Brazilian Journal of Microbiology (2007) 38:531-537
ISSN 1517-8382

A BRAZILIAN *BACILLUS THURINGIENSIS* STRAIN HIGHLY ACTIVE TO SUGARCANE BORER *DIATRAEA SACCHARALIS* (LEPIDOPTERA: CRAMBIDAE)

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Submitted: January 04, 2007; Approved: July 29, 2007.

ABSTRACT

The control of the major sugarcane pest, *Diatraea saccharalis*, is limited by the stem location of the caterpillar. As part of a long-term project towards the development of an alternative and efficient delivery system of Cry proteins to control the sugarcane borer, the current work describes the selection and characterization of a Brazilian *B. thuringiensis* strain with prominent activity towards *D. saccharalis.* Strain S76 was eleven-fold more active than the HD-1 Lepidoptera-standard strain, as estimated by the LC_{50} of 13.06 µg/L and 143.88 µg/L, respectively. We observed bipiramidal and cuboidal crystals similar to those found in other *B. thuringiensis* strains with entomopathogenic activity against Lepidoptera and Diptera. In addition, smaller and spherical crystalline inclusions were also observed. The plasmid profile of strain S76 is similar to that of HD-1. PCR amplifications of S76 DNA using *cry* specific primers confirmed the presence of *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa1*, and *cry2Ab2*, but not *cry1Ad, cry2Ac and cry9* type genes. No differences that could explain the superior activity of S76 when compared to HD-1, the Lepidoptera standard strain, were observed. Nevertheless, its higher entomopathogenic activity has pointed this strain S76 as a potential source of *cry* genes to control sugarcane borer, an important pest that affects sugarcane, a crop that occupies a planted area of about 6 million ha in Brazil.

Key words: Biological control; *Bacillus thuringiensis kurstaki*; Cry toxins; Spherical crystal; *Diatraea saccharalis*.

INTRODUCTION

Diatraea saccharalis, the sugarcane borer, is the major pest of sugarcane crops and its caterpillar-feeding behaviour, inside the stems, hampers control. Synthetic pesticides are inappropriate due to poor penetration and environmental impairment. To avoid or minimize the damage produced by *Diatraea* spp, biocontrol programs based on egg and larva predators, and on parasitoids have been used (21). The National Program for controlling *Diatraea* spp. in Brazil began in the 1970's and is considered the World's largest biocontrol program based on *Cotesia flavipes*, a larval parasitoid. Nevertheless, fluctuations in larva control efficiency as a result of geographical and growing season peculiarities, a large number of natural enemies required for field release, expertise (crucial to monitor these populations in large affected areas), and the occurrence of a hyperparasitoid to *C. flavipes* cocoons on several Brazilian cane crops (19) threaten this strategy. An attack of 10% in the sugarcane crop, grown in the state of São Paulo – Brazil, by the borer *D. saccharalis* represents a yield loss in the order of US$ 100 million a year due only to the secondary damages caused by the red rot fungus that weakens the plant (13). Therefore, a highly efficient control of *Diatraea saccharalis* is imperative especially if the 6 million ha planted with sugarcane in Brazil are considered.

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The uniquely specific Cry proteins of *Bacillus thuringiensis* are accumulated in the cytoplasm of the cells during sporulation. After ingestion by target insects, these large crystals, consisting of one or more inactive precursors, are solubilized and proteolyticaly converted into functional toxins by insect mid-gut pH and proteases, respectively (14). These proteins, also known as delta-endotoxins, are active against several insect orders including nematodes, mites, and protozoa, and are safe for non-targeting organisms such as humans, plants and other invertebrates (32). Distinction in toxin processing by different larval gut proteases may provide *B. thuringiensis* with selectivity observed in some insect species. The current nomenclature is based solely on hierarchical clustering using amino acid sequence identity (12) see also http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt. The extraordinary diversity of *B. thuringiensis* strains and toxins is, at least in part, due to a high degree of genetic plasticity. Most *cry* genes, which code for these toxins, reside on large (>30 MDa) plasmids (10,22,25) that can harbour one or several delta-endotoxin genes, with some being conjugative in nature (23). This plasticity is further enhanced by mobile elements, often found flanking *cry* genes (27).

Bioinsecticides based either on a spore and crystal mixture or the heterologous expression of *cry* genes in microorganisms (18, 31) as well as in plants (33) have been efficiently used for controlling important agricultural pests and mosquitoes. Early attempts to control the sugarcane borer with *B. thuringiensis* subsp. *kurstaki* strain HD-1 were carried out with success in the United States (11). More recently, a screening of Mexican *B. thuringiensis kurstaki* strains against the sugarcane borer resulted in a selection of the strain GM-34 with a LC_{50} value of 33.21 µg/mL (29). This bacterium has been used as an active ingredient in the insecticidal formulation prepared to control the sugarcane borer in that country (30). On the other hand, Bohorova (5) did not identify strains bearing activity exceeding 60% mortality, including HD-1 standard strain when tested against lepidopteran insects.

Bioassay analyses have allowed the selection of Cry1Ab proteins with important lethality towards the sugarcane borer (2,7). As a consequence, the corresponding *cry1Ab* gene was used to develop sugarcane plants resistant to stem borer attack (7,8). Among the Cry proteins, the Cry1, Cry2 and Cry9 groups exhibit the strongest activity against Lepidoptera (35). In *B. thuringiensis* with this biological activity, the *cry1* gene family is the most abundant, followed by the *cry2* and *cry9* gene families (35).

The objective of the current work was to evaluate the susceptibility of *D. saccharalis* to Brazilian *B. thuringiensis* strains and the selection of a strain with remarkable activity towards the sugarcane borer. In addition, the Cry protein profile and ultra-structure along with the plasmid profile and *cry* gene content of the S76 strain were characterized.

MATERIALS AND METHODS

Strains and growth conditions

Five *B. thuringiensis* strains used in this work are new Brazilian strains stored at *Bacillus* spp. Collection (Embrapa Genetic Resources and Biotechnology - Brazil). They are S48 and S105 subspecies unknown, S76 and HD-1 subsp. *Kurstaki*, S90 and S135 subsp. *Tolworth* (28) Strain HD-1 is a standart strain against lepidopteran insects, kindly provided by Pasteur Institute – Paris – France. The cultures were grown to mid-log phase or induced to sporulation in BHI (Acumedia, UK) or HCT culture media (15), respectively, at 30ºC and 200 rpm.

Toxicity Bioassays

Bioassays against *D. saccharalis* larvae were carried out using 500 µg L^{-1} of spore-crystal complex diluted in the soy/cane artificial diet dispensed in dishes. The control did not contain the spore-crystal complex. Twenty larvae on 2nd instar were used, with 5 repetitions for each treatment. The insects were maintained in the laboratory, at 26 ± 2ºC, 70 ± 10% RH, and a photoperiod of 14:10 (L:D). Mortality rates were evaluated 5 days after exposing the larvae to the spore-crystal complex. The mortality assay was calculated according to the Abbot's formula (1). The bioassays were repeated at least 3 times to confirm the results. The strain that exhibited the highest toxic activity was selected for further studies.

Lethal Median Concentration

Serial dilutions of the spore-crystal suspensions were incorporated into the artificial diet. The dry mass concentrations of each dilution was determined and the LC_{50} value was calculated. The HD-1 standard strain for lepidopteran insects was used as a positive control. The treatments were applied in the same way as the toxicity bioassays and the LC_{50} values and confidence limits were obtained using the "Micro Probit 3.0" program (20).

Crystal purification

Cells from 1 L of sporulated culture were harvested by centrifugation and crystals purified on a step sucrose gradient, as previously described (17), in a Beckman (USA) ultracentrifuge, model L8-80M. The band containing crystals was washed 3 times in 50 mM Tris-HCL, pH 7.5, resuspended in 3 mL of distilled water containing 1.0 mM PMSF, and stored at -20ºC.

Scanning electronic microscopy (SEM)

Purified crystals were dried on a metal support, at room temperature, and covered with gold for 60 sec at 40 mA, in a Bal-Tec Sputter-coater, SCD 050 model. Samples were observed and photographed in Stereoscan 200 scanning electron microscope.

SDS-PAGE

Twenty µL spore crystals mixture (17) were analysed on 10% SDS-PAGE under standard conditions.

Plasmidial Profile

Plasmidial DNA and agarose gel analysis was performed as described by De-Souza (16).

PCR

The synthetic oligonucleotide primers used in this work are shown in Table 1. From each *B. thuringiensis* strain, 50 ng of DNA (17), were mixed with (all reagents from Gibco, BRL, USA): 100 rmol of each primer, 1 U of *Taq* DNA polymerase, 200 µM of each dNTP, and 1.5 mM of $MgCl_2$. Amplifications were performed on a Programmable Thermal Controller – PTC-100™ (MJ Research, Inc., USA) according to the authors (Table 1). From each amplification, 10 µL were analysed on agarose gel under standard conditions.

Table 1. General and specific primers used in this study.

Primer pair	Target Gene	Expected Product length (kb)	Reference
SB-1/U8-15c	*Cry1Aa*	1500	
SB-2/U3-18c	*Cry1Ab*	858	(6)
RB-19/U8-15c	*Cry1Ac*	653	
Un2d/EE-2Ar	*Cry2Aa1*	498	
Un2d/EE-2Abr	*Cry2Ab2*	546	(4)
Un2d/EE-Acr	*Cry2Ac*	725	
spe-cry9A	*Cry9A*	571	
spe-cry9B	*Cry9B*	402	(9)
spe-cry9C	*Cry9C*	306	

RESULTS AND DISCUSSION

Toxicity Bioassays

The strain S76 assured 100% mortality within the first 72 hours to the sugarcane borer when incorporated in the diet at a concentration of 500 µg L^{-1} of spore-crystal complex. The remaining strains (S48, S90, S105 and S135) provided less than 3% mortality, significantly less than strain HD-1, the Lepidoptera standard, which promoted 69% mortality (Fig. 1).

Lower activities (maximum 60%) were also observed when the five strains were submitted to bioassays against 2nd instar larvae of *Anticarsia. gemmatalis*, *Plutella. xylostella*, and *Spodoptera Frugiperda* (data not shown). Mortality higher than 60% was also reported when several *B. thuringiensis* strains, including subspecies *kurstaki* strains, were evaluated

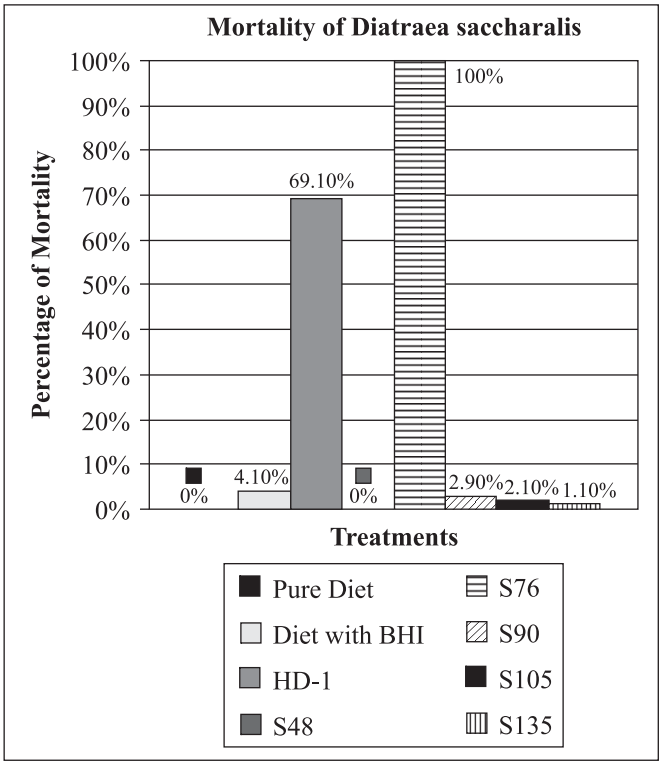

Figure 1. Mortality of *D. saccharalis* larvae fed with artificial diet containing different *B. thuringiensis* strains. Bioassays were carried out using 500 µg L^{-1} of spore-crystal complex. The mortality test was corrected with the Abbot's formula (1).

against larvae of *D. saccharalis* (5). These authors also observed higher entomopathogenic activity of these strains when compared to the strain HD-1. Toxicity of HD-1 strain against *D. saccharalis* was earlier reported by Charpentier (11). Therefore, these results corroborate with the strategy to select a new and more efficient strain to control the sugarcane borer. Although biological control using parasitoids has been applied with success to control the stemborer and transgenic sugarcane plants are being developed to control these insects, the use of alternative methods such as the highly efficient *B. thuringiensis* strain S76 is an interesting option that should be explored. The toxicity of sporulated S76 cultures against 2nd instar larvae of *D. saccharalis* was determined and compared with that of HD-1 (Table 2). Strain S76 was about eleven-fold more toxic than HD-1, as estimated by the LC_{50} values of 13.06 µg/L and of 143.88 µg/L for S76 and HD-1 strains, respectively. A Mexican *B. thuringiensis kurstaki* strain GM-34 with a much lower LC_{50} (33.21 µg/mL) (29) has been used in the insecticidal formulation against the sugarcane borer in Mexico (30). Comparison of both strains suggests that the Brazilian isolate is much more efficient than the Mexican bacterium in the control of *D. saccharalis*. Therefore, considering the bioinsecticide potential of strain S76

Table 2. Dose-response insecticidal actives of *B. thuringiensis* S76 and HD-1 strains against *D. saccharalis*.

Strain	LC_{50}[a] (μg L^{-1})[b]	Slope ± SE	X^2 (0.95)[c]
S76	13.06 (10.58-15.94)[2]	1.42 ± 0.09	9.23
HD-1	143.88 (105.22-206.91)[2]	0.87 ± 0.06	10.08

[a] LC_{50}, lethal median concentration; [b] Number in parentheses indicate 95% confidence interval; [c] Chi-Square statistic. The LC_{50}, values and confidence limits were obtained by the probit analysis (20).

to control *D. saccharalis* larvae in sugarcane field in Brazil, it was selected for further characterization of Cry toxin production and the respective *cry* gene contents.

Scanning Electronic Microscopy and Protein Profile

SEM analysis revealed large amounts of bipiramidal (Fig. 2A) and cuboidal (Fig. 2B) shapes, usually present in *B. thuringiensis* strains with Lepidoptera- and Diptera-killing activities, including the HD-1 strain (32,36). An additional smaller and spherical shaped crystal was also observed in strain S76 (Fig. 2A). Although *kurstaki* strains are commonly reported to bear bipiramidal and cuboidal crystals, these findings are in agreement with the recent observation of Silva (34), who found a similar crystal content in two other *B. thuringiensis kurstaki* strains. Wasano (36) also observed spherical crystals among crystalline inclusions from *B. thuringiensis* strains with activity to Lepidoptera larvae. These authors showed that the delta-endotoxins, or a polypeptide with a molecular weight of 130 kDa, were the main components of these crystals, indicating that the spherical morphology may represent a change in the delta-endotoxins crystallization process.

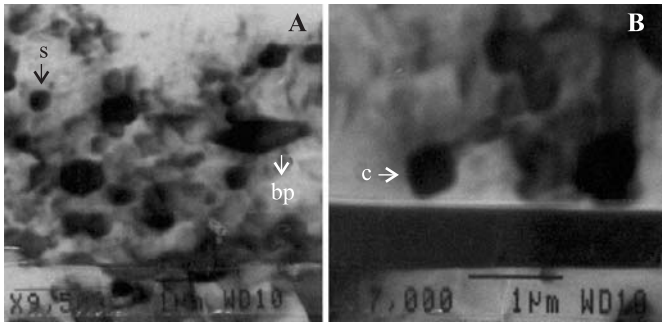

Figure 2. Cry proteins ultra-structural analysis. Scanning electronic micrograph (SEM) of *B. thuringiensis kurstaki* S76 crystals purified on sucrose gradient (72-79%). (A) bp and s indicate bipiramidal and spherical morphologies (9.500x), respectively. (B) cuboidal morphology (17.000x).

Spore-crystal mixtures of strain S76 were analysed on 10% SDS-PAGE and compared to strain HD-1 (Fig. 3). Two major polypeptides of ca. 130 kDa (Cry1A-type) and 70 kDa (Cry2-type), usually associated with strains producing bipiramidal (Fig. 2A) and cuboidal crystal morphology respectively (Fig. 2B), were observed for both strains. Huber (24), reported that the majority of the crystals from *B. thuringiensis*, toxic to insects of the Lepidoptera order, are composed of proteins of approximately 130 kDa, which corresponds to the size of delta-endotoxins from the Cry1 class. The 70-kDa polypeptide may corresponds to delta-endotoxins from the Cry2 class, which have a molecular mass 71 kDa. However, other smaller peptides with apparent molecular weights of 38 kDa could also be seen (Fig. 3). Whether these peptides are related to spherical crystal and S76 activity remains to be investigated.

Genetic characterization

Generally, Lepidoptera-specific *B. thuringiensis* isolates bare *cry1* (6), *cry2* (4), and *cry9* genes (9) and the HD-1 standard strain carries *cry1* and *cry2* type genes (32). To determine the S76 *cry* gene content, PCR with the primers described in Table 1 was performed. Both S76 and HD-1 carry a similar toxin gene profile, composed of *cry1Aa*, *cry1Ab* and *cry1Ac* (Fig. 4A) and *cry2Aa*, *cry2Ab* (Fig. 4B) genes.

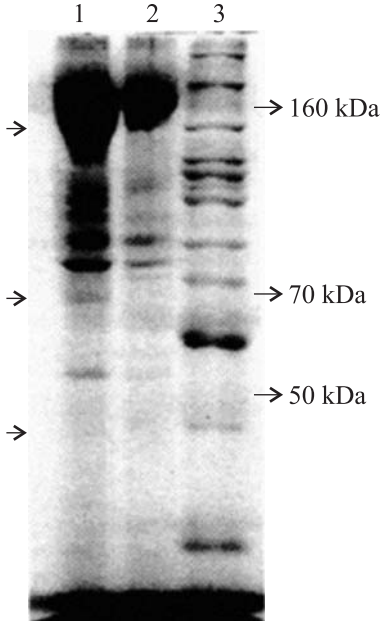

Figure 3. Cry protein profile. Spore crystal mixtures from *B. thuringiensis* were resolved on 10% SDS-PAGE and stained by Coomassie blue R. Lane 1: HD-1. Lane 2: S76. Lane 3: Markers (Benchmark protein ladder; Invitrogen); bars, on the left, indicate molecular weights (kDa). Arrows the right indicate polypeptides ca. 130; 70, and 38 kDa.

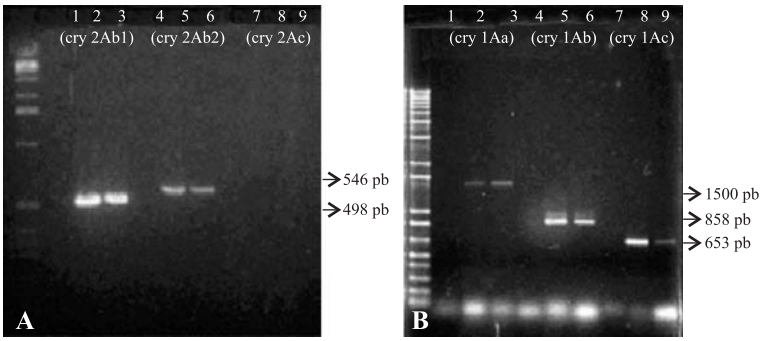

Figure 4. *cry* genes content *B. thuringiensis kurstaki* S76. PCR products amplified using S76 and HD-1 (control) DNA and analysed by electrophoresis. Arrows and numbers indicates product length in bp 1% agarose gel. **(A)** *cry1A*-type genes and **(B)** *cry2A*-type genes. Lanes 1, 4 and 7: no DNA; Lanes 2, 5 and 8: S76 strain; Lanes 3, 6 and 9: HD-1 strain; (Molecular marker (1 kb ladder; Gibco BRL, USA).

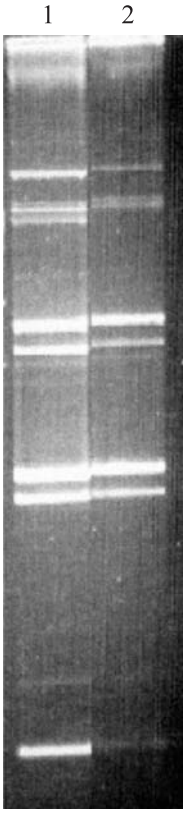

Figure 5. Strain S76 plasmidial profile. Plasmids were extracted by alkaline lysis and purified on 0.5% agarose gel. Lane 1: HD-1, *kurstaki* standard strain; Lane 2: S76.

For neither strain amplification was observed when *cry1Ad* or *cry2Ac* specific primers were used. To investigate the gene(s) responsible for spherical crystal production, other rounds of PCR, using primers described in Table 1, were also performed. No PCR product was obtained when primers specific to *cry9* gene-types, which are commonly associated with spherical crystal morphology, were used (data not shown). Similar results were obtained by Silva (34) for *B. thuringiensis* strains of the subsp. *kurstaki*, with activities against the *Spodoptera frugiperda* lepidopteran. The *cry* genes so far described are present on large (>30 MDa) plasmids (10,22,25,32) that can harbour one or several delta-endotoxin genes. *B. thuringiensis kurstaki* contains multiple protoxin genes confined on few plasmids. A 110 MDa plasmid bears *cry1Aa*, *1Ac*, and *cry2* genes while a 44 MDa plasmid carries the *cry1Ab* gene. It is also know that the 44 MDa plasmid is unstable, although it can be maintained in the population by conjugation (3). The plasmid profile of S76 was compared to that of HD-1. As shown in Fig. 5, strain S76 displays a similar number and size of extrachromosomal elements to that of HD-1. Probably, strain S76 harbours the *cry* genes located in a similar manner. The data did not point to differences that could explain the superior activity of S76 when compared with HD-1, the Lepidoptera standard strain.

Sequencing of 44 and 110 MDa plasmids to analyse their nature and expression studies involving the *cry* genes S76 strain are in progress for our research group. Apart from contributing to the plasmidial biology of Gram positive bacteria, especially the *B. thuringiensis* resident plasmids, the search for novel *B. thuringiensis* strains with much higher insecticidal activities than those available in the market is very important. Defining the specific toxicity of *cry* genes products present in strain S76 and determining their correspondence to novel *cry* genes open new opportunities in the biological sugarcane borer control program.

ACKNOWLEDGEMENTS

This work was funded by Embrapa Agrobiology, UFRJ, Embrapa Genetic Resources and Biotechnology, CNPq and Faperj, Brazil. PMG was supported by Capes/UFRJ.

RESUMO

Uma Estirpe Brasileira de *Bacillus thuringiensis* com elevada atividade para a broca da cana-de-açúcar *Diatraea saccharalis* (Lepidoptera: Crambidae)

Diatraea saccharalis é o inseto-praga que provoca os maiores danos a cultura da cana-de-açúcar, e seu controle é limitado pela localização do ataque no interior do colmo das plantas. Como parte de um projeto a longo prazo com o objetivo de desenvolver uma alternativa eficiente para o controle da broca

da cana utilizando as proteínas Cry de *Bacillus thuringiensis*, o presente trabalho descreve a seleção e caracterização de uma estirpe desta bactéria com atividade larvicida para *D. saccharalis*. A estirpe brasileira S76, foi selecionada pela alta atividade letal contra larvas da broca, dez vezes maior do que a estirpe comercial HD-1 de *B. thuringiensis*, com resultados da CL_{50} de 13.06 µg/L e 143.88 µg/L, respectivamente. Foram observados cristais bipiramidais e cuboides similares aos encontrados em outras estirpes *de B. thuringiensis* com atividade entomopatogenica para lepidópteros e dípteros. Adicionalmente, foram visualizadas pequenas inclusões cristalinas esféricas. O perfil plasmidial da estirpe S76 foi similar ao observado na estirpe HD-1. Amplificações por PCR confirmaram a presença dos genes *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa1* e *cry2Ab2*, porém não foram detectados os genes *cry1Ad*, *cry2Ac* e *cry9* na estirpe S76. Não foi observada nenhuma diferença para explicar a maior atividade da estirpe S76 quando comparada a HD-1. Entretanto, os resultados indicam a estirpe S76 como fonte potencial de genes *cry* para controlar *D. saccharalis*, praga importante que afeta plantas de cana-de-açúcar, cultura esta que ocupa uma área plantada de 6 milhões ha no Brasil.

Palavras-chave: Controle biológico; *Bacillus thuringiensis kurstaki*; Toxinas Cry; Cristais esféricos; *Diatraea saccharalis*.

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