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Effects of *Bacillus thuringiensis* toxin Cry1Ac and *Beauveria bassiana* on Asiatic corn borer (Lepidoptera: Crambidae)

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ABSTRACT

In this study, interactions between Cry1Ac, a toxic crystal protein produced by *Bacillus thuringiensis* (Berliner), and *Beauveria bassiana* on the mortality and survival of *Ostrinia furnacalis* was evaluated in the laboratory. The results showed that Cry1Ac is toxic to *O. furnacalis*. Not only were larval growth and development delayed, but pupation, pupal weight and adult emergency also decreased when larvae were fed on artificial diet containing purified Cry1Ac toxin. When third instars *O. furnacalis* were exposed to combination of *B. bassiana* (1.8×10^5, 1.8×10^6 or 1.8×10^7 conidia ml^{-1}) and Cry1Ac, (0.2 or 0.8 $\mu g\, g^{-1}$), the effect on mortality was additive, however, the combinations of sublethal concentrations showed antagonism between Cry1Ac (3.2 or 13 $\mu g\, g^{-1}$) and *B. bassiana* (1.8×10^5 or 1.8×10^6 conidia ml^{-1}). When neonates were reared on sublethal concentrations of Cry1AC until the third instar, and survivors exposed *B. bassiana* conidial suspension, such treatments showed additive effect on mortality of *O. furnacalis* except for the combination of Cry1Ac (0.2 $\mu g\, g^{-1}$) and *B. bassiana* (1.8×10^6 conidia ml^{-1}) that showed antagonism.

1. Introduction

The Asiatic corn borer, *Ostrinia furnacalis* (Guenée), is a major pest of corn in China (Zhai, 1992). This insect, at times, causes significant economic damage in cotton by chewing holes in the leaves, tunneling into stems, branches, flower buds, flowers, and green bolls (Cao et al., 1994; Wu and Guo, 2005). Control of the pest with chemical insecticides is difficult because of the cryptic nature of larval behavior, which makes the timing of insecticide application crucial for success. Because of its high efficacy, specificity, and improved toxin delivery system, Bt cotton has been increasingly adopted by farmers in China as an alternative to chemical sprays against lepidopterous pests, including *O. furnacalis* (Huang et al., 2003; Yang et al., 2005). However, field studies have demonstrated that 5–20% of larvae of *O. furnacalis* could survive on Bt cotton in the latter part of the growing season due to the diminishing levels of Bt toxins in plant tissues as the plant ages (He et al., 2006).

The fungus, *Beauveria bassiana* (Balsamo) Vuillemin (Hyphomycetes), is a ubiquitous and important entomopathogen of *O. furnacalis* (Feng et al., 1985; Cherry et al., 1999) and several other insect species (Fargues and Remaudiere, 1977; Inglis et al., 2001; Lacey et al., 2001). It can be used effectively to suppress *O. furnacalis* populations (Anderson and Lewis, 1991; Lewis and Bing, 1991; Feng et al., 1994; Lewis et al., 1996, 2002). Commercial-scale use of this pathogen as biopesticide for *O. furnacalis* control has been developed in China (Zhang et al., 1990; Wang et al., 2000; Ji, 2001). Despite its efficacy and general environmental safety, *B. bassiana* is often mixed with chemical and microbial pesticides presumably to enhance its efficacy against target insect pests (Inglis et al., 2001). Lewis et al. (1996) observed that application of *Bacillus thuringiensis* (Berlinger (please check)) (Bt) to whorl-stage and pollen-shedding stage of maize enhanced the suppression of European corn borers, *Ostrinia nubilalis* (Hübner) by endophytic *B. bassiana*. In other instances, there was no apparent advantage of the co-treatment, and independent action of both microbes was observed (Lewis and Bing, 1991). However, *B. thuringiensis* formulation contains bacterial spores and several different endotoxins, including Cry1Aa, Cry1Ab, CrylAc, active against Lepidoptera (Rukmini et al., 2000; Huang et al., 2002) that may maximize the toxicity of Bt preparation to *O. nubilalis* (Mohd-Salleh and Lewis, 1982). The Cry1Ac endotoxin crystal protein is expressed by most Bt crystal protein-engineered cotton varieties planted in China (Wu et al., 2002). Hence, increasing use of Bt cotton could expose *O. furnacalis* to the Cry1Ac δ-endotoxin for a longer duration. The continuous production of Bt toxin in transgenic plants leads to a season-long selection for insect resistance to the Bt insecticidal protein, and

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the large-scale use of Bt crops is likely to cause an evolution of Bt toxin resistance in the insect pests (Gould, 1998; Bates et al., 2005). The rate of resistance in an insect population could be influenced by sublethal effects of δ-endotoxin that alter the interactions of insects with other inimical agents (Gould et al., 1991). Additionally, such sublethal exposure results in greater susceptibility of survivors to entomopathogenic fungi, this could increase the utility of fungi for control of the *O. furnacalis* or enhance the potential for a disease epizootic to occur (Benz, 1987; Watanabe, 1987).

It was, therefore, important to assess the interactions of *O. furnacalis*, to *B. bassiana* and Cry1Ac. The objective of this research was to examine the effect of purified Cry1Ac on growth, development, and survival of *O. furnacalis* and the interaction between *B. bassiana* and Cry1Ac.

2. Materials and methods

2.1. Production of test organisms

2.1.1. Insects

Ostrinia furnacalis used in the study originated from egg masses obtained from a laboratory colony maintained on artificial diet at the Institute of Plant Protection of the Chinese Academy of Agricultural Sciences, Beijing. The original colony was collected from corn fields in Hebei Province in 1995 and was never exposed to Bt, entomopathogenic fungi or other insecticides. Eggs were held at 28 °C, 80% RH, and 14:10 (L:D) photoperiod until eclosion.

2.1.2. B. bassiana culture and conidia suspension preparation

Beauveria bassiana (strain Bb18), was isolated from infected *O. furnacalis* larvae collected from Jilin Province. Conidia were obtained from 15-day-old sporulating cultures maintained on PDA medium in Petri dishes at 25 °C in darkness. Conidia suspensions were prepared by scraping conidia from the surface of the culture into a sterile aqueous solution of 0.2% Tween 80 in deionized water. The conidial suspension was filtered through several layers of cheesecloth into glass beakers and mixed with a sterile spatula until homogeneity. Conidial suspensions of 1.8×10^8, 1.8×10^7, 1.8×10^6, 1.8×10^5, and 1.8×10^4 conidia ml^{-1} were prepared by serial dilution. Conidial concentration was verified by a Neubauer hemocytometer counts under 15 to 40× phase contrast microscopy. Spore viability was assessed by examining 100 conidia through 40× magnification and spore concentration was adjusted to reflect viability of >95% prior to performing bioassay.

2.1.3. Cry1Ac toxin and diet preparation

Cry1Ac toxin was purified from the HD-73 strain of *B. thuringiensis* subsp. *kurstaki*, provided by the Institute of Microbiology, Chinese Academy of Science. The strain was grown in LB medium (tryptone 1%, yeast extract 0.5%, NaCl 1%, pH 7–7.2) for 3–4 days at 28 °C at which time the separation of crystals from spores was confirmed by microscopic observation. The spores were centrifuged at 8000*g* for 10 min at 4 °C. The supernatant was decanted and the pellet washed five times with distilled water. The pellet was then kept in an alkaline buffer (Caps 0.1 M, β-mercaptoethanol 10 mM, pH 10.5) at 28 °C for 1 h until the crystal protein had dissolved. The solution was then centrifuged at 8000*g* for 15 min. The supernatant was collected and its pH adjusted to 5–6 with 1 M HCl and stored overnight at 4 °C to precipitate the toxin. The precipitate was then collected by centrifugation and washed three times with distilled water. The Cry1Ac toxin was lyophilized and dissolved in double distilled water.

The larvae were reared using an artificial diet based on soybean powder, maize powder, yeast, JSMD, antiscorbutic vitamin, and glucose (Song et al., 1999); formaldehyde, sorbic acid and erythromycin were omitted. A stock suspension of Cry1Ac toxin was serially added to aliquots of the liquid artificial diet before solidification, and mixed in a blender for about 30 s. The final Cry1Ac concentrations used were 0, 0.05, 0.2, 0.8, 3.2, 12.8, 51.2 μg g^{-1} diet. After the diet had solidified at room temperature for ≈6 h, ≈5 g of diet from each mixture were placed into 15-ml glass test-tube plugged with cotton ball.

2.2. Bioassays

2.2.1. General experimental design

Three bioassay procedures were used. One larva of *O. furnacalis* was placed onto the surface of the diet within each glass test-tube. Each treatment was replicated four times using 15 larvae per replicate. A completely randomized design was used for this study. All the treatments were held at 25 ± 1, 90% RH and 14:10 (L:D) h photoperiod.

2.2.2. Growth, development and mortality of larvae on the diet with Cry1Ac

Neonates were reared on diet with Cry1Ac toxin at the concentrations of 0, 0.05, 0.2, 0.8, 3.2, 12.8 μg g^{-1}, until larvae either died or pupated. Larval instar, larval weight, and larval survival were determined on the 10th day. Surviving larvae from each treatment were assessed for instar based on body length, and the number of head capsule exuvae found in each glass test-tube. Additionally, larval period, pupal weight, pupation rate, pupal period, adult emergence rate and adult longevity were all recorded. Living first instars were recorded as *dead* because they effectively have negligible contribution to plant damage and build-up of subsequent *O. furnacalis* populations. Other larvae were considered dead if they were unable to move in a coordinated manner when probed with a blunt probe. In the second experiment, third instars were individually fed on diets containing 0, 0.2, 0.8, 3.2, 12.8, 51.2 μg Cry1Ac g^{-1} until they either died or pupated. Only larval survival on 10th day was recorded.

2.2.3. Larvae exposed to Cry1Ac and B. bassinia simultaneously

Larvae of *O. furnacalis*, which had entered the third instar within the 24-h period, were dipped into *B. bassiana* conidia suspension in serial concentrations of 1.8×10^7, 1.8×10^6, and 1.8×10^5 conidia ml^{-1} for approximately 30 s (Tefera and Pringle, 2003a). The larvae were air-dried for 10 min in sterile Petri dishes. They were fed a diet containing Cry1Ac at concentrations of 0.2, 0.8, 3.2 and 12.8 μg g^{-1} until either died or pupated. Mortality, pupation, and adult emergence rates were recorded. Larvae killed by *B. bassiana* typically turned salmon colored prior to development of mycosis which coincided with apparent slow movement and cessation of feeding. Dead larvae were destructively sampled to confirm presence or absence of *B. bassiana* infection.

2.2.4. Effect of B. bassiana on Cry1Ac-stressed larvae

Neonates were reared in 0.05, 0.2, 0.8 μg Cry1Ac g^{-1} diet until third instar and within the 24-h period into the instar, survivors were exposed *B. bassiana* (1.8×10^8, 1.8×10^7, 1.8×10^6 conidia ml^{-1}) for approximately 30 s. The treated larvae were dried in sterile Petri dishes for 10 min, and allowed to continue feeding on the Cry1Ac diet at the same concentration. Mortality, pupation, and adult emergence rates were recorded. The bioassay also included some larvae infected only by *B. bassiana* or Cry1Ac toxin, and a control.

2.3. Statistical analysis

Distributions of the five instars were analyzed in a 6 by 5 contingency table for independence between Cry1Ac concentration in diet and larval development (CHISQ option of the SAS procedure