Biological Control 50 (2009) 157–163

Contents lists available at ScienceDirect

Biological Control

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

Selection and characterization of the *Bacillus thuringiensis* strains toxic to *Spodoptera eridania* (Cramer), *Spodoptera cosmioides* (Walker) and *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae)

Karen Bianchi dos Santos [a], Pedro Neves [a], Ana Maria Meneguim [b], Rachel Bianchi dos Santos [a], Walter Jorge dos Santos [b], Gislaine Villas Boas [a], Vinicius Dumas [c], Erica Martins [c], Lílian Botelho Praça [c], Paulo Queiroz [c], Colin Berry [d], Rose Monnerat [c,*]

[a] *Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, km 445, CEP 86047-902, Londrina/PR, Brazil*
[b] *Instituto Agronômico do Paraná, Rodovia Celso Garcia Cid, km 375, CEP 86047-902, Londrina/PR, Brazil*
[c] *Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica, W3 norte final, CEP 70770-900, Brasília/DF, Brazil*
[d] *Cardiff University, Park Place, Cardiff, CF10 3TL Wales, UK*

ARTICLE INFO

Article history:
Received 24 November 2008
Accepted 26 March 2009
Available online 2 April 2009

Keywords:
Bacillus thuringiensis
Spodoptera eridania
Spodoptera cosmioides
Spodoptera frugiperda
Cry protein

ABSTRACT

Among the pests of cultivated plants, the *Spodoptera* species complex is one of the most important. This genus is composed of polyphagous insects that attack, among other crops, cotton, corn, soy and castor bean. In Brazil *Spodoptera frugiperda* has been recognized as a primary pest in cotton, and *Spodoptera eridania* (Cramer) and *Spodoptera cosmioides* (Walker) species are important pests in cotton and soy. This study was performed in 2008 and was focused on selecting and characterizing *Bacillus thuringiensis* strains highly pathogenic to *S. cosmioides*, *S. eridania* and *S. frugiperda* and identifying Cry proteins toxic to these species. One hundred strains that exhibited toxicity to Lepidoptera were evaluated through bioassay and the most toxic ones were characterized through morphologic, biochemical and molecular analyses and LC_{50} values were determined. Since the *cry1Aa*, *cry1Ab*, *cry1Ac* and *cry2Aa* genes were present in most of the toxic strains, recombinant Bt producing toxins encoded by representative genes from these families, were individually tested. The Cry proteins showed different levels of toxicity to the three *Spodoptera* species, with Cry1Aa and Cry1Ab the most toxic to *S. cosmioides*, Cry2Aa the most toxic to *S. eridania* and Cry1Aa, Cry1Ab and Cry2Aa most toxic to *S. frugiperda*. Cry1Ac, a component of some transgenic cotton varieties such as Bollgard I, extensively used in Brazil, presented low toxicity to the three species studied.

1. Introduction

The species complex of the genus *Spodoptera* (Lepidoptera: Noctuidae) has shown a high damage potential on several cultivated plants. These insects attack cotton, corn and soy crops and have frequently appeared in castor bean, sorghum, tomato, vegetables and fruit cultures, causing significant feeding damage to different organs of the plants (King and Saunders, 1984; Nora et al., 1989; Bavaresco et al., 2003; Santos, 2007). In Brazil, *Spodoptera frugiperda* (Smith) is considered as a primary pest in corn and cotton (Santos, 1997, 2007; Silvie et al., 2001) and the species *Spodoptera eridania* (Cramer) and *Spodoptera cosmioides* (Walker) have been reported as important pests in cotton and soy (Santos, 2007). The occurrence of these species in agricultural systems composed of soy, corn and cotton, which provide a continuous food source, has demanded frequent insecticide applications, increasing the control cost, and creating the potential for resistance, as well as causing damage to the environment and risk to the agricultural operators.

Among the alternative control measures available to decrease environmental impact problems, is the use of the Gram-positive bacterium *Bacillus thuringiensis* (Berliner). During the sporulation process, this bacterium produces crystal inclusions composed of Cry proteins that are toxic to a limited number of target insect larvae but which cause no harm to non-target insects, vertebrates or to the environment (Krieg and Langenbruch, 1981; Höfte and Whiteley, 1989; Monnerat and Bravo, 2000). Because of this, commercial products made of *B. thuringiensis* are used in the control of pest insects and disease vectors (Borem, 2005; Vilas-Boas et al., 2007) and some toxin genes have been inserted in transgenic plants including cotton, corn and soy, often referred to as Bt plants (Barroso and Hoffmann, 2007).

Although *S. frugiperda* is an important pest in America, there have been few studies performed to select *B. thuringiensis* strains toxic to this species (Hernandez, 1988; López-Edwards et al.,

* Corresponding author. Fax: +55 61 34484673.
E-mail address: rose@cenargen.embrapa.br (R. Monnerat).

1049-9644/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.biocontrol.2009.03.014

1999; Monnerat et al., 2007). Furthermore, there are no reports detailing the screening of strains of this bacterium against other members of the genus such as *S. eridania* and *S. cosmioides*. For this reason, the present study focused on selecting and characterizing *B. thuringiensis* strains pathogenic to *S. eridania*, *S. cosmioides* and *S. frugiperda*; determining the level of susceptibility of theses species to some individual Cry proteins and, thereby, determining the possible impact of bio-insecticides or Bt plants on this insect complex.

2. Materials and methods

2.1. Insects

The colonies of *S. eridania*, *S. cosmioides* were started with individuals originally collected from Brazilian cotton cultures from the states of Mato Grosso and Bahia, respectively. The rearing of *S. frugiperda* was started with individuals originally from cotton culture from the state of Paraná. The three species were reared on an artificial diet based on beans, yeast extract and flour extract (Parra, 2001). The rearing was performed in the Agricultural Institute of Parana in an acclimatized chamber at 27 ± 2 °C, 60 ± 10% humidity and photophase of 14 h. To obtain the eggs, the adults were placed in plastic cages (10 cm diameter by 20 cm high), closed on the upper extremity with tissue and on the lower extremity with a Petri dish. The adults were fed daily with an aqueous solution of 10% (v/v) honey (Santos et al., 2005). From this mass rearing, we obtained eggs that originated the 2nd generation laboratory caterpillars used in bioassays.

2.2. Bacillus thuringiensis strains

One hundred *B. thuringiensis* strains, belonging to the Entomopathogenic banks of the State University of Londrina and Embrapa Genetic Resources and Biotechnology (Monnerat et al., 2007) isolated from soil and water samples from different regions of Brazil and previously identified as pathogenic to Lepidopteran species of insects, were used in this work.

The HD-1 strain of *B. thuringiensis* subspecies *kurstaki* and *B. thuringiensis* subspecies *israelensis* IPS-82 was obtained from the Collection of *B. thuringiensis* and *Bacillus sphaericus* at the Institut Pasteur, Paris.

2.3. Bioassays against S. eridania, S. cosmioides and S. frugiperda

Two types of bioassays were performed (i) selective: to identify pathogenic strains; and (ii) dosing: to determine the lethal concentration needed to kill 50% of the tested insects (LC_{50}). The selective bioassays were performed by spreading 150 μl of the final whole culture of each *B. thuringiensis* strain (obtained through the culture in NYSM medium (Yousten, 1984) in a shaker at 30 °C, 200 rpm for 72 h), onto the surface of artificial diet (Parra, 2001) previously poured into 100 ml plastic cups and allowed to solidify to give a surface area of 18 cm^2.

After drying the spore/crystal mixture on the diet, 10 s instar caterpillars were placed in each cup, in a total of three replicates per strain. The insects were then left in an acclimatized chamber at 27 ± 2 °C, 60 ± 10% humidity and photophase of 14 h. As a control, three cups were maintained without the bacterium. The procedure of the selective bioassay was the same for each of the three species tested and was repeated twice. The quantification of the dead caterpillars was performed on the fifth day after the beginning of the bioassay. The strains that caused at least 70% mortality were selected for further study.

The 50% lethal concentration (LC_{50}) was determined using lyophilized suspensions of spores and crystals. The strains were cultivated according to the method described above, centrifuged at 14,000*g* for 30 min at 4 °C, frozen overnight and lyophilized in a Labconco Lyphlock model lyophilizer for 18 h. Then, decreasing concentrations (between 100 and 0.01 μg/ml) of the lyophilized material diluted in sterile water were prepared and 150 μl of these suspensions were spread over the artificial diet previously poured in 100 ml cups, as described for the previous assay. In the *S. frugiperda* bioassays the caterpillars were individualized to prevent cannibalism. The bioassays were repeated three times and kept in an acclimatized chamber at 27 ± 2 °C, 60 ± 10% humidity and a photophase of 14 h. The mortality data were evaluated on the fifth day after the beginning of the assay and submitted to Probit analysis (Finney, 1971), to determine the LC_{50} values.

2.4. Characterization of the B. thuringiensis strains toxic to S. frugiperda, S. cosmioides and S. eridania

The strains that caused at least 70% mortality to the three species of *Spodoptera* were characterized according to their morphology, protein profile and presence of *cry* genes.

2.5. Morphological characterization

The morphological characterization of the strains was performed through scanning electron microscopy. The strains were cultivated in NYSM agar medium at 30 °C for 72 h, then a loop full of the strain was collected and diluted in sterile water. A 100 μl of this dilution was deposited over metallic supports for drying during 24 h, at 37 °C, covered with gold for 180 s by a Emitech metalizator model K550 and observed in a scanning electron microscope Zeiss model DSM 962.

2.6. Characterization of proteins through SDS–PAGE

The characterization of the strains was performed by protein electrophoresis in polyacrylamide gel (SDS–PAGE 10%). The proteins were obtained according to the protocol described by Lecadet et al. (1991), from material grown in NYSM for 72 h at 200 rpm and 30 °C. The HD-1 strain of *B. thuringiensis* subsp. *kurstak*i was used as control.

2.7. Molecular characterization

The selected strains were characterized according to the presence of genes encoding Cry proteins. For this, PCR tests were performed using specific primers designed to amplify the *cry1*, *cry2*, *cry3*, *cry4* genes and specific to identify *cry1*, *cry2*, *cry4* and *cry9* genes (Ceron et al., 1994, 1995; Bravo et al., 1998; Ibarra et al., 2003; Lima et al., in press).

The DNA extraction was accomplished by adapting the protocol described by Sambrook et al. (1989). The PCRs were performed in 0.2 ml polypropylene tubes in a MJ Research, Inc. thermocycler (PTC-100™). From each sample 2 μl of genomic DNA was transferred to a polypropylene tube containing 0.5 μM of each specific primer, 0.2 mM of dNTP mix, Taq buffer 1× and 2.5 U of Taq DNA polymerases in a total volume of 50 μl. The PCR products were visualized in 1.5% agarose gels.

The HD-1 strain of *B. thuringiensis* subspecies *kurstaki* and *B. thuringiensis* subspecies *israelensis* IPS-82 were used as controls.

2.8. Individual recombinant strains expressing Cry proteins

Mixtures of spores and crystals of Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa proteins were obtained from recombinant *B. thuringiensis*

strains that express individually the *cry1Aa*, *cry1Ab*, *cry1Ac* and *cry2Aa* genes. The recombinant strains were constructed by cloning genes in the shuttle vector pHT315 (Arantes and Lereclus, 1991) and *B. thuringiensis* 4Q7 cells were transformed as previously described (Lereclus et al., 1989). The purification protocol and the bioassay protocol were the same as that used for the bioassays using *B. thuringiensis* strains.

3. Results and discussion

3.1. Selective bioassay of the B. thuringiensis strains against S. eridania, S. cosmioides and S. frugiperda

From the one hundred *B. thuringiensis* strains tested through the selective bioassays, only seven (S997, S1905, S608, BR9, BR10, BR37 and BR45), besides the standard HD-1 *B. thuringiensis* subspecies *kurstaki* (Btk) presented toxicity above 70% to *S. eridania*, *S. cosmioides* and *S. frugiperda* species. Although there are reports in the literature that the *Spodoptera* species presented low susceptibility to the Btk based products (Morales and Novoa, 1992; Navon, 1993; Bohorova et al., 1997; Nyouki et al., 1996), our bioassays showed that the Btk HD-1 strain was highly pathogenic to the *Spodoptera* species studied here.

The LC_{50} of the seven *B. thuringiensis* strains varied between 0.09 and 1.6 μg/ml to *S. cosmioides*, between 0.22 and 9.1 μg/ml to *S. eridania* and between 0.35 and 2.2 μg/ml to *S. frugiperda* (Table 1). The lowest lethal concentrations to the *S. cosmioides* were caused by the BR37 and S1905 strains. The BR10, BR37, BR45 and S608 isolates were the most toxic to *S. eridania*, and BR9, BR37, BR45 S608 and S1905 were the most toxic to *S. frugiperda* (Table 1).

3.2. Characterization of B. thuringiensis strains toxic to S. frugiperda, S. cosmioides and S. eridania

The morphological characterization through scanning electron microscopy allowed the detection of different crystal protein inclusions. Strains S608, BR37 and BR45 produced bi-pyramidal and spherical crystals (Fig. 1). The BR9 and BR10 strains, produced three different types of crystals: bi-pyramidal, spherical and cuboidal (Fig. 1). Strains S1905, S997 and HD-1 also presented these three types of crystals, as described previously (Praça et al., 2004; Monnerat et al., 2007). Microscopic examination of the crystal morphology of a strain may provide some indication of its insecticidal activity (Martin and Travers, 1989; Karamanlidou et al., 1991; Meadows et al., 1992; Tailor et al., 1992; Lereclus et al., 1993; Habib and Andrade, 1998; Saadoun et al., 2001). The bi-pyramidal crystals may be related to the presence of Cry1 proteins, efficient against Lepidoptera and Coleoptera insects, while cuboidal crystals are associated with the Cry2 proteins, which show activity against lepidopterans and dipterans (Silva et al., 2004).

The analysis of the proteins of the spore–crystal mixtures by SDS–PAGE showed the presence of two main polypeptides of approximately 130 and 65 kDa (Fig. 2). These molecular masses are typical of lepidopteran active crystal-proteins from the Cry1 and Cry2 classes (Schnepf et al., 1998; Lereclus et al., 1993), consistent with the results of our bioassays.

Using PCR with specific primers designed to detect *cry1*, *cry2*, *cry3*, *cry4* genes and specific for identification of *cry1*, *cry2*, *cry4* and *cry9*, it was possible to determine which *B. thuringiensis cry* genes were present in the strains. The gene profile presented in Table 2 shows that strains S608, BR9 and BR10 carried *cry1Aa*, *cry1Ab*, *cry1Ac* and *cry2Aa* genes, a profile similar to that previously reported for the strains S997 (Praça et al., 2004) and S1905 (Monnerat et al., 2007). Strain BR37 carried eight genes, seven of them belonging to the *cry1* group, while strain BR45 had only the *cry1Ab*, *cry1E* and *cry2Aa* genes. It is interesting to note that strain BR10 also contained a *cry3* gene and Cry3 proteins are associated with toxicity to coleopterans (Monnerat and Bravo, 2000). Bourgouin et al. (1988) reported that some *B. thuringiensis* strains may carry only one gene, while others may have multiple different genes. In the case of strain BR37, there appear to be at least seven genes. The genes that were found most commonly in the new strains reported here were *cry1Ab* and *cry2Aa*, which were found in all of the strains. The *cry1Aa* gene was found in six strains, while *cry1Ac* and *cry1B* were found in five. The genes that were found less frequently were *cry1C*, *cry1D* and *cry1F* (once only) and *cry1E* (occurring twice).

3.3. Toxicity of purified Cry proteins of B. thuringiensis

Due to the frequency at which the *cry1Aa*, *cry1Ab*, *cry1Ac* and *cry2Aa* genes were found in the strains toxic to *S. frugiperda*, *S. eridania* and *S. cosmioides*, tests were performed to evaluate the toxicity of mixtures of spores and crystals of Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa proteins.

The bioassays showed that there was a statistically significant difference in the LC_{50} of the Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa purified proteins when tested in *S. eridania*, *S. frugiperda* and *S. cosmioides* (Table 3). The Cry1Aa and Cry1Ab proteins were the most toxic to *S. frugiperda*, Cry1Ab was the most toxic to *S. cosmioides* and Cry2Aa the most toxic to *S. eridania*. It can be noted that Cry2Aa showed low toxicity to *S. cosmioides*, Cry1Ac low toxicity to *S. frugiperda* and Cry1Aa, Cry1Ab and Cry1Ac low toxicity to *S. eridania* (Table 3).

Several studies performed with *S. frugiperda* have shown that this insect presents variations in the susceptibility to different *B. thuringiensis* toxins, probably related to the genetic variability among different populations of the insect. Bohorova et al. (1997)

Table 1
LC_{50} from the seven *B. thuringiensis* strains against second instar caterpillars of *S. cosmioides*, *S. eridania* and *S. frugiperda* after 5 days of bioassay.

Strain	*S. cosmioides*		*S. eridania*		*S. frugiperda*	
	LC_{50} (μg/ml)	Fiducial limit (95%)	LC_{50} (μg/ml)	Fiducial limit (95%)	LC_{50} (μg/ml)	Fiducial limit (95%)
HD-1	0.56 b*	0.26–1.04	3.4 c	2.17–4.72	1.4 b	0.69–2.80
BR9	1.0 b	0.7–2.30	1.6 b	1.30–2.14	1.0 ab	0.47–4.66
BR10	1.6 b	0.70–3.56	0.41 a	0.20–0.67	1.6 b	0.64–2.33
BR37	0.09 a	0.03–0.16	0.28 a	0.08–0.61	0.63 ab	0.46–1.03
BR45	0.90 b	0.60–1.28	0.49 a	0.32–0.72	0.35 a	0.18–0.60
S608	0.61 b	0.30–1.16	0.22 a	0.16–0.29	0.87 ab	0.34–1.93
S997	–	–	3.2 bc	2.08–4.78	2.2 b	0.81–5.39
S1905	0.40 ab	0.16–0.78	9.1 c	4.43–18.79	0.76 ab	0.40–1.60

–, Not tested.
* Measures followed by the same letter, in the column, do not differ among themselves by the fiducial limit, according to the Probit analysis.

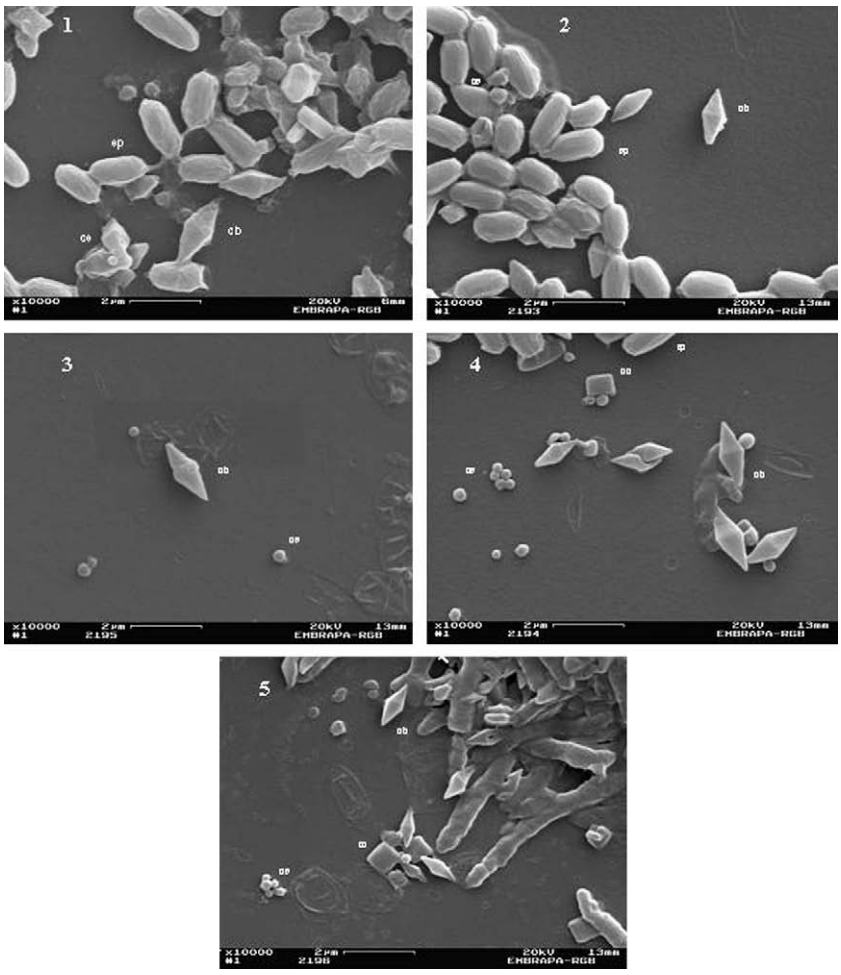

Fig. 1. Scanning electron microscopy of spore–crystal mixtures of *B. thuringiensis* strains. 1, S608; 2, BR37; 3, BR45; 4, BR9; 5; BR10; cb, bipyramidal crystal; ep, spore; ce, spherical crystal; cc, cuboidal crystal.

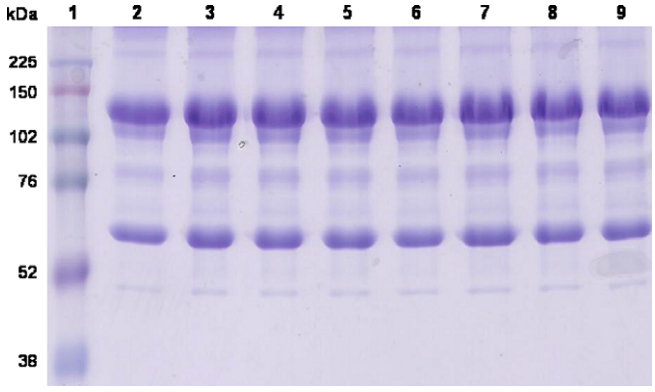

Fig. 2. Protein profile produced by the strains toxic to *Spodoptera frugiperda, S. exigua and S. cosmioides*. 1, Rainbow molecular weight marker (GE); 2, S1905; 3, S997; 4, HD-1; 5, S608; 6, BR37; 7, BR9; 8, BR45; 9, BR10.

Table 2
Cry genes and protein profile present in *B. thuringiensis* strains.

Strains	Gene profile
S1905	*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B* and *cry2Aa*
S997	*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B* and *cry2Aa*
HD-1	*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa*, *cry2Ab*
S608	*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B* and *cry2Aa*
BR37	*cry1Aa*, *cry1Ab*, *cry1Ad*, *cry1C*, *cry1D*, *cry1E*, *cry1F* and *cry2Aa*
BR9	*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B* and *cry2Aa*
BR45	*cry1Ab*, *cry1E* and *cry2Aa*
BR10	*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B*, *cry2Aa* and *cry3*

reported that purified Cry1Ab protein was more toxic to *S. frugiperda* than the Cry1Aa, Cry1Ab and Cry1Ac proteins all together. Waquil et al. (2004) observed that the Cry1Ab protein caused the inhibition of the accumulation of biomass from the caterpillars of *S. frugiperda* by approximately 90%. Van Rie et al. (1990) confirmed that species of *Spodoptera* are tolerant to the Cry1Aa and Cry1Ab proteins. Aranda et al. (1996) described that the Cry1Ab protein produced by the HD-1 strain was not toxic to *S. frugiperda* and Luttrell et al. (1999) observed low efficiency of Cry1Ac protein in the control of *S. frugiperda* while Aguiar et al. (2006) reported that the Cry2Aa protein presented toxicity to *S. frugiperda*. All the authors worked with different populations of this insect. Monnerat et al. (2006) have demonstrated toxicity differences of several *B. thuringiensis* strains in *S. frugiperda* populations native in Mexico, Brazil and Colombia and showed that this susceptibility occurred due to the high genetic variability among the populations that resulted in groups that did not present receptors to the Cry1D toxin (the Brazilian population) or Cry1B (the Mexican population) and were, therefore, insensitive to the respective toxins, although, the Cry1C toxin showed average toxicity to the three populations tested.

Table 3
Results of the dosing bioassays performed with purified Cry proteins effective on *S. cosmioides*, *S. eridania* and *S. frugiperda*.

Cry proteins	*S. cosmioides*		*S. eridania*		*S. frugiperda*	
	LC_{50} (μg/ml)	Fiducial limit (95%)	LC_{50} (μg/ml)	Fiducial limit (95%)	LC_{50} (μg/ml)	Fiducial limit (95%)
Cry1Aa	0.58 ab*	0.14–1.52	75 c	42.83–121.4	0.32 a	0.14–0.72
Cry1Ab	0.37 a	0.12–0.82	62 c	37.34–188.22	0.88 a	0.40–1.74
Cry1Ac	2.8 b	1.20–6.26	21 b	14.29–33.97	11 b	4.78–30.65
Cry2Aa	24 c	8.91– 171.1	1.0 a	0.42–2.36	1.9 ab	0.70–5.30

* Measures followed by the same letter, in the columns, do not differ among themselves by the Fiducial limit.

All of these data must be carefully analyzed before selecting a bio-larvicide or a *B. thuringiensis* transgenic plant. An important issue is the susceptibility of different *S. frugiperda* populations and the presence of different species of the *Spodoptera* complex. At present, bio-insecticides are available for sale, which contain Btk HD-1 expressing Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa proteins or which contain Bt subsp. *aizawai* (Bta), which produce Cry1Ab, Cry1C and Cry1D proteins, which are toxic in variable levels to *Spodoptera* complex caterpillars in a manner that depends on the target species and on the different populations of *S. frugiperda* that may be present in the field. Producers from the Distrito Federal region in Brazil have described a variable efficiency (sometimes high, sometimes low) against *S. frugiperda* from the products made of Btk and a good efficiency of Bta (unpublished reports). Since both products both produce Cry1Ab protein among their arsenal of toxins and we show Cry1Ab to be potent against *S. frugiperda*, it is possible that these reports of problems may be related to the formulation of the product and the application technology, which is not well developed in Brazil, rather than the efficiency of the bacterium itself.

In Brazil, transgenic plants expressing the Cry1Ac toxin are also available (registered or in the process of registration), such as Bollgard I (Macintosh et al., 1990; Stewart et al., 2001, 2000; Armstrong et al., 2007), DP90 and NUOPAL (Vohlk et al., 2007), Bollgard II (which was developed through the incorporation of the Cry2Ab toxin in Bollgard cotton) (Greenplate et al., 2000a,b), DP50 (which expresses a greater concentration of Cry2Ab and Cry1Ac toxic proteins (Bollgard II) (Santos, 2007), WideStrike (expressing Cry1Ac and Cry1F) and VipCot (expressing the VIP3 protein, also derived from *B. thuringiensis*) (ICAC, International Cotton Advisory Committee, 2004).

Vohlk et al. (2007) evaluated the efficiency of DP90 and NUOPAL transgenic cotton, presently registered in Brazil, which express the *cry1Ac* gene. In the evaluation, damage caused by *Pseudoplusia includens* (Lepidoptera: Noctuidae) and species of *Spodoptera* (mainly *S. eridania)*, were noted. According to the authors, it was necessary to apply insecticide on these varieties due to damage caused by these pests in order to ensure their control. In another field study performed with DP90B and NUOPAL cultures, Miranda et al. (2007) monitored the efficiency of these cultures on *Alabama argilacea* (Lepidoptera: Noctuidae) and *Heliothis virescens* (Lepidoptera: Noctuidae) but again, additional insecticide applications for the control of *S. cosmioides* were also necessary. The studies of Miranda et al. (2007) and Vohlk et al. (2007) confirmed in Brazil the low efficiency of Cry1Ac on *S. cosmioides* and *S. eridania* in crops that produce this protein. The data obtained in this work showed that Cry1Ac has low toxicity to these species and, thus, explain the results obtained by the authors listed above.

The expression of the Cry2Ab protein in Bollgard II promotes an increase in the insecticidal activity compared to Bollgard I cotton. Studies confirm the greater efficiency of Bollgard II over Bollgard (Penn et al., 2001; Voth et al., 2001), because it contains the Cry2Ab and Cry1Ac proteins. Stewart et al. (2001) noted that no *H. virescens*, *S. frugiperda* and *S. exigua* caterpillars survived to the pupal stage when fed with Bollgard II, however, there was survival of caterpillars of these species reared on Bollgard. In Brazil, field research with the DP50 variety that expresses a greater concentration of the Cry2Ab and Cry1Ac toxic proteins (Bollgard II – Monsanto) (Santos, 2007), were found to be efficient for the control of *S. frugiperda* (Stewart et al., 2001, 2000). Bollgard II is not only effective against pests controlled with Bollgard, but it satisfactorily controls *Helicoverpa zea* (Lepidoptera: Noctuidae), *S. frugiperda*, *S. eridania* and *Trichoplusia ni* (Lepidoptera: Noctuidae) (Jackson et al., 2001; Chitkowski et al., 2003; Degrande and Fernandes, 2006; Li et al., 2007).

The data obtained in our study demonstrate the great importance of always performing susceptibility studies on each primary or secondary target insect when choosing a strain or toxin for use in the field. Secondary or less important pests of the cotton crop may become primary pests, if the pest control is not well performed. Using the results with the toxins studied in this work we can understand the different species susceptibilities and, for example, avoid reliance on use of Cry1Aa or Cry1Ab toxins to prevent selection of *S. eridania* and promoting its "status" from secondary pest to primary pest. Similarly, Cry2Aa can be seen to control *S. cosmioides* poorly and care must be taken to include other toxins against this pest.

On the other hand, the construction of "pyramidal" transgenic plants expressing more than one toxin may be a good alternative for the control of *Spodoptera* complex species: in the case of the species studied here, a good option would be the use of *cry1Aa* or *cry1Ab* genes (encoding the toxins active against *S. frugiperda* and *S. cosmioides*) and *cry2Aa* (encoding the toxin active against *S. eridania*). In this context, biological products made of native Bt strains that express these toxins would also be a recommended strategy.

For Integrated Pest Management (IPM), the formulation of new insecticides made of *B. thuringiensis* strains and the development of plants that express different Cry proteins are highly important for the reduction of the potential resistance development of *Spodoptera* spp. The usage of bio-insecticides and plants that express Cry protein diversity minimizes the selection pressure that induces the resistance to *Spodoptera* complex in parallel areas and soy, corn and cotton producing areas. The presence and the expression of multiple toxins makes possible the development of plants that express *cry* genes as well as the formulation of bio-insecticides as an instrument of Integrated Pest Management.

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