Susceptibility of Four Tropical Lepidopteran Maize Pests to *Bacillus thuringiensis* CryI-Type Insecticidal Toxins

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J. Econ. Entomol. 90(2): 412–415 (1997)

ABSTRACT The relative susceptibility of 4 tropical maize pests, *Spodoptera frugiperda* (J. E. Smith), *Diatraea grandiosella* Dyar, *D. saccharalis* (F.), and *Helicoverpa zea* (Boddie), to the lepidopteran-specific CryI-type proteins produced by *B. thuringiensis* is presented. The toxin with the highest potency against *H. zea* larvae was the CryIAc toxin. *S. frugiperda* larvae were susceptible to CryID and CryIF toxins. The CryIB toxin showed to be highly toxic against *D. grandiosella* and *D. saccharalis*. This information will establish a basis for selecting *B. thuringiensis* strains producing the appropriate CryI proteins to be used for the biological control of these tropical pests.

KEY WORDS *Spodoptera frugiperda, Diatraea grandiosella, Diatraea saccharalis, Helicoverpa zea, Bacillus thuringiensis,* δ-endotoxin

Bacillus thuringiensis (BERLINER) is a gram-positive bacterium that produces crystallic protein inclusions during sporulation. This inclusions are formed by proteins called δ-endotoxins or insecticidal crystal proteins (ICP), which are toxic to insect larvae, many of which are disease vectors or major crop pests. The use of *B. thuringiensis* as a microbial insecticide has several advantages over the use of chemical control agents; *B. thuringiensis* strains are highly specific for certain hosts and are not toxic to other insects, and plants and vertebrates and are completely biodegradable so no residual toxic products accumulate in the environment. Preparations of *B. thuringiensis*-based insecticides applied to the foliage are washed off by rain and may be inactivated by sunlight; therefore, only relatively short-term protection from pest populations is obtained from single applications.

It has been estimated that up to 15% of crops worldwide are lost because of insect damage alone (Boulter et al. 1989). Insecticidal crystal proteins from *B. thuringiensis* have been used as biopesticides during the past 30 yr and a wide array of commercial products are now available and used in the field. Furthermore, some ICP genes have been introduced into the plant genome, with a high level of protection from insect attack (Vaeck et al. 1987, Perlak et al. 1990).

Bacillus thuringiensis strains can be isolated from soil samples from all over the world on plant surfaces, dead insects, and stored grains. Currently, 45 different serotypes and 8 nonflagellated biotypes have been cataloged (Lecadet et al. 1994). Numerous ICP genes have been cloned, sequenced, and classified, based on their homology and specificity (Höfte and Whiteley 1989). To date, 11 subgroups of *cryI* genes specific to lepidopteran pests have been characterized and identified. Each CryI toxin has a narrow range of toxicity against different lepidopteran insects (Feitelson et al. 1992). Five *cryIII* genes specific against coleopteran pests have been described. Both insect orders contain some of the most devastating agricultural pests.

The search for novel *B. thuringiensis* ICPs is a priority worldwide. This toxin will provide different alternatives for insect control and for coping with the problem of insect resistance. In this sense, it is important to have a methodology that allows for accurate identification of the most active toxins against selected pests. The analysis of toxic activity through bioassay allows the identification of the powerful toxins against a particular insect.

Maize (*Zea mays* L.) is one of the principal crops in Mexico and other developing countries, where it is used for human and animal nutrition. The crop is a target of many different pests including 4 lepidoptera species: *S. frugiperda, D. grandiosella, D. saccharalis,* and *H. zea.* The objective of the current study was to determine the relative susceptibility of these 4 tropical pests to the lepidopteran specific proteins produced by *B. thuringiensis*.

Materials and Methods

Purification of Insecticidal Crystal Proteins. Insecticidal crystals containing CryIAa, CryIAb,

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0022-0493/97/0412–0415$02.00/0

and CryIAc protoxins were purified from *B. thuringiensis* HD1 strain. CryIAc protoxin was purified from *B. thuringiensis* subsp. *kurstaki* HD73 strain. CryIE toxin was purified from *B. thuringiensis* HD125 strain and CryIF from *B. thuringiensis* 1B181 strain. CryIAb, CryIC, and CryID protoxins were obtained as recombinant proteins expressed in *Escherichia coli*. The *cryIAb* gene was cloned from *B thuringiensis* subsp. *berliner* 1715 (Höfte et al. 1986). The *cryID* gene was cloned from *B. thuringiensis* subsp. *aizawai* HD68 (Höfte et al. 1990). The *cryIC* gene was cloned from *B. thuringiensis* subsp. *entomocidus* 60.5 (Honée et al. 1988). *E. coli* transformant strains were kindly supplied by M. Peferoen (Plant Genetic Systems, Gent, Belgium). CryIB protoxin was purified from the Mexican isolated *B. thuringiensis* subsp. *thuringiensis* IB43 strain. This strain was characterized by enzyme-linked immunosorbent assay (ELISA) using a specific monoclonal antibody against CryIB toxin and polymerase chain reaction (PCR) analysis using specific primers for the *cryIB* gene described in Cerón et al. (1994).

Crystalline inclusions produced in *B. thuringiensis* strains were purified from spores and cell debris by centrifugation in discontinuous sucrose gradients as described by Thomas and Ellar (1983). Cells were grown in nutritive medium (Difco, Detroit MI) until complete sporulation was achieved. Spores and crystals were centrifuged at 6,000 × *g* for 10 min at 4°C. The pellet was washed 4 times with deionized water. The final pellet was resuspended in 50 mM Tris·HCl (pH 7.5). Spores and crystals were layered on top of a 30-ml discontinuous sucrose gradient, composed of 6 ml each of 67, 72, 79, 84, and 90% (wt:vol) sucrose in 50 mM Tris HCl, (pH 7.5). Centrifugation was carried out at 80,000 × *g* for 14 h at 4°C. Crystals formed a major band at the interface between 72 and 79% sucrose or between 79 and 84% sucrose, while the spores formed a discrete pellet at the bottom of the tube. The crystal band was removed and washed 3 times in 50 mM Tris·HCl (pH 7.5) and centrifuged at 15,000 × *g* for 5 min at 4°C to remove the sucrose. The final pellet was resuspended in deionized water. Purity of the crystal preparation was monitored by phase contrast microscopy. If necessary, a 2nd centrifugation in the discontinuous sucrose gradients was performed. Crystal inclusion bodies were solubilized in 0.1 M $NaHCO_3$, 10 mM β-mercaptoethanol, pH 9.5 for 4 h at 37°C. After centrifugation at 15,000 × *g* for 10 min, protein concentrations were determined by using the Bradford procedure (Bradford 1976) with bovine serum albumin as a protein standard and sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described by Laemmli (1970)

The insecticidal proteins produced in the recombinant *E. coli* strains were purified from 500 ml of saturated culture in LB medium (Sambrook et al. 1989) supplemented with the corresponding antibiotic. Cells were suspended in 50 mM Tris HCl (ph 7.5), 50 mM EDTA, 15% sucrose, treated with lysozime (100 mg/ml) for 30 min at 37°C and sonicated on ice 4 times for 1 min. The cell debris was washed twice with 200 ml of 50 mM Tris·HCl, 150 mM NaCl (ph 7.5), containing 2% triton X-100, incubated for 30 min in the same buffer at 0°C, centrifuged at 15,000 × *g* for 10 min, and washed twice with 50 mM Tris HCl, 150 mM NaCl, (ph 7.5). *B. thuringiensis* protoxins present in the pellet were solubilized in 0.1 M $NaHCO_3$, 10 mM β-mercaptoethanol pH 9.5 for 4 h at 37°C and centrifuged at 15,000 × *g* for 10 min. Solubilized protein was loaded on a Sephacryl S-300 column (100 × 2.5 cm) and eluted with a flow rate of 40 ml/h.

Toxicity Assays. Larvae of *S. frugiperda, D. grandiosella, D. saccharalis,* and *H. zea* were obtained from colonies established in the Entomological Rearing Facility at CIMMYT (Mexico) where the bioassay were done.

Artificial diets, prepared as described by Mihm (1982, 1983a, b) were used for all tests. Each toxin was diluted in water, mixed with a vortex mixer for 1 min, sonicated, and added to the diet at the rate of 10 or 100 mg/g diet. The mixture was poured into each well of a 32-well microtiter plates (Cell Wells, Corning Glass Works, Corning, New York). The diet was allowed to solidify and a single larvae (1-d-old larvae of *S. frugiperda, D. grandiosella,* and *D. saccharalis* and 2-d-old larvae of *H. zea*) was added to each well. Microtiter plates were covered with polyester film lidding material and incubated in a growth chamber at 27°C, 65 ± 5% RH, and a photoperiod of 16:8 (L:D) h. Thirty-two insects were evaluated per toxin and each treatment was replicated at least 3 times. Mortality was assessed after 7 d.

Mortality of the control larvae reared on a toxin-free diet and under the same conditions was recorded and used to correct the mortality test with Abbott formula (Abbott 1925) The LC_{50} values and confidence limits were obtained by probit analysis (Finney 1971).

Results and Discussion

Bioassays with neonate lepidopteran larvae were performed with the CryI type ICPs. It has been reported that these proteins are highly specific against lepidoteran insects. The percentage of mortality obtained with 10 mg of each toxin applied per gram of artificial diet is presented in Fig. 1.

Helicoverpa zea is a serious pest of highland maize crops. We found that the toxin with the highest potency against *H. zea* larvae was the CryIAc toxin (Fig. 1; Table 1). This insect was also susceptible to the CryIAb toxin (Fig. 1). These data are in agreement with previous bioassays that showed that CryIAc has a LC_{50} values of 464 ng/cm^2 on *H. zea* larvae (Garczynski et al. 1991). However, there is noinformation regarding the sus-

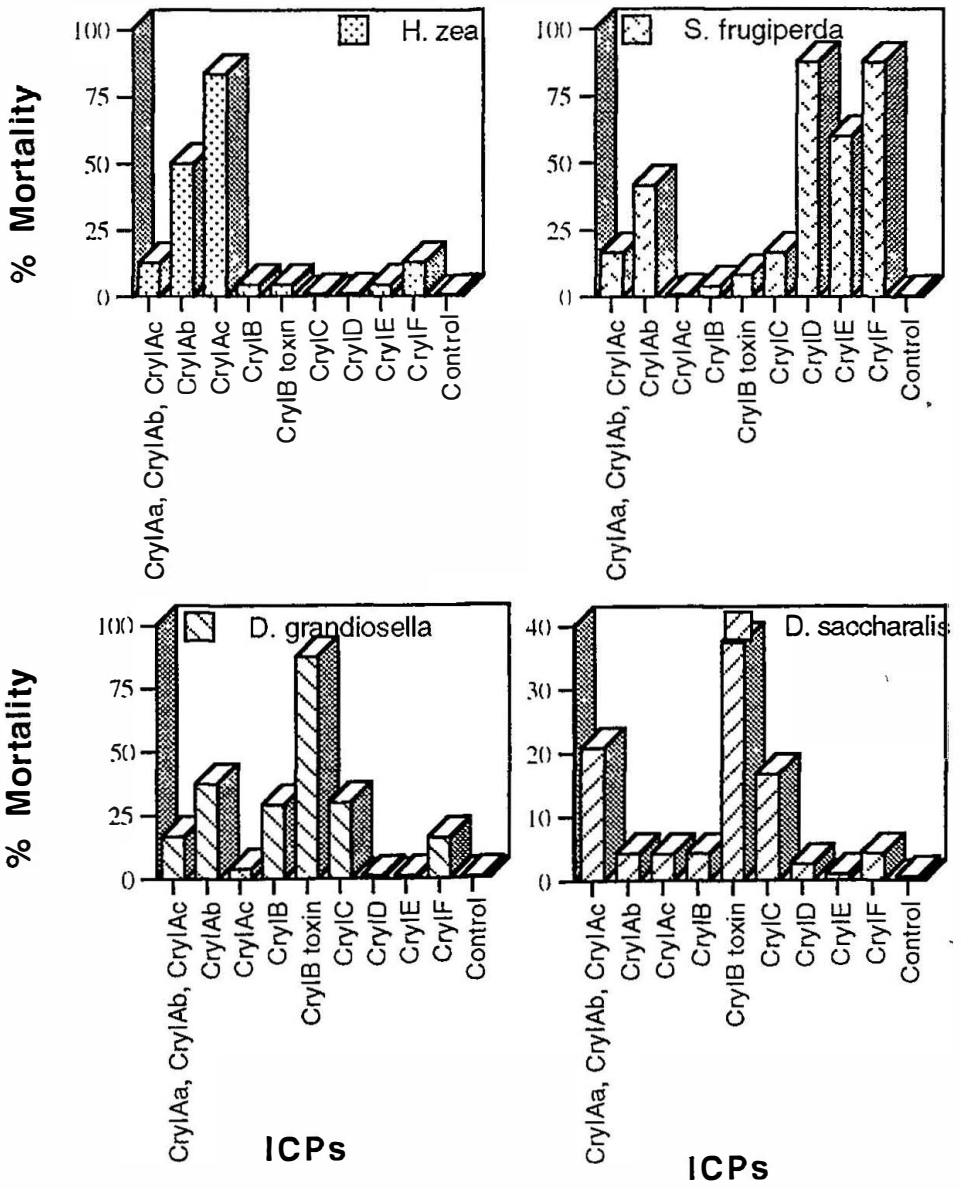

Fig. 1. Response of 4 different lepidopteran insects to CryI insecticidal crystal proteins from *B. thuringiensis*. Percentage mortality obtained with 10 mg of each toxin applied per gram of artificial diet.

ceptibility of this insect toward other Cry toxins. Other *Heliothis* species are also highly susceptible to CryIAc. For example, *H. virescens,* which is a cotton crop pest that was shown to be highly susceptible to CryIAc and CryIAb toxins, presented LC_{50} of 2 and 7 ng, respectively, of toxin applied over the surface of 1 cm^2 of artificial diet (Van Rie et al. 1989).

Table 1. Toxicity of Cry toxins on lepidopteran insects

Insect	ICP	LC_{50}	CI_{95}	*n*
D. grandiosella	CryIB	5.2	3.6–5 5	7
D saccharalis	CryIB	113.6	45.8–318.9	3
S. frugiperda	CryID	1.54	0.4	4
H zea	CryIAc	8.2	7.	3

Data are expressed in micrograms of toxin applied per gram of artificial diet. 95% CI were calculated from probit analysis. *n,* Number of replications.

Information regarding the susceptibility of *S. frugiperda* to the Cry protein family is limited. The larval stage of this insect is an important pest of maize, cotton, alfalfa, clover, peanuts, and many garden crops. The CryIC toxin has been reported to be toxic against *S. exigua* (Visser et al. 1990) and *S. littoralis* (Van Rie et al. 1990) with LC_{50} values of 68 and 93 ng/cm^2, respectively. CryID and CryIE have been reported to be toxic to *S. littoralis* with LC_{50} of 423 and 88 ng/cm^2, respectively (Van Rie et al. 1990). In this study, we analyzed the susceptibility of *S. frugiperda* larvae to the family of CryI toxins and we found that the toxins with the highest activity were CryID and CryIF toxins (Fig. 1).

There is little information regarding the control of *D. grandiosella* or *D. saccharalis* by *B. thuringiensis* ICPs. We found that both types of larvae are highly susceptible to CryIB toxin (Fig. 1). Table 1 shows the LC_{50} and the confidence intervals

for the *D grandiosella* and *D saccharalis* bioassays. The CryIB protein was highly toxic to *D grandiosella,* LC_{50} of 5 1 mg/g artificial diet was obtained CryIB toxin was found to be ≈20 times less active against *D saccharalis*

Our findings indicate that no single ICP is active against all pests species However, these results will be important in selecting *B thuringiensis* strains producing the appropriate CryI protein to be used for the biological control of each of these tropical pests or the different ICPs combinations needed to control maize pests, where mixed insect populations attack the crop Also, the knowledge of which toxin has the higher activity against a selected pest will set the basis for selecting which genes will be most appropriate in transgenic plants generation

Acknowledgments

We thank M Peferoen for providing recombinant *E coli* strains and Myriam Ortíz for strain maintenance This work was supported in part by UNDP, DGAPA IN-214294 and CONACyT 400344-5-4311N

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Received for publication 1 March 1996, accepted 9 December 1996