Biological Control 47 (2008) 89–96

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Interactions between Cry1Ac, Cry2Ab, and Cry1Fa *Bacillus thuringiensis* toxins in the cotton pests *Helicoverpa armigera* (Hübner) and *Earias insulana* (Boisduval)

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ARTICLE INFO

Article history:
Received 11 April 2008
Accepted 8 July 2008
Available online 16 July 2008

Keywords:
Earias insulana
Helicoverpa armigera
Bacillus thuringiensis
Bt cotton
Cry toxins
Interaction
Synergy
Pest control

ABSTRACT

One of the most effective strategies recommended to delay insect resistance to *Bt*-plants is concurrent expression of several toxins in the same plant. A new generation of *Bt*-cotton, including Bollgard II and WideStrike, has been developed to simultaneously express two different Cry toxins, Cry1Ac and Cry2Ab, and Cry1Ac and Cry1Fa, respectively. The aim of this study was to determine the individual and combined toxic effect of Cry1Ac, Cry2Ab, and Cry1Fa in the cotton pests *Helicoverpa armigera* and *Earias insulana*, as well as the nature of the interactions between these toxins, as determined by mean lethal concentration (LC_{50}) values and larval growth inhibition studies. Singly, all three assayed toxins were more toxic against *E. insulana* than against *H. armigera* larvae. Toxin Cry1Ac was significantly more toxic than the other two on *H. armigera*, while toxin Cry1Fa was the least toxic and caused no significant mortality. When combined, Cry1Ac and Cry1Fa showed an additive interaction in all proportions analyzed for both pest species, whereas Cry1Ac and Cry2Ab interacted synergistically in mixtures comprising 1:1 or 1:4 of each toxin against *H. armigera*. In *E. insulana*, there was no synergism between Cry1Ac and Cry2Ab but both these toxins showed a high insecticidal activity when administered individually and in mixtures. This study suggest that each particular toxin or toxin combination expressed in transgenic *Bt*-cotton should be carefully selected depending on the most important pest species present in each geographical area.

1. Introduction

The advance of biotechnology has allowed the development of insect-resistant transgenic plants expressing *Bacillus thuringiensis* (*Bt*) *cry* genes. *Bt*-plants now include several major crops including maize and cotton (Schnepf et al., 1998). The first generation of *Bt*-cotton, known as Bollgard I or Ingard (Monsanto Co., St. Lois, MO), expressed toxin Cry1Ac, and provided effective control against important cotton pests from North America, such as *Heliothis virescens* (Fabricius) and *Pectinophora gossypiella* (Saunders) and a moderate control against *Helicoverpa zea* (Boddie) (Halcomb et al., 1996; MacIntosh et al., 1990). In Australia, it offered protection against *H. armigera* (Hübner) and *H. punctigera* (Wallengren), for much of the growing season (Fitt, 2004). However, the widespread use of *Bt*-plants poses strong selection pressure that can accelerate the development of insect resistance and limit the long-term use of this technology and of all other *Bt*-based bioinsecticides.

One of the most effective strategies to delay the development of insect resistance to *Bt*-plants is the simultaneous expression of several toxins in the same plant, known as gene pyramiding (Roush, 1998). A second generation of *Bt* cotton simultaneously expressing two different Cry toxins has been developed recently. Bollgard II (Monsanto Co., St. Lois, MO) produces toxins Cry1Ac and Cry2Ab, WideStrike Insect Protection (Dow Agroscience, Indianapolis, IN) expresses toxin Cry1Fa in combination with Cry1Ac, and VipCot, which expresses Cry1Ab and Vip3A (Syngenta Biotechnology, Research Triangle Park, NC). Other *Bt* cotton varieties include different combinations of *Bt* toxins, such as a hybrid Cry1Ab/Cry1Ac.

Lepidopteran species such as *H. armigera* and *Earias insulana* (Boisduval) constitute some of the most serious pests in cotton crops in many countries of southern Europe, Asia, Africa, and Australia (Fitt, 1989; Reed, 1994). Several countries in these regions have introduced Bollgard II and this variety is now grown over a wide area in Australia, India, and South Africa.

The expression of multiple toxins in a single plant is likely to result in interactions between toxins in the target insect that might result in an increase or decrease in the degree of pest control. Synergistic interactions between *Bt* toxins were described first between Cry and Cyt toxins of *B. thuringiensis* serovar *israelensis* in dipterans (Bravo et al., 2007). Subsequently, both synergistic and antagonistic interactions between *Bt* toxins have been observed in lepidopterans (Del Rincón-Castro et al., 1999; Lee et al., 1996;

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doi:10.1016/j.biocontrol.2008.07.003

Sayyed et al., 2001). In *H. armigera*, synergistic interactions have been observed between toxins Cry1Aa and Cry1C (Xue et al., 2005), or Cry1Ac and Cry1Fa (Chakrabarti et al., 1998), whereas toxins Cry1Ac and Cry2Aa appeared to interact antagonistically (Liao et al., 2002). No interactions between Cry toxins have been reported in *Earias* spp.

The aim of this study was to determine the individual toxic effect of Cry1Ac, Cry2Ab, and Cry1Fa toxins on the cotton pests *H. armigera* and *E. insulana*, and the nature of the interactions between these toxins (synergistic, antagonistic or additive), as determined by concentration–mortality responses and larval growth inhibition studies.

2. Materials and methods

2.1. Insects

Two colonies of *E. insulana* and one colony of *H. armigera* were used in this study. One of the colonies of *E. insulana* was established in Spain from pupae obtained from a continuous laboratory culture maintained since 2001 in the Plant Protection Institute, Giza, Egypt. A second colony was started with field collected larvae from cotton fields in Córdoba, Spain, during the summer of 2005. The *H. armigera* colony was established in Spain from pupae supplied by Prof. Robert Possee (Centre for Ecology and Hydrology, Oxford, U.K.). These colonies were continuously maintained in our laboratory in a growth chamber at 27 ± 1 °C, 60% RH, and a photoperiod of 14:10 h (L:D) using an artificial diet (Greene et al., 1976).

2.2. Bacillus thuringiensis strains

Cry1Ac4 (EG11070), Cry1Fa1 (EG11069) and Cry2Ab2 (EG7699) toxins were produced in recombinant *B. thuringiensis* strains (name given in parenthesis), expressing just one type of Cry protein. Strains EG11070 and EG11069 were obtained from Ecogen Inc. (Langhorne, PA) and the EG7699 strain from Monsanto Co. (Chesterfield, MO) by way of Prof. J. Ferré (Departamento de Genética. Universidad de Valencia, Spain).

2.3. Production of Cry toxins

The recombinant strains were initially grown for 48 h at 28 °C on in CCY-agar medium plates, for sporulation, supplemented with 3 μg/ml chloramphenicol. One colony was removed and resuspended in sterile double-distilled water and incubated for 30 min at 70 °C to synchronize spore germination. Spores were cultured in CCY (casein hydrolysate/yeast-containing medium) containing 3 μg/ml chloramphenicol, at 28 °C and constant shaking during 48 h. When most cells had lysed, 1 M NaCl was added to inhibit protease activity. Cultures were centrifuged in a fixed angle rotor for 15 min at 9000*g* at 4 °C and the pellets were suspended in double-distilled water and centrifuged twice more. Mixtures of spores and crystals were completely disaggregated by sonication on ice for 30 s. Crystals were separated from spores by sucrose density gradient centrifugation, as described by Luthe (1983). Solutions of 79% and 67% (w/v) sucrose were prepared in double-distilled water and then placed in a 38.5 ml centrifuge tube. A 10 ml volume was carefully layered onto the sucrose gradient and centrifuged for 16 h at 70,000*g* at 4 °C. The crystal-containing band was collected, washed three times with double-distilled water at 12,000*g* for 15 min and 4 °C. The purified crystals were solubilized by gentle agitation in a solution of 50 mM Na_2CO_3 pH 11.3 and 10 mM dithiothreitol (DTT), for 2 h at room temperature. Unsolubilized crystals were removed by centrifugation at 9000*g* for 10 min at 4 °C. Protein activation was carried out with trypsin type I from bovine pancreas (Sigma Chemical Co., St. Louis, MO) in a proportion of 0.1 mg trypsin per mg protoxin. The solution was incubated at 37 °C for 1 h with constant shaking and the completion of the reaction was checked by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS–PAGE). Trypsin activated Cry proteins were purified by anion-exchange chromatography with the MonoQ HR5/5 column by fast-protein liquid chromatography (Pharmacia, Uppsala, Sweden). Protein concentration was determined by densitometry on a UV transiluminator using the Chemi Doc E.Q. program (Bio-Rad, Hercules, CA) including bovine serum albumin (BSA) as a standard.

2.4. Toxicity bioassays

The toxicity of individual and mixtures of Cry toxins against *H. armigera* and *E. insulana* was assessed by concentration–mortality assays and larval growth inhibition studies. Mortality bioassays involved incorporation of the toxins into the insect artificial diet, as previously described (MacIntosh et al., 1990). The toxin solution was mixed with the artificial diet at 50 °C and then dispensed into 24 multiwell-plates. Toxin concentrations in *H. armigera* ranged from 0.5 to 16 μg/ml for Cry1Ac and from 4 to 128 μg/ml for Cry1Fa and Cry2Ab and in *E. insulana* from 0.075 to 2.4 μg/ml for all three toxins. The same concentrations were used for the mixtures Cry1Ac + Cry1Fa and Cry1Ac + Cry2Ab, which were tested at three different proportions: 1:1, 1:4 and 4:1; e.g. for the mixture Cry1Ac + Cry1Fa in *H. armigera*, 16 μg/ml of Cry1Ac were mixed with 16 μg/ml of Cry1Fa to produce the highest concentration used in the bioassay and serial dilutions were performed. These particular ratios were chosen because mean Cry2Ab2:Cry1Ac ratio in the crop season is 3.5:1 (http://www.epa.gov/opp00001/biopesticides/ingredients/factsheets/factsheet_006487.htm) and also because the first generation of *H. armigera* and *E. insulana* larvae appear in the month of June, when the mean levels expression of Cry2Ab are 3.6-fold greater those of Cry1Ac (Adamczyk et al., 2001). Mortality bioassays in *E. insulana*, for mixtures Cry1Ac:Cry1Fa were performed on the Egyptian population and for mixtures of Cry1Ac + Cry2Ab on the Spanish population, because the Egyptian population succumbed due to a bacterial infection halfway through the study. Groups of 24 neonate larvae were treated with each concentration of *Bt* alone or in mixtures. Negative control insects were mock-infected and positive controls comprised neonate larvae of known susceptible species to these toxins, i.e., *Plutella xylostella*, *Spodoptera exigua*, and *Lobesia botrana*. Each bioassay was performed three times. Insects were incubated at 25 °C, 60% RH, and a 14:10 (L:D) photoperiod. Mortality was recorded after 7 days.

To determine larval growth inhibition, sub-lethal toxin concentrations were used following the method described above. The sub-lethal concentrations used for singly assayed toxins against *H. armigera* ranged from 16 to 8000 ng/ml for Cry1Ac and Cry2Ab, and from 250 to 8000 ng/ml for Cry1Fa. For *E. insulana* (Spanish population), each of the three toxins were administered in the range 5–150 ng/ml. For the assays with combined toxins, identical sub-lethal concentrations those of the most active toxin, Cry1Ac, were used. The live weight of surviving larvae (weighed in groups of four) was recorded after 7 days. Since Cry1Ac and Cry2Ab toxins caused similar mortality, but differed in their effect on growth inhibition in *E. insulana*, a new series of bioassays were performed using the two highest sub-lethal concentrations (75 and 150 ng/ml) of each toxin that were administered to groups of 40 larvae per concentration. A total of four assays were performed and mortality and individual weights of the surviving larvae were recorded after 7 days.