Original Article

SELECTION AND CHARACTERIZATION OF *Bacillus thuringiensis* ISOLATES WITH A HIGH INSECTICIDAL ACTIVITY AGAINST *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

SELEÇÃO E CARACTERIZAÇÃO DE ISOLADOS DE Bacillus thuringiensis COM ALTA ATIVIDADE INSETICIDA CONTRA Spodoptera frugiperda (Lepidoptera: Noctuidae)

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ABSTRACT: *Spodoptera frugiperda* (SMITH, 1797) (Lepidoptera: Noctuidae) affects diverse crops of great economic interest, for instance, it can cause severe yield losses in maize, rice and sorghum. In this study, a selection and characterization of *Bacillus thuringiensis* (BERLINER, 1911) isolates with a high insecticidal activity against *S. frugiperda* was performed. Fifty-two crystal-forming *B. thuringiensis* isolates that were identified from 3384 *Bacillus*-like colonies were examined and screened by PCR for the presence *cry* genes (*cry*1, *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1D, *cry*2 and *cry*2Ab). Four isolates that showed high toxicity towards *S. frugiperda* were shown to harbor *cry*2 genes. The crystals were analyzed by electron microscopy and showed bipyramidal and cuboidal shapes. Furthermore, these four isolates had lethal concentration (LC_{50}) values of 44.5 ng/cm^2 (SUFT01), 74.0 ng/cm^2 (SUFT02), 89.0 ng/cm^2 (SUFT03) and 108 ng/cm^2 (SUFT 04) to neonate *S. frugiperda* larvae. An ultrastructural analysis of midgut cells from *S. frugiperda* incubated with the SUFT01 spore-crystal complex showed disruptions in cellular integrity and in the microvilli of the midgut columnar cells. The isolates characterized in this work are good candidates for the control of *S. frugiperda*, and could be used for the formulation of new bioinsecticides.

KEYWORDS: Cry protein. *Spodoptera frugiperda*. Entomopathogen. Microbial insecticide. Insect control.

INTRODUCTION

The Lepidopteran insect *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae) affects diverse crops of great economic interest, for instance, it can cause severe yield losses in maize, rice and sorghum (Gallo et al., 2002). Since 35% of crops are lost to pest damage, an efficient pest control program is an important component in any effort to increase crop yields. The main trouble with the control of this pest is due to this insect behavior, which remains within the plant cartridges, reducing the contact with insecticides applied for its control (BRAGA MAIA et al., 2013). Moreover, in the conventional agricultural systems, the effects caused by changes in biodiversity and instability between trophic levels make the control of *S. frugiperda* increasingly difficult and costly (SANTOS-AMAYA et al., 2016; BOREGAS et al., 2013). The chemical control of insects in agriculture has been estimated to cost US$ 1391 miillion dollars annually in the united states (PIMENTEL, 2005), thus sustainable insect pest control is of vital importance.

Applications of insecticides and growing resistant cultivars are considered effective control methods. However, insecticides are considered generally, harmful to the environment (TODOROVA; KOZHUHAROVA, 2010) and can result in insecticide resistance in the insect pest population. There is an urgent demand to supplement existing management strategies with new methods that improve the insect pest control.

Biological control through the application of bioactive agents or microorganisms that are entomopathogenic to insect pests is an attractive alternative and a sustainable strategy for plant protection (MASSON et al., 1998). The biological control of *S. frugiperda* has been attempted using

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various microorganisms. *Bacillus thuringiensis* (BERLINER, 1911) (Bt) produces an array of Cry proteins with are potentially toxic to *S. frugiperda* (SANTOS et al., 2009; VALICENTE et al., 2010). The interest in this microorganism stems from its potential as an economical, effective, species-specific and environmentally safe pesticide (ARRIETA; ESPINOSA, 2006).

The accumulation of Cry proteins in a *Bt* cell can constitute 20–30% of the dry weight of the sporulated cell and each crystal protein has in its own insecticidal spectrum (AGAISSE AND LERECLUS, 1995). Some Cry proteins display toxicity to more than one insect order. For example, Cry1I is active against both Lepidoptera and Coleoptera (TAILOR et al., 1992), whereas Cry1B shows toxicity against Lepidoptera, Coleoptera and Diptera (HONGYU et al., 2000). Therefore, Cry proteins have been classified on the basis of their host specificity and their amino acid composition (SCHNEPF et al., 1998; ASOKAN et al., 2012).

The diversity and distribution of Bt and its *cry* genes have been described in several reports (CHAK et al., 1994; BRAVO et al., 1998; WANG et al., 2003). Depending on the soil type, different microbial communities are found. Several studies have demonstrated the toxicity of *B. thuringiensis* against *S. frugiperda* obtained from different types of environment. According to observations by Praça et al. (2004) of the 300 Bt isolated from soil samples, only one was effective against *S. frugiperda* with an LC_{50} of 90.24 ng / cm^2. In contrast, have found a larger number of Bt isolates with toxicity against *S. frugiperda* from the surface of leaves (phylloplane) (65%) and fewer were obtained soil samples (4.7%) in Colombia (Jara et al (2006). According to observations by Armengol et al. (2007), from 445 Bt isolates of different geographic locations from Colombia only 9.7% were toxic against *S. frugiperda*. The selection of new isolates of *Bt* that are toxic against *S. frugiperda* and could serve as a source of new *cry* genes, which could be introduced into economically important plant genomes that need protection against this important pest.

In the present study, soil samples from different regions of the state of Tocantis, Brazil, were used for the screening of *Bt* isolates toxic to *S. frugiperda.* New *Bt* isolates were characterizated and shown potential for further research that may lead to the development of new biopesticides.

MATERIAL AND METHODS

Isolation of *B. thuringiensis*

Bacillus-like colonies were isolated from soil samples from different parts of Tocantins State, Brazil, using the method described by Monnerat et al. (2001). The isolates were obtained from rural region the Darcinópolis (6°43'7" S: 47°45'10" W), Guaraí (8°50'4" S: 48°30'36" W), Santa Rita (10°51'44" S: 48°54'27" W), Cariri (11°53'25" S: 49°9'49" W), Gurupi (11º43'45" S; 49º04'07" W), Sucupira (12°0'57" S: 48°56'6" W), Sandolândia (12°31'37" S: 49°56'7" W), Araguaçú (12°55'51" S: 49°48'59" W), São Salvador (12°44'36" S: 48°14'20" W) and Palmeirópolis (13°2'36" S: 48°24'11" W) (Figure 1). The soil samples were screened for Bt on petri dishes with a selective NYSM medium (KALFON et al., 1983) containing 100 mg/L penicillin G grown for 24 h at 30 ± 0.5°C at 180 rpm. After this period, each sample was individually analyzed and identified by phase contrast microscopy (× 1000), to verify the presence of inclusion bodies and crystals that allow for the differentiation between *B. thuringiensis* and *Bacillus cereus* (FRANKLAND; FRANKLAND, 1887)**.**

Insects

S. frugiperda population with three generations was maintained at the Integrated Pest Management Laboratory of the Federal University of Tocantins. The insects were reared on an artificial diet based on beans, wheat germ and agar, in a controlled room under the following conditions: 26 ± 1°C, 70 ± 10% relative humidity and a light: dark period of 12:12 h. The adults were fed on an artificial diet according to the procedures described by Martins et al. (2008).

Insect bioassays

For selective bioassays, a *Bt* suspension was spread on top of the insect diet in 24-well plates (TPP, Techno Plastic Products AG, Switzerland) and one neonate *S. frugiperda* larvae was introduced in each well. Sterile distilled water was used as control, and the mortality was recorded 24 and 48 h after inoculation. Insect mortality rate was determined by Abbott's formula: $\%M = (T–I)/T \times 100$, where %M = percent insect mortality, T = the number of insects in the control treatment without the application of Bt, and I = the number of insects treated with Bt (ABBOTT, 1925).

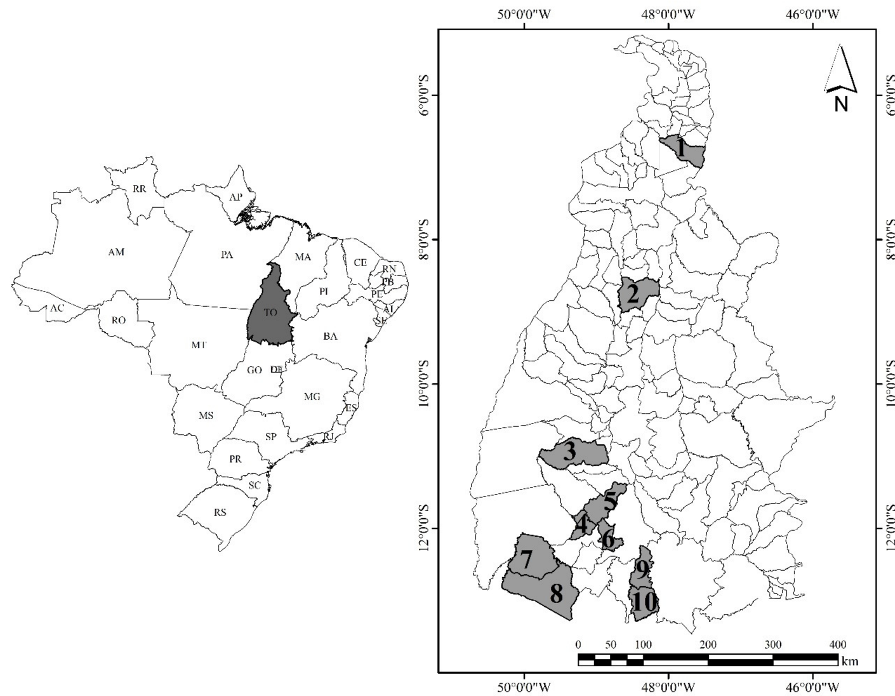

Figure 1. Tocantins State, Brazil, Map indicating the nine cities where the *B. thuringiensis* isolates were obtained. 1- Darcinópolis; 2- Guaraí; 3- Santa Rita; 4- Cariri; 5- Gurupi ; 6- Sucupira; 7- Sandolândia; 8-Araguaçú ; 9- São Salvador; 10: Palmeirópolis.

Dose-response bioassays for the determination of lethal concentration 50 (LC_{50}) were performed with 24 neonate *S. frugiperda* larvae as described by Monnerat et al. (2007), using different doses of dry spores and crystals (2, 23, 43, 63, 83, 103, 123, 143, 163, 183, 203, 243 and 263 ng / cm^2). The insects were kept in an incubator with a photoperiod of 12 / 12 h (light/dark) at 26°C. Mortality was recorded at 24 and 48 h, and the LC_{50} was obtained by a Probit analysis, (FINNEY, 1971), using Polo Plus software (LeOra Software Berkeley, CA, USA). *Bacillus thuriengiensis* subsp. *Kurstaki* HD1 (Btk) was used as reference strain and positive control.

Cry gene analysis

DNA from different isolates of Bt was purified and used as the template for polymerase chain reaction (PCR) with oligonucleotide primer pairs designed to amplify the *cry*1 and *cry*2 genes (Cerón et al., 1995; Ben-Dov et al., 1997; Bravo et al., 1998; Lima et al., 2008) (Table 1). PCR was carried out using Taq DNA polymerase (Invitrogen), and the PCR program was performed as follows: 1 min at 95°C; 30 cycles at 95°C for 1 min, annealing temperature (established as described in Table 1) and 72°C for 1 min, and a final step at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 1, 2% agarose gels in TBE buffer (400 mM Tris, 10 mM boric acid, and 100 mM EDTA; pH 8.0) at 120 V for 30 min.

Protein analysis by SDS–PAGE

Proteins from spore/crystal mixtures were obtained according to the protocol described by Lecadet et al. (1992). Proteins were suspended in a small volume of phosphate-buffered saline (136 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 8 mM Na_2HPO_4 and 4.2 mL H_2O, pH 7.4), and fractionated by electrophoresis in 12% SDS-PAGE gels (Sambrook et al., 2001).

Ultrastructural characterization of spores and Cry proteins

The ultrastructural characterization of the spores and Cry proteins from *B. thuringiensis* isolates was performed by scanning electron microscopy. The isolates were cultivated in NYSM agar medium at 30°C for 72 h and then a loop of the isolate was collected and diluted in sterile water. A volume of 100 µL of this dilution was deposited over metallic supports to be dried for 24 h at 37°C, covered with gold for 180 s using an Emitech apparatus (model K550), and observed in a Zeiss scanning electron microscope (model DSM 962) at 10 or 20 Kv.

Table 1. Primers that recognize the *cry*1, *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1D, *cry*2 *and cry*2Ab genes of *Bacillus thuringiensis* and the expected PCR amplicon size.

Gene	Sequence of primer (F)	Sequence of primer (R)	Prod. (bp)	Ann. temp. (°C)	Reference
cry1	CTGGATTTACAGGTGGGGATAT	TGAGTCGCTTCGCATATTTGACT	558	52	(Bravo et al., 1998)
*cry1*Aa	TGTAGAAGAGGAAGTCTATCCA	TATCGGTTTCTGGGAAGTA	272	48	(Ceron et al., 1995)
*cry1*Ab	TGTAGAAGAGGAAGTCTATCCA	TATCGGTTTCTGGGAAGTA	284	48	(Ceron et al., 1995)
*cry1*Ac	TGTAGAAGAGGAAGTCTATCCA	TATCGGTTTCTGGGAAGTA	272	48	(Ceron et al., 1995)
*cry1*D	TGTAGAAGAGGAAGTCTATCCA	TGTAGAAGAGGAAGTCTATCCA	284	48	(Ceron et al., 1995)
cry2	GTTATTCTTAATGCAGATGAATGGG	GAGATTAGTCGCCCCTATGAG	498	54	(Ben-Dov et al., 1997)
*cry*2Ab	GGGATCCATGAATAATGTATTGAATAGTGGAAG	GGGATCCTTAATAAAGTGGTGGAAGATTAGTTGGC	1990	52	(Lima et al., 2008)

F = specific forward primers; R = specific reverse primers, Ann. Temp – Annealing temperature (°C).

Analysis of *S. frugiperda* midguts

Neonate *S. frugiperda* larvae were fed an artificial diet containing a suspension of Bt. Inoculated larvae were collected 24 h after diet ingestion, and the midguts were removed and processed for transmission electron microscopy, according to the protocol described by Martins et al. (2008). Ultra-thin sections were cut in an ultramicrotome (Leika ultracut UCT) and observed in a JEOL 1011 at 80 kV.

Colony forming units (CFUs) and δ-endotoxin production

The CFU and δ-endotoxin production of the Bt isolates were assessed in CCY medium (Stewart et al., 1981) after 72 h at 30°C in a rotary shaker set at 200 rpm (ZOUARI et al., 2002; AZZOUZ et al., 2014). The number of spores and the δ-endotoxin production were determined as described by Ghribi et al. (2007).

RESULTS AND DISCUSSION

Isolation of *B. thuringiensis* isolates

In total, 52 crystal-forming *B. thuringiensis* isolates were identified from 3384 *Bacillus*-like colonies isolated from soil samples (Table 2). Only 37 showed any toxicity to *S. frugiperda* larvae (Table 3). The occurrence of Bt having higher levels of toxicity towards *S. frugiperda* was more prevalent in soil samples from the south-west region of Tocantins State (Araguaçu and Sandolândia), including four isolates that induced more than an 80% mortality in neonate *S. frugiperda* larvae. Isolates obtained from Palmas, Guaraí were less toxic to *S. frugiperda,* with induced mortalities of less than 15% (Table 3). In this study, 71.2% of Bt isolates showed toxicity against *S. frugiperda*. As can be seen from these results, the Bt isolates toxic to *S. frugiperda* can be found in different sample types and regions. These isolates have the potential to encode new combinations of *cry* genes or new *cry* genes.

Table 2. *Bacillus thuringiensis* (Bt) isolates from soil samples from Tocantins State, Brazil.

Origin[a]	No. of samples Collected	No. of Bt colonies screened	No. of Bt isolates obtained	Bt isolation index[b]
GUR 1	8	123	3	0.024
GUR 2	7	146	1	0.007
GUR 3	7	80	1	0.013
GUR 4	4	73	1	0.014
GUR 5	4	98	1	0.010
GUR 6	6	79	1	0.013
GUR 7	6	67	1	0.015
GUR 8	10	123	2	0.016
GUR 9	14	176	1	0.006
ARA 1	10	78	2	0.026
ARA 2	14	129	3	0.023

ARA 3	7	103	1	0.010
ARA 4	10	75	3	0.040
ARA 5	10	87	1	0.011
PAL 1	4	108	1	0.009
PAL 2	3	176	1	0.006
PAL 3	4	144	1	0.007
PAL 4	4	128	1	0.008
PAL5	7	88	1	0.011
SS 1	23	45	1	0.022
SS 2	14	96	1	0.010
SU 1	23	156	1	0.006
SU 2	6	145	2	0.014
DAR 1	4	98	1	0.010
DAR 2	7	95	1	0.011
DAR 3	8	82	1	0.012
CAR 1	9	64	2	0.031
CAR 2	8	36	1	0.028
SR 1	4	29	1	0.034
SR 2	3	45	1	0.022
GUA 1	6	56	2	0.036
SAN 1	8	72	1	0.014
SAN 2	10	70	1	0.014
Total	303	3.384	52	0.015

[a] First column shows the district of origin of each isolate -GUR: Gurupi; ARA: Araguaçú; PALM: Palmerópolis; SS: São Salvador; SU: Sucupira; SAN: Sandolândia; CAR: Cariri; DAR: Darcinopolis; SR: Santa Rita; GUA: Guarai); [b] Bt isolation index calculated by dividing the population of crystalliferous *Bt* isolates by the total *Bacillus* population of each sample collected.

In this work, Bt isolates were found in both clay and sandy soils. Our data also indicated the occurrence of Bt isolates with higher toxicities to *S. frugiperda* in clay soil samples (Table 3). Intensive screening programs have isolated and characterized new isolates with different combinations of crystal proteins resulting in the discovery of new toxins and toxins with a broader activity spectrum (BRAVO et al., 1998).

Table 3. List of *Bacillus thuringiensis* (*Bt*) isolates that showed toxic activity against *Spodoptera frugiperda*. The data show the polypeptide and gene (*cry*1 and *cry*2) profiles, crystal morphology and % mortality rate for the spore/crystal mixture of each isolate in a selective bioassay.

N.	Origin[a]	Soils	Bt isolate	Polypept. (kDa)	Crystal morphol.	Mort. (%)	*cry* genes
1	GUR 1	clay	SUFT 5	Nd	NF	30%	*cry*1
2	GUR 2	clay	SUFT 9	Nd	NF	50%	*cry*1
3	GUR 3	clay	SUFT 10	130	NF	40%	*cry*1, *cry*1Aa
4	GUR 4	clay	SUFT 11	130	NF	40%	*cry*1
5	GUR 5	clay	SUFT 14	130	NF	45%	*cry*1, *cry*1Aa, *cry*1Ab, *cry*1Ac
6	GUR 6	clay	SUFT 12	140/70	Bip	55%	*cry*1, *cry*1Aa, *cry*1Ac
7	GUR 7	clay	SUFT 23	130/70	Bip	45%	*cry*1
8	GUR 8	clay	SUFT 41	70	Bip	50%	*cry*1, *cry*1Ab, *cry*1Ac
9	GUR 9	clay	SUFT 08	70	Bip	45%	*cry*1,*cry*1Ac
10	ARA 1	clay	SUFT 16	130	Bip	50%	*cry*1,*cry*1Aa, *cry*1Ac
11	ARA 2	clay	SUFT 06	130	Bip/Cub	25%	*cry*1
12	ARA 3	clay	SUFT 07	Nd	NF	25%	*cry*1
13	ARA 4	clay	SUFT 02	140/70	Bip/Cub	85%	*cry*1, *cry*1Aa, *cry*1Ab, *cry*1Ac *cry*1D, , *cry*2, *cry*2Ab
14	ARA 5	clay	SUFT 04	70	Cub	80%	*cry*1, *cry*1Aa, *cry*1Ab, *cry*1Ac,

							*cry*2
15	PAL 1	clay	SUFT 24	Nd	NF	20%	*cry*1
16	PAL 2	clay	SUFT 26	Nd	NF	20%	*cry*1
17	PAL 3	clay	SUFT 32	70	NF	20%	*cry*1, *cry*1Ac
18	PAL 4	clay	SUFT 31	130/70	Bip	10%	*cry*1,*cry*1Ab, *cry*1Ac
19	PAL5	clay	SUFT 44	130/70	Bip	60%	*cry*1, *cry*1Ab, *cry*1Ac
20	SS 1	sandy	SUFT 24	130	Bip	50%	*cry*1, *cry*1Ac
21	SS 2	sandy	SUFT 18	140	Bip	45%	*cry*1, *cry*1Ab,*cry*1Ac
22	SU 1	clay	SUFT 19	70	Bip	45%	*cry*1, *cry*1Ab,*cry*1Ac
23	SU 2	clay	SUFT 15	70	Bip	45%	*cry*1,*cry*1Aa
24	DAR 1	clay	SUFT 13	130/70	Bip/Cub	45%	*cry*1, *cry*1Ab
25	DAR 2	sandy	SUFT 49	Nd	NF	15%	*cry*1
26	DAR 3	sandy	SUFT 37	Nd	NF	35%	*cry*1
27	CAR 1	sandy	SUFT 36	Nd	NF	30%	*cry*1
28	CAR 2	sandy	SUFT 39	Nd	NF	15%	*cry*1
29	SR 1	sandy	SUFT 42	130	Bip	25%	*cry*1, *cry*1Ac
30	SR 2	sandy	SUFT 34	Nd	NF	15%	*cry*1
31	GUA 1	sandy	SUFT 38	Nd	NF	10%	*cry*1
32	SAN 1	clay	SUFT 01	140/70	Bip/Cub	90%	*cry*1, *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1D, *cry*2, *cry*2Ab
33	SAN 2	clay	SUFT 03	140/70	Bip/Cub	80%	*cry*1, *cry*1Aa *cry*1Ab, *cry*1Ac, *cry*2

Nd= not determined, NF= not found, Bip = bipyramidal, Cub =cuboidal.

Insect bioassays

The four isolates that produced 80% or more larval mortality in the selective assay were selected for analysis using the dose-response bioassay. SUFT01 showed the highest toxicity with an LC_{50} of 44.5 ng / cm^2, while isolates SUFT02, SUFT03 and SUFT04 had LC_{50} values of 74.0, 89.0 and 108 ng / cm^2, respectively (Table 4). *S. frugiperda* has shown a variable susceptibility to different *Bt* toxins, probably related to the genetic variability among different populations of the insect (MONNERAT et al., 2007).

At the same time, some Cry proteins were shown to be non-toxic to *S. frugiperda* (ARANDA et al., 1996; LUTTRELL et al., 1999). Santos et al. (2009) evaluated the effects of one *B. thuringiensis* subsp. *aizawai* and several *B. thuringiensis* subsp. *kurstaki* isolates against *S. frugiperda*. They found that, in general, most isolates of *Bt* were not toxic or showed low toxicity towards *S. frugiperda* larvae. Dias et al. (1999) also found that out of 25 *Bt* isolates, only eight had a high toxicity towards *S. frugiperda* larvae.

Table 4. LC_{50} values of *Bacillus thuringiensis* (*Bt*) isolates that have activity against *Spodoptera frugiperda* larvae.

Bt isolate	n[a]	Slope ± SE	LC_{50} (FL[b]) (ng/cm^2)[c]	LC_{95} (FL[b]) (ng/cm^2)[c]	χ^{2}[d]	*P*
SUFT01	25	1.08 ± 0.12	44.5 (22.7-61.0)	104.5 (91.0-142.23)	1.717	0.16
SUFT02	25	2.04 ± 0.21	74.0 (31-150)	194.0 (171-225)	3.944	0.21
SUFT03	25	1.48 ± 0.20	89.0 (72.5-106)	198 (172.5-211.2)	0.325	0.23
SUFT04	25	1.34 ± 0.21	108 (72-220)	218 (199-241)	1.960	0.29

[a] n = The total number of larvae tested in bioassays; [b] FL = Fiducial Limits; [c] LC_{50} and FL calculated by Probit analysis; [d] χ *2* = chi-square test; *P=;* The results of three different bioassays.

Identification of *cry* genes

Predictions of insecticidal activities were made based on the *cry* gene content of the isolates as determined by PCR analysis. All isolates were shown to have *cry* genes when screened with a general *cry*1-specific primer pair designed by Bravo et al. (1998) (Table 2, Figure 2). Using specific *cry* primers, the *cry1* gene was detected in all isolates, the *cry*1Ac gene was the most present with 45.9% (17 isolates), followed by the *cry*1Ab gene with

32.4% (12 isolates), *cry*1Aa with 18.9% (9 isolates), *cry*1D with 5.4% (2 isolates), *cry2* with 10,8% (4 isolates) and *cry2Ab* with 5,4% (2 isolates) (Figure 2). Different Bt screening programs worldwide have identified the *cry*1 genes as the most common *cry*-type gene (BRAVO et al., 1998; URIBE et al., 2003; BERÓN; SALERMO, 2006; SU et al., 2007; GAO et al., 2008; SANTOS et al., 2009; VALICENTE et al., 2010).

Isolates that harbored *cry1*, *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1D, *cry*2 and *cry*2Ab were more toxic to *S. frugiperda* (Table 3). Valicente et al. (2010) suggested that the toxicity of different Brazilian *B. thuringiensis* isolates to *S. frugiperda* was related to their *cry*1 gene content. The *cry* genes frequencies and profiles differ among worldwide Bt collections based on region, moreover, they reported that the genes most frequently found in Minas Gerais, Amazonas, Paraná and São Paulo State, Brazil were *cry*1D, *cry*1G, *cry*1B and *cry*1E, respectively.

Since the toxic activity of any Bt isolate is due to the combination of different Cry proteins, we attempted to identify some common *cry*1 and *cry*2 genes present in a collection of *Bt* isolates, which had any level of toxicity to *S. frugiperda*, from Tocantins State (Brazil). The high toxicity of the four isolates of Bt may be associated with the presence of both the *cry*1 and *cry*2 genes (Table 3). In addition, several factors could be implicated in the insecticidal activity of the four isolates such as: different expression levels of *cry1*A and *cry2* genes and the specific proportions of delta-endotoxins present in the crystal; mutations in *cry* genes and the presence of unknown Cry toxins in the crystal (CHENG et al., 1999; TOUNSI et al., 2006; HIRE et al., 2008). Moreover, the toxicity of Bt isolates against *S. frugiperda* is associated combinations of *cry* genes. Bravo et al. (1998), showed the combination of *cry*1D and *cry*1C genes exhibit high toxicity LC_{50} against *S. frugiperda* below 35 ng / cm^2. Similar results were observed by Jara et al. (2006) with combinations of *cry*1Aa, *cry*1Ac, *cry*1D and *cry*1b genes showing LC_{50} of 29 ng / cm^2. In the present study we observed that Bt isolated with higher toxicity (SUFT01) against *S. frugiperda* had combinations of *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1D, *cry*2 and *cry*2Ab genes and LC_{50} of 44.5 ng / cm^2 (Table 4).

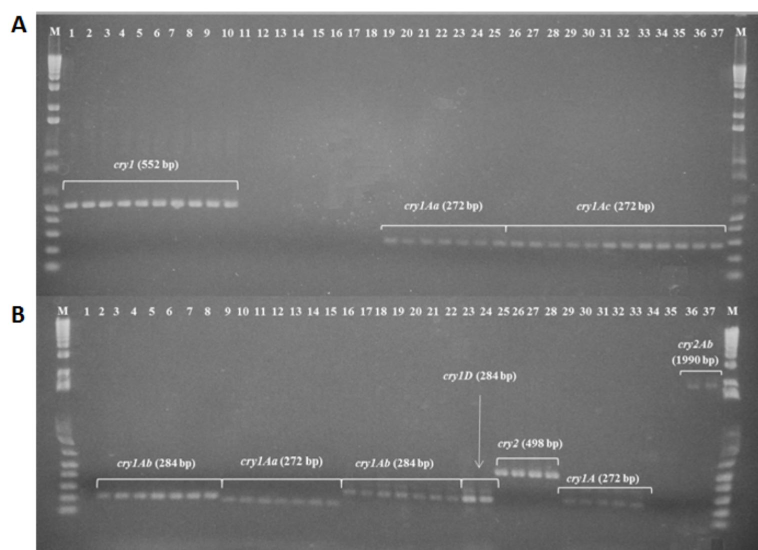

Figure 2. Screening of 37 *Bacillus thuringiensis* (Bt) isolates for the presence of *cry*1 and *cry*2 genes from PCR amplification. **A:** PCR amplification of *cry*1-specific oligonucleotides in lanes 1 to 10 (~552 bp); *cry*1Ab-specific oligonucleotides in lanes 11 to 18 (no PCR amplification); *cry*1Aa-specific oligonucleotides in lanes 19 to 25 (~ 272 bp) and *cry*1Ac-specific oligonucleotides in lanes 26 to 37 (~ 272 bp). **B.** PCR amplification of *cry*1Ab genes in lanes 2 to 8 (~ 284 bp); *cry*1Aa genes in lanes 9 to 15 (~ 272 bp) ; *cry*1Ab genes in lanes 16 to 22 (~ 284 bp); *cry*1D genes in lanes 23 to 24 (~ 284 bp); *cry*2 genes in lanes 25 to 28 (~ 498 bp); *cry*1Ac genes in lanes 29 to 33 (~ 272 bp) ; *cry*1Ac-specific oligonucleotides in lanes 34 and 35 (no PCR amplification produced); *cry*2Ab -specific oligonucleotides in lanes 36 and 37 (1990 bp).

Crystal protein patterns and morphologies

The protein profiles of purified crystals from isolates SUFT01, SUFT02, SUFT03 and SUFT04 were analyzed using SDS-PAGE and showed polypeptides of ~ 70 and 140 kDa (Table 3,

Figure 3). An ultrastructural analysis of thespore/crystal mixtures from SUFT01, SUFT02 and SUFT03 isolates showed the presence of bipyramidal and cuboidal crystals (Figure 4A, B and C). The SUFT04 isolate showed only cuboidal crystals (Figure 4D). The different profiles could be the result of different toxins being produced in these isolates.

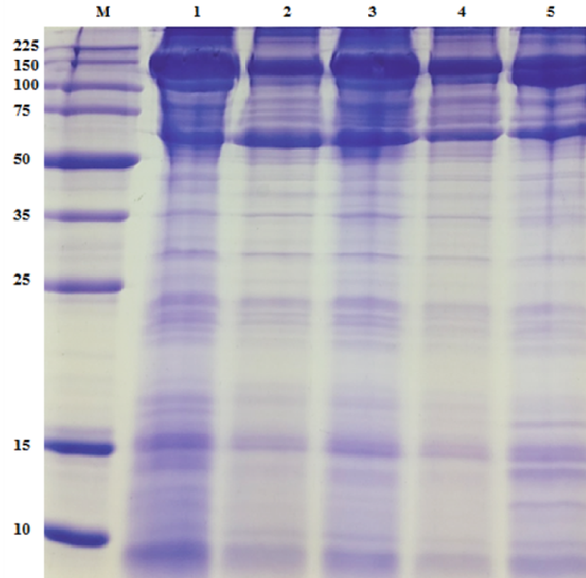

Figure 3. SDS-PAGE of *Bacillus thuringiensis* crystal proteins from selected isolates. Spore/crystal preparations from sporulated cultures were subjected to electrophoresis in bis-acrylamide gels. M – M1, Molecular weight marker; 1- HD1 – strain of *B. thuringiensis*; 2, SUFT01 isolate; 3, SUFT02 isolate; 4, SUFT03 isolate; and 5, SUFT04 isolate.

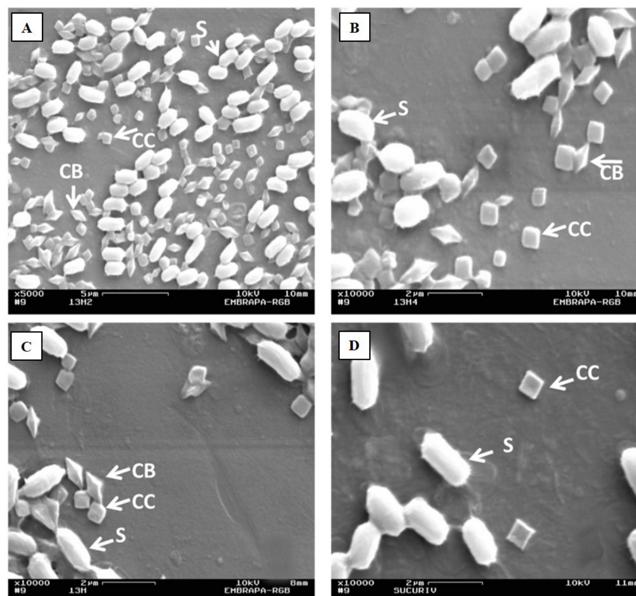

Figure 4. Ultrastructural analysis of the spore/crystal mixtures obtained from selected isolates of *Bacillus thuringiensis* obtained from Tocantins State, Brazil. **A.** SUFT01 isolate, **B.** SUFT02 isolate, **C.** SUFT03 isolate, and **D.** SUFT04 isolate. Arrows: S, spores; CC, cuboidal crystals; and CB, bipyramidal crystals.

The analysis of the Bt isolates revealed that from 37 isolates analyzed, 12 formed bipyramidal crystals (Table 3), similar to isolates active against Lepidoptera (PRAÇA et al., 2004). However, 5 isolates that exhibited toxicity to *S. frugiperda* showed cuboidal and bipyramidal crystal inclusions (Table 3). The presence of a particular crystal type is not always associated with the toxicity towards an

insect order. For example, Cry1I is active against both Lepidoptera and Coleoptera (TAILOR et al., 1992), whereas Cry1B shows toxicity against Lepidoptera, Coleoptera and Diptera (HONGYU et al., 2000). SUFT04 isolate formed cuboidal crystals and the presence of ~ 140 kDa band in SDS-PAGE. The ~140kDa band is usually associated with the formation of bipyramidal crystals. This absence of bipyramidal crystals could be due to the expression of a mutated version of a Cry protein or interaction of more than one Cry protein to produce the cuboidal crystal or even to the presence of a different Cry or other unrelated protein of the same size.

Several Bt toxins have already been identified and their toxicity to *S. frugiperda* has been demonstrated. However, new toxins are still being discovered. That diversity of these toxins includes activity against protozoans, mites, sheep lice, bark beetles, cockroaches, grasshoppers, tephritid fruit flies, moth flies and delphacids (FRANKENHUYZEN, 2009). Specific toxins responsible for those activities have not yet been identified or are still being characterized (SONG et al., 2008).

CFUs and δ-endotoxin production

The CFU and δ-endotoxin production of the selected isolates showed that SUFT01 produced 2,678.90 ± 31.6 mg L^{-1} δ-endotoxin, which was similar quantity to the 2,602.25 ± 14.27 mg L^{-1} δ-endotoxin produced by SUFT03, but superior to the 2,469.74 ± 19.7, 2,337.12 ± 8.45 and 2,425.17 ± 26.70 mg L^{-1} δ-endotoxin produced by SUFT02, SUFT04 and HD1, respectively (Table 5). SUFT01 produced 87.12 (± 9.01) × 10^9 CFUs /L, which was fewer than the 120 (± 12.14) and 101 (± 1.28) × 10^9 CFUs /L produced by SUFT02 and SUFT03, respectively, while lower CFU numbers, 60.45 (± 4.25) and 76.56 (± 10.01) × 10^9 CFUs /L, were produced by SUFT04 and HD1, respectively (Table 5). A careful study of additional fermentation conditions revealed that the complexes of SUFT01 and SUFT02 spores and crystals showed high levels of insecticidal activities than when was performed the selective bioassays (100% mortality).

Table 5. δ-endotoxin production of selected *Bacillus thuringiensis* (Bt) isolates on a CCY medium at 72 hours.

Bt isolate	Toxins (mg/L)	CFUs (10^9 spores/L)	Activity against *Spodoptera frugiperda* larvae (120 ng/cm^2)
SUFT01	2.678,90 ± 31.6 a	87.12 ± 9.01 a	100 ± 0.0 b
SUFT02	2.469,74 ± 19.7 b	120 ± 12.14 a	100 ± 0.0 b
SUFT03	2.602,25 ± 14.27 a	101 ± 1.28 b	70 ± 10.05 a
SUFT04	2.337,12 ± 8.45 c	60.45 ± 4.25 b	55.12 ± 5.01 c
HD1	2.425,17 ± 26.70 c	76.56 ± 10.01 b	92.01 ± 12.0 b

Results are expressed as means ± standard error. Data represent the means of six replicates. Means in the same column followed by different letters are significantly different ($p < 0.05$). Analysis of variance by Tukey's test.

The high toxicity of SUFT01 may be associated with the *cry* gene copy number and could be a factor in δ-endotoxin overproduction. Other studies also demonstrated that the *Bt* isolates with greater *cry*1A gene copy numbers produced more δ-endotoxin than did the reference strain HD1 (SAADAOUI et al., 2009).

Additionally, Azzouz et al. (2014) showed that δ-endotoxin production by two Bt isolates that had high insecticidal activity against *Spodoptera littoralis* (BOISDUVAL, 1833) (Lepidoptera: Noctuidae) produced 43.71 and 80.81% more δ-endotoxin than HD1. The spore and δ-endotoxin production of Bt isolates obtained in this work indicates the potential for using formulations, at proper concentrations, to control *S. frugiperda* (GONZÁLEZ-CABRERA et al., 2011).

Analysis of the integrity of *S. frugiperda* midguts

Isolates from the SUFT01 spore-crystal complex were highly toxic to *S. frugiperda* midgut cells, causing disruptions to the cellular integrity and the microvilli of the midgut columnar cells (Figure 5A). After 24 h, an extensive disintegration in the columnar cells of the *S. frugiperda* midgut could be observed by transmission electron microscopy (Figure 5B). The disorganization of the cell cytoplasm caused by loss the microvilli in cells treated with Bt crystal and spore suspensions were visualized (Figure 5B). Endomembrane dilation, plasma membrane blebbing and cell fragmentation were observed, and they became more frequent as the post-inoculation time increased. Martins et al. (2008) described the effects of recombinant Cry1Ia protein action in the midgut of cotton boll weevil ,*Anthonomus grandis* (Boheman) (Coleoptera: Curculionidae) larvae, reporting morphologic changes in the midgut cells that were similar to those described in this work. Monnerat et al. (2007) obtained 27 isolates of Bt that killed 100% of *S. frugiperda*, *Anticarsia gemmatalis* (HÜBNER, 1818) and *Plutella xylostella* (LINNAEUS, 1758)

larvae after 5 days in selective bioassays, and 19 isolates that exhibited LC_{50} values that were lower than those of the standard strain Btk HD-1. From our results, four isolates showed promise in controlling *S. frugiperda* larvae with LC_{50} values of 44.5, 74.0, 89.0 and 108 ng / cm^2. However, the isolates SUFT01, SUFT02 and SUFT03 showed LC_{50} values that were less than the 285 ng / cm^2 value for the Btk HD-1 strain reported in the work of (MONNERAT et al., 2007).

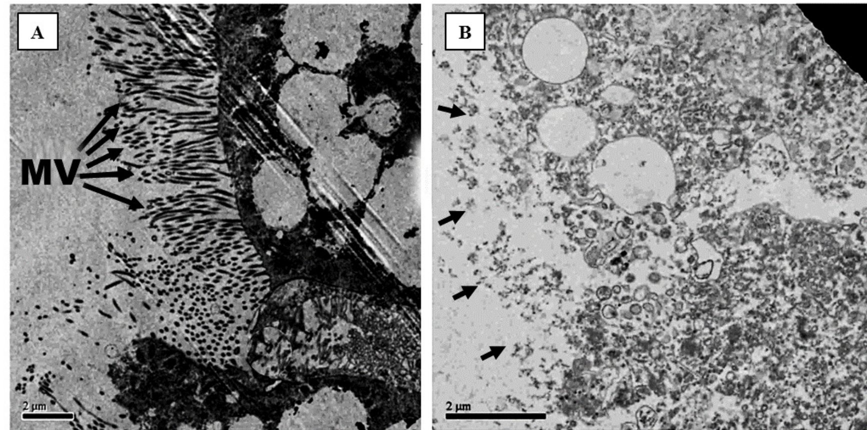

Figure 5. Toxicity effect of SUFT01 isolate suspensions in insect diets on *Spodoptera frugiperda* larvae midgut columnar cells. **A.** Midgut columnar cell of a *S. frugiperda* larva without the Bt treatment. Arrows indicate intact microvillus (**MV**). **B.** Midgut columnar cell of a larva fed an artificial diet containing the Bt suspension (24 h after inoculation). Arrows indicate the disorganization of the cell cytoplasm caused by loss of the microvillus in cell.

The search for alternative methods to control insect pests is necessary, mainly to reduce the use of conventional chemical control methods. Thus, new Bt isolates represent a source of new toxin genes with the potential to be introduced into the genome of plants of economic interest. Currently, numerous studies have been reported in several transgenic plants containing the *cry* genes of *Bt*, such as potato (VALDERRAMA et al., 2007; KUMAR et al., 2010), tomato (LI et al., 2007), cotton (WU et al., 2005), rice (YE et al., 2003; YOUNG-JUN et al., 2004; GAO et al., 2010), corn (BELTAGI, 2008) and soybean (WALKER et al., 2000). Bt containing transgenic crop plants covered over 179,7 million hectares (corn, soybeans, cotton and potatoes) in worldwide and the highest increase was in Brazil, with 2 million hectares (James, 2015), this signifies the importance of this technology in controlling insect pests (ZHAO et al., 2005).

CONCLUSION

The high level of insecticidal activity of the isolates described in this work makes them excellent candidates for the control of *S. frugiperda*, and could provide alternatives in controlling insect pest populations that have developed resistance to chemical insecticides. Moreover, screening for new Bt isolates and their *cry* genes is important for the construction of cry gene databases for a possible future use in economically important transgenic crops.

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RESUMO: *Spodoptera frugiperda* (SMITH, 1797) (Lepidoptera: Noctuidae) afeta diversas culturas de grande interesse econômico, por exemplo, pode causar severas perdas em milho, arroz e sorgo. Neste estudo, foi realizada uma seleção e caracterização de isolados de *Bacillus thuringiensis* (BERLINER, 1911) com elevada atividade inseticida contra *S. frugiperda.* Cinquenta e dois isolados formadores de cristal *B. thuringiensis* que foram identificados a partir de 3384 colônias foram examinados e testados por PCR para a presença dos genes cry (cry1, cry1Aa, cry1Ab, cry1Ac, cry1D, cry2 e cry2Ab). Quatro isolados que apresentaram alta toxicidade contra *S. frugiperda* foram mostrados para abrigar os genes cry2. Os cristais foram analisados por microscopia eletrônica e mostraram formas bipiramidais e cúbicas. Os valores da

concentração letal (CL_{50}) destes quatro isolados foram de 44,5 ng / cm^2 (SUFT01), 74,0 ng / cm^2 (SUFT02), 89,0 ng / cm^2 (SUFT03) e 108 ng / cm^2 (suft 04) para larvas recém-eclodidas de *S. frugiperda*. Uma análise ultra-estrutural das células do intestino médio de *S. frugiperda* incubadas com complexo esporo-cristal do isolado SUFT01 mostrou rupturas na integridade celular e microvilosidades das células cilíndricas do intestino médio. Neste estudo, o alto nível de atividade inseticida de isolados os torna excelentes candidatos para o controlo de *S. frugiperda*, e pode proporcionar alternativas no controle destas populações de pragas, bem como a formação de novos bioinsecticidas.

PALAVRAS-CHAVE: Cry proteína. Lepidópteros. Entomopatógeno. Inseticida microbiano. Controle de insetos.

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