on neonate and second instar SAB larvae. Respective regression equations for neonates and second instars are:  $Y = -1.80 + 2.78X$ ,  $Y = -5.84 + 2.75X$ .



**Fig. 2.** Effects of sublethal Cry3Aa protoxin (10 µg/ml) on (A) larval weight after 8 d incubation and (B) developmental time from second instar larvae to prepupae. Different letters indicate significant differences ( $\pm$ SE).



**Fig. 3.** Mortality ( $\pm$ SE) of second instar SAB exposed to  $1 \times 10^7$  conidia/ml *B. bassiana* with or without pretreatment of a sublethal dose of Cry3Aa protoxin (10 µg/ml).

78.33  $\pm$  3.37% than other three treatments (all  $P < 0.001$ ). The mortality of Cry3Aa-treated larvae (3.73  $\pm$  0.55%) and controls (3.33  $\pm$  1.27%) were not significantly different ( $P = 0.96$ , Fig. 3).

#### 4. Discussion

Our results suggest that the susceptibility of SAB neonate larvae to Cry3Aa toxins decreases in later instars, as reported by McGaughey (1978) for two Lepidoptera pests. We found that second instar SAB larvae survive much higher doses of Cry3Aa toxin, with a 30-fold increase in the LD<sub>50</sub>. However, a sublethal dose of Cry3Aa prolongs the development time and reduces the weight of SAB larvae, as was reported for the Western corn rootworm, *Diabrotica virgifera virgifera*, and transgenic Cry3Bb corn (Meissle et al., 2009). This suggests that it would be difficult to achieve control of older SAB larvae in the field with only Cry3Aa sprays.

Although second instar SAB are susceptible to *B. bassiana*, time to kill is long and significant damage to asparagus plants would occur. We found that pretreatment of SAB larvae with Cry3Aa toxin increased susceptibility to fungal infection, promoting faster larval mortality. These observations agree with the findings of Wraight and Ramos (2005), in which synergism was observed between Bt

toxins and *B. bassiana* against Coleoptera. Lawo et al. (2008) indicated that sublethal Cry2Aa intoxication of the bollworm, *Helicoverpa armigera*, enhanced the effectiveness of the fungus, *Metarhizium anisopliae*. In contrast, Meissle et al. (2009) found only an additive effect of *M. anisopliae* and Bt maize on *D. v. virgifera* larvae and adults.

The observed enhancement of *B. bassiana* efficacy by Bt toxin, which may be termed synergism in the sense of piperonyl butoxide synergism of pyrethroids, may be explained by several mechanisms. It is well known that the molting process can remove fungal inoculum from an insect and thus prevent infection (Wraight and Ramos, 2005). Our data demonstrates that sublethal Cry3Aa doses retard larval development in SAB, and this may increase susceptibility to *B. bassiana* by prolonging the interval between molts. Thus, the delayed development by Cry3Aa may promote successful fungal penetration. Alternatively, Furlong and Groden (2003) demonstrated that starvation increased the susceptibility of *Leptinotarsa decemlineata* to *B. bassiana* infection. Interruption of larval feeding by Bt intoxication may lead to starvation stress and cause detrimental effects on host physiology and immune response that may lead to the observed Bt-*B. bassiana* synergism.

In summary, our results suggest that the combination of Cry3Aa toxin and *B. bassiana* may result in a cost-effective strategy for asparagus crop pest management. Combined use of both agents represents an environmentally safe strategy to avoid safety issues related to the use of synthetic pesticides on asparagus plants. Field trials in asparagus fields will determine the efficacy of the combined use of Cry3Aa and *B. bassiana* as a tool in sustainable pest management strategies.

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