Two new Brazilian isolates of *Bacillus thuringiensis* toxic to *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)

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Abstract

Bacillus thuringiensis is a bacterium used for biopesticides production and pest-resistant plants due to the synthesis of protein crystals by *cry* genes, which are effective in controlling several insect orders such as Lepidoptera. This work aimed at the evaluation and characterisation of two new *B. thuringiensis* isolates active against *A. gemmatalis* (Hübner 1818) larvae, which is the soybean major pest. The results showed that *Bt117-4* isolate amplified fragments corresponding to *cry2* and *cry9* genes, and synthesised protein fragments equivalent to 130, 90 and 45 kDa. The *Bt3146-4* isolate amplified DNA fragments corresponding to *cry9* gene and synthesised protein fragments of 70, 58 and 38 kDa. Transmission electron microscopy revealed the presence of protein crystals in both isolates. CL_{50} with Cry purified proteins from *Bt117-4* and *Bt3146-4*, corresponded to 0.195 and 0.191 µg larvae^{-1}, respectively. The two *B. thuringiensis* isolates selected in this study were effective to control velvetbean caterpillar at laboratory conditions. Field tests should be carried on to develop new biopesticides formulation as well for *cry* genes resource for *Anticarsia gemmatalis* resistant transgenic plants.

Keywords: Lepidoptera, velvetbean caterpillar, *cry* genes, *Bacillus thuringiensis*.

Dois novos isolados brasileiros de *Bacillus thuringiensis* tóxicos para *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)

Resumo

Bacillus thuringiensis é uma bactéria utilizada na produção de biopesticidas e de plantas resistentes às pragas por causa da síntese de cristais proteicos pelos genes *cry*, os quais são eficazes no controle de diversas ordens de insetos, como os lepidópteros. O presente trabalho objetivou a avaliação e a caracterização de dois novos isolados de *B. thuringiensis* ativos contra lagartas de *A. gemmatalis* (Hübner 1818), que é a principal praga da cultura da soja. Os resultados obtidos revelaram que o isolado *Bt117-4* amplificou fragmentos correspondentes aos genes *cry*2 e *cry*9, sendo que os fragmentos proteicos sintetizados foram equivalentes a 130, 90 e 45 kDa. O isolado *Bt3146-4* amplificou fragmentos de DNA que correspondem ao gene *cry*9 e sintetizou fragmentos proteicos de 70, 58, e 38 kDa. Os dados de microscopia eletrônica de transmissão revelam a presença de cristais proteicos em ambos os isolados. A CL_{50}, com proteínas Cry purificadas de *Bt117-4* e *Bt3146-4*, correspondeu a 0,195 e 0,191 µg lagarta^{-1}, respectivamente. Os dois isolados de *B. thuringiensis* selecionados neste trabalho mostraram-se eficientes no controle da lagarta-da-soja em laboratório, sendo recomendada sua avaliação a campo para posterior aplicação na formulação de biopesticidas ou como fonte de genes *cry* para a obtenção de plantas geneticamente modificadas resistentes à Anticarsia *gemmatalis*.

Palavras-chave: Lepidoptera, lagarta-da-soja, genes *cry*, *Bacillus thuringiensis*.

1. Introduction

Biopesticides based on *Bacillus thuringiensis* have become a reliable tool in agricultural pest control, forestry and health-related conditions (Lopes et al., 2010). This gram-positive bacterium has been isolated worldwide and characterised by different techniques, including PCR screening to detect the *cry* genes. The efficiency of the insecticidal properties of *B. thuringiensis* is due to the synthesis of insecticidal crystal proteins (Cry proteins) encoded by the *cry* genes. Currently, more than 560 *cry* genes have been identified and classified into 68 classes based on the homology of their proteins (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt). Cry proteins have already been described as toxic to several insect from Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, and Malophaga orders (Zhong et al., 2000; De Maagd et al., 2001; Castilhos-Fortes et al. 2002; Pinto et al. 2003; Martins et al., 2004; Nazarian et al., 2009; López-Pazos et al., 2009) and also to nematodes (Marroquin et al., 2000; Jouzani et al., 2008).

Cry proteins are produced in inactive form and, after ingestion by the susceptible insect, the crystal protein is solubilised by the intestinal alkaline pH. After solubilisation, the protoxins are activated by intestinal proteases, yielding the activated form of Cry toxins. Then, the toxins bind to receptors of high affinity sites on the surface of epithelial cells of the midgut microvilli, which are turgid and burst due to formation of pores in the apical membranes (Grochulski et al., 1995; Bravo et al., 2004; Fiuza, 2004; Knaak et al., 2010).

In Brazil, *Anticarsia gemmatalis* Hübner 1818 (Lepidoptera, Noctuidae) is considered the key pest of soybean (Panizzi and Corrêa-Ferreira, 1997; Levy et al., 2004; Monnerat et al., 2007). *A. gemmatalis*, also known as velvetbean caterpillar, attack the aerial parts of plants (Gallo et al., 2002), hampering their development and thus causing losses in grain production (Morales et al., 1995). The control of this pest can occur naturally under favourable conditions, through presence of natural enemies. When this does not happen, the use of insecticides with high applications per crop, is designed to avoid loss in yield (Moscardi, 1993). Currently, many adverse factors interfere in the velvetbean caterpillar control, such as the environmental impact that pesticides cause to other unrelated species and even to its natural enemies. In this research, the delta-endotoxins of two new isolates of *B. thuringiensis* that synthesize insecticidal proteins active against the target insect were tested, as the variability of *cry* genes and morphological crystals structure from these isolates can be applied in soybean pest management programs, aiming for the *A. gemmatalis* control.

2. Material and Methods

2.1. B. thuringiensis isolates

The *B. thuringiensis* isolates tested in this work belong to the Bank of Entomopathogenic Bacteria from the Laboratory of Microbiology and Toxicology (UNISINOS). They were isolated from soil samples collected in free biopesticides agricultural areas of southern Brazil. The isolates *Bt117-4* and *Bt3146-4*, which showed a mortality of 78% and 100%, respectively, were previously evaluated against *third-instar larvae* of *A. gemmatalis* using bacterial suspensions with 1.10^9 cells mL^{-1}. The mortality was evaluated within three different generations, using 20 larvae per treatment, until the 7th day after treatment application, and then corrected by Abbott's formula (1925). The strain *B. thuringiensis kurstaki* HD1 (Institut Pasteur, Paris) was used as positive control.

2.2. Electron microscopy

The bacterial and paraesporal inclusion body ultrastructures were evaluated at the Electron Microscopy Centre (UFRGS, Brazil). The selected isolates in bioassays were grown in solid Usual Medium (UM) (De Barjac and Lecadet, 1976) supplemented with glucose, and bacterial aliquots were fixed in Karnowsy's pH 7.4 (0.1 M phosphate buffer, 8% paraformaldehyde and 25% glutaraldehyde) and post-fixed in osmium tetroxide (2 %). The samples were dehydrated in increasing acetone solutions (30-100%) and subsequently impregnated with resin (Arauditidurcopan ACM). The blocks were cut with ultramicrotome (Leica) and compared with 7% uranyl acetate for subsequent observation in transmission electron microscopy (Zeiss EM900).

2.3. Cry genes

B. thuringiensis isolates were cultured for 12 hours at 30 °C on Nutrient Agar (3 g L^{-1} peptone, 5 g L^{-1} meat extract, 0.5 g L^{-1} albumin extract, 0.006 g L^{-1} MnCl2, 0.08 g L^{-1} $CaCl_2$, 0.07 g L^{-1} $MgCl_2$ and 1.5 g L^{-1} agar), then were subjected to total DNA extraction, according to the method described by Hansen and Hendriksen (2001). The primers for amplification of DNA fragments corresponding to *cry1* were: Un1(d), 5'-CATGATTCATGCGGCCAGATAAAC-3', Uni1(r), 5'-TTGTGACACTTCTGCTTCCCATT-3' (Ben-Dov et al., 1997); *cry*2: Un2(d), 5'-GTTATTCTTAATGCAGATGAATGGG-3' Un2(r), 5'-CGGATAAAATAATCTGGGAAATAGT-3' (Ben-Dov et al., 1997), *cry*9: Un9(d), 5'-CGGTGTTACTATTAGCGAGGGCGG-3', Un9(r), 5'-GTTTGAGCCGCTTCACAGCAATCC-3' (Ben-Dov et al., 1999); to specific *cry* genes: Cry2Aa: Un2(d) 5'-GTTATTCTTAATGCAGATGAATGGG-3', EE-2Aa(r) 5'-GAGATTAGTCGCCCCTATGAG-3'; Cry2Ab: EE-2Ab(r) 5'-TGGCGTTAACAATGGGGGGAGAAAT-3'; Cry2Ac: EE-2Ac(r) 5'-GCGTTGCTAATAGTCCCAACAACA-3' (Ben-Dov et al., 1997) e Cry9A: EB-9A(d) 5'-GGTTCACTTACATTGCCGMGTTAGC-3', Un9(r) 5'-GTTTGAGCCGCTTCACAGCAATCC-3'; Cry9B: EB-9B(d) 5'-GCAAATGCATTTAGCGCTGGTCAA-3'; Cry9C: EB-9C(d) 5'-CCACCAGATGAAAGTACCGGAAG-3'; Cry9D: EB-9D(d) 5'-GCAATAAGGGTGTCGGTCACTGG-3' (Ben-Dov et al., 1999).

Each Polymerase Chain Reaction (PCR) was performed with a final volume of 25 µL, using: 1 µL of DNA sample with reaction buffer (10 mM TrisCl, 1mM EDTA); 0.2 mM dNTP; 0.2 a 0.5 µM of each *primer* and 0.5 U of Taq DNA polimerase (GIBCO-BRL). The amplification was performed in thermal cycler set at 35 cycles for each reaction, with 1 minute denaturation at 95 °C, annealing to primers for 1 minute at 52 °C and extension for 1 minute at 72 °C. A 5 minutes final extension at 72 °C was added. The strains *B. thuringiensis kurstaki* HD1 and *B. thuringiensis aizawai* type 1 were used as positive control and reactions without DNA addition as negative controls. PCR products amplified were analysed in agarose gel (1.5%).

2.4. Purification of ICPs

To purify the Insecticidal Crystal Proteins (ICPs) from *B. thuringiensis*, the selected isolates were grown in UM for 48 hours at 28 °C and 180 rpm. The suspensions with spores, crystals and cells were homogenised in phosphate buffer, pH 6.0 (0.1 M NaH_2PO_4.H2O; 100 mM phenylmethanesulfonyl fluoride) and centrifuged at 28.000 g at 4 °C. The suspensions were washed twice with autoclaved distilled water by centrifugation at 7.000 g for 15 minutes, and then the crystal purification was performed in sucrose gradient from 67 to 78%. The pellet containing the purified crystals was solubilised according to Fiuza (1995) and 15 µL of the Cry proteins were analysed by SDS-PAGE (Laemmli, 1970) and stained with Coomassie blue. The protein concentration was determined according to the Bradford method (1976). The molecular weight was estimated by comparison with the marker Hig Range (Gibco-BRL).

2.5. Insects

A. gemmatalis larvae were collected from soybean fields in southern Brazil. After the quarantine period, healthy adults were introduced into the insect rearing. In addition to the specimens obtained in the field, two other lots were supplied by the Entomology Laboratory of the National Center for Soybean Research (Embrapa-Soja, Londrina-PR). *A. gemmatalis* was maintained in the laboratory at 25 ± 2 °C, 12 hours photoperiod, 70% relative humidity (RH), and the larvae were reared on an artificial diet described by Greene et al. (1976).

2.6. Bioassays

Cry proteins from *B. thuringiensis* isolates were evaluated in second-instar larvae of *A. gemmatalis*. The range of tested protein concentrations was established in a pilot test. After, 10 µL of six different concentrations (3.0, 1.5, 0.75, 0.37, 0.18 and 0.09 µg mL^{-1}) were applied on soybean leaf discs (0.8 cm diameter) and placed individually in acrylic mini-plates. Each treatment consisted of 30 insects and three replicates. As the insect was consuming the entire soybean disc, it was replaced by another disc without the addition of *B. thuringiensis* protein.

In control, the suspensions were replaced by sodium phosphate buffer 100 mM, pH 6.0. A total of 1260 insects were used, where the replicates consisted of different insect generations. The experiments were kept in the same rearing conditions and evaluations were performed daily until the 5th day after treatment. The mean lethal concentration (LC_{50}) was estimated by Probit analysis using Polo-PC program LeOra Software, 1987 (Haddad, 1998).

3. Results

3.1. Characterisation of B. thuringiensis isolates

The transmission electron microscopy analysis showed the presence of protein crystals in both isolates, where the *Bt117-4* isolate crystal (Figure 1a) had a cuboidal aspect, while the *Bt3146-4* (Figure 1b) showed a spherical shape.

3.2. Crystal protein analysis

The protein profile analysis from *Bt117-4* and *Bt3146-4* isolates, held on SDS-PAGE (10%), revealed that *Bt117-4* isolate synthesizes protein fragments of ~130, 90 and 45 kDa and *Bt3146-4* synthesises peptides of ~70, 58, and 38 kDa (Figure 2).

3.3. Cry genes

PCR amplification (Figure 3) did not show fragments with molecular weights expected for *cry1* genes (Ben-Dov, et al., 1997) in both isolates. However, isolates evaluation with primers corresponding to cry9 genes (Ben-Dov et al., 1999) revealed 400 bp fragments, which correspond to this class of genes. In *Bt117-4* isolate, a 725 pb fragment was amplified, corresponding to molecular weight of *cry2*

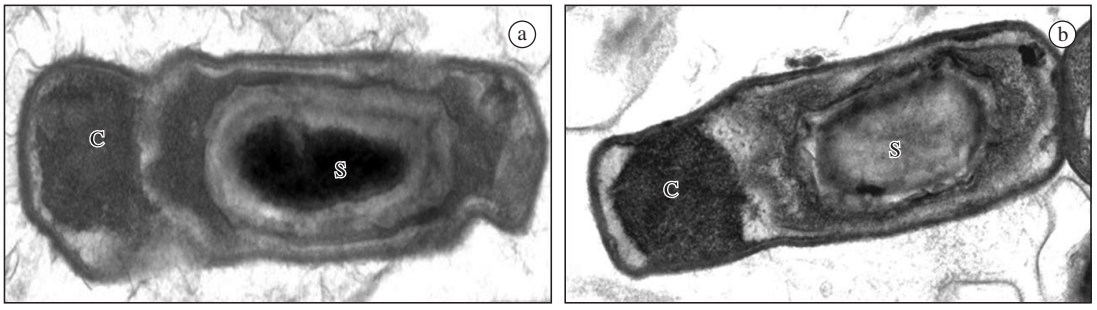

Figure 1. Transmission electron microscopy of *Bacillus thuringiensis* isolates: (a) *Bt117-4*, (b) *Bt3146-4*, (s) spore and (c) crystal. Magnification 40.000×.

genes (Ben-Dov, et al., 1997), which was not amplified in the *Bt3146-4* isolate. The primers analysis specific for *cry2* and *cry9* genes is showed in Table 1.

3.4. CL_{50} of Cry proteins

The *Bt117-4*, *Bt3146-4* and Bt *kurstaki* HD1 strains showed a LC_{50} of 0.195, 0.191 and 0.082 µg/larvae, respectively, on the fifth day after the treatment (DAT) (Table 2).

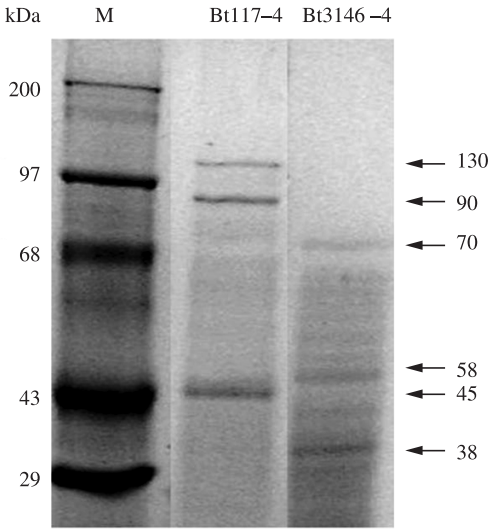

Figure 2. SDS-PAGE (10%) analysis of solubilised toxins from *Bacillus thuringiensis* isolates. *M* indicates de molecular mass marker. Sizes of protein bands (kDa) are indicated by arrows.

4. Discussion

Some research (Bobrowski et al., 2001; Silva et al., 2004; Monnerat et al., 2007; Silva-Werneck and Ellar, 2008; Gobatto et al., 2010) has shown the possibility of finding *B. thuringiensis* native isolates with toxicity to *A. gemmatalis*. The bioassays screening results with new *B. thuringiensis* isolates are useful for local and regional insect pest management. For example, in studies with *B. thuringiensis kurstaki* strains, Mascarenhas et al. (1998) and Dias et al. (1999) found different mortality rates for the same species.

Regarding the crystal shape analogy and its specific toxicity to insect orders, Taylor et al. (1992), Bernhard et al. (1997), and Habib and Andrade (1998) observed that the crystal shape can provide information on the insecticidal activity of a strain. In this study, the toxic isolate *Bt117-4* showed cuboidal crystals, which are known to contain proteins toxic to Lepidoptera (Dankocsik et al., 1990; Wu et al. 1991; Monnerat et al., 2007; Silva-Werneck and Ellar, 2008).

The SDS-PAGE protein profiles analysis of *Bt117-4* and *Bt3146-4* isolates showed that the solubilised samples are composed of different protein fragments (Figure 2) and this may be related to the different gene profiles obtained from the two analysed samples. Cry9 proteins have around 130 kDa, and Cry2 proteins are known to have a molecular weight below 90 kDa (Crickmore et al., 1998). These protein profiles were observed in the *Bt117-4* isolate and it can be related to the *cry9* and *cry2* genes, detected by PCR. Regarding the *Bt3146-4* isolate, its proteins may be codified by new cry genes that were not identified with the

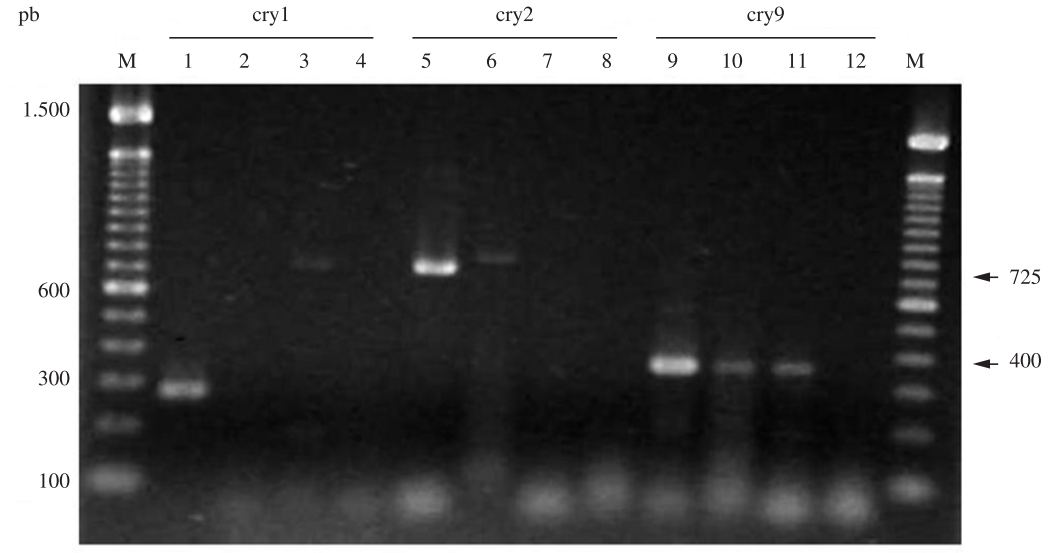

Figure 3. Agarose gel (1.5%) electrophoresis of PCR products amplified from *B. thuringiensis* isolates, with primers to *cry1*, *cry2* and *cry9* genes. Lanes: (1, 5) *B. thuringiensis kurstaki* HD1; (9) *B. thuringiensis aizawai* type 1; (2, 6, 10) *Bt117-4*; (3, 7, 11) *Bt3146-4*; (4, 8, 12) negative control. M, molecular weight marker (100 bp, Gibco BRL). Arrows indicates expected product length.

Table 1. Profile of *cry* genes in *Bacillus thuringiensis* isolates, determined by PCR.

Genes	*B. thuringiensis* isolates			
	***Bt* 117-4**	***Bt* 3146-4**	***Bt kurstaki* HD1**	***Bt aizawai* type 1**
cry2Aa	+	−	+	+
cry2Ab	+	−	+	−
cry2Ac	+	−	+	−
cry9A	+	+	−	+
cry9B	−	−	−	+

Table 2. Mean Lethal Concentration (LC_{50}) of *Bacillus thuringiensis* Cry proteins for *Anticarsia gemmatalis* second-instar larvae.

Isolates	LC_{50} (μg larvae^{-1})		
	5 DAT*	**Confidence limits (95%)**	**Slope ± SEM**
Bt 117-4	0.195	(0.024-0.373)	0.575 ± 0.205
Bt 3146-4	0.191	(0.071-0.405)	0.750 ± 0.211
Bt kurstaki HD-1	0.082	(0.056-0.105)	0.512 ± 0.672

*DAT = days after treatment.

used set of primers of this study. The protoxins from the *Bt3146-4* isolate did not present the corresponding bands of Cry9 proteins in SDS-PAGE, which could indicate that *cry9* genes detected by PCR are silent in this isolate.

Similar data were described by Pinto and Fiuza (2003) in tests performed with *B. thuringiensis* strains active against Lepidoptera, Coleoptera and Hymenoptera.

PCR analyses showed that *Bt117-4* isolate harbours more than one class of *cry* genes, which DNA amplified fragments correspond to *cry2* and *cry9* genes. Estruch et al. (1997) and Liao et al. (2002) described that the action spectrum of different *B. thuringiensis* strains depends on the Cry proteins combination present in the crystal, which are encoded by different *cry* genes.

Bravo et al. (1998), by analysing *cry* genes diversity, found several combinations of these genes, however only 2.6% of the evaluated Mexican strains had *cry9* genes.

On the other hand, Pinto et al. (2003) showed that the *B. thuringiensis* strains isolated from southern Brazil presented a high frequency of *cry9* genes (47%). The data described by these authors and the results presented here were congruent, once the *B. thuringiensis* strains were obtained from the same region. The isolates evaluated in this study revealed the simultaneous presence of *cry2* and *cry9* genes, differing from Wang et al. (2003) who did not find both *cry2* and *cry9* genes in the same isolate. In another study, Ben-Dov et al. (1999) described strains from Israel, Kazakhstan, and Uzbekistan that had simultaneously *cry9* genes with *cry1* or *cry2* genes.

The most toxic proteins evaluated for Lepidoptera are encoded by *cry1*, *cry2*, and *cry9* (Bravo et al., 1998; Crickmore et al., 1998). In order to control *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae), Pang et al. (2002) showed that a genetically modified *B. thuringiensis* strain, with the *cry9Ca* gene insertion, was four times more efficient than the *B. thuringiensis kurstaki* HD-1 strain, traditionally used in their control.

Several studies aiming to evaluate new *B. thuringiensis* strains against lepidopterans have been performed worldwide (Zhong et al. 2000; Uribe, et al. 2003; Polanczyk et al., 2003; Knaak et al. 2010). Despite the mode of action of this pathogen showing similarity to all Lepidoptera, the discovery of new *B. thuringiensis* strains becomes important to study specific toxicity to target insects. As a consequence, these new strains might promote the production of specific biopesticide formulated with different *B. thuringiensis* strains, which may contain new genes that encode new insecticidal proteins. These genes can also be used for the development of new insect resistant plants (De Guglielmo-Cróquer et al., 2010).

The current results show that *Bt117-4* and *Bt3146-4* isolates are highly pathogenic, and can be used in soybean genetic transformation to obtain *A. gemmatalis* resistant plants, or in commercial formulations of potentially active biopesticides to the target species studied.

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