Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9275
ISSN on-line: 1807-8621
Doi: 10.4025/actasciagron.v39i4.32707

Isolation and characterization of *Bacillus thuringiensis* strains active against *Elasmopalpus lignosellus* (Zeller, 1848) (Lepidoptera, Pyralidae)

Janaina Zorzetti[1*], Ana Paula Scaramal Ricietto[1], Fernanda Aparecida Pires Fazion[1], Ana Maria Meneguim[2], Pedro Manuel Oliveira Janeiro Neves[1] and Gislayne Trindade Vilas-Bôas[1]

*[1]Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, Km 445, 86047-902, Londrina, Paraná, Brazil. [2]Instituto Agronômico do Paraná, Londrina, Paraná, Brazil. *Author for correspondence. E-mail: zorzettijanaina@gmail.com*

ABSTRACT. *Elasmopalpus lignosellus* (Zeller, 1848) (Lepidoptera, Pyralidae) is an insect pest of 60 economically important crops, including sugarcane, wheat, soybean, rice, beans, sorghum, peanuts, and cotton. The aim of this work was to select and characterize *Bacillus thuringiensis* isolates with insecticidal activity against *E. Lignosellus* that could be used as an alternative method of control. Selective bioassays were done to evaluate the toxicity of 47 isolates against first instar larvae of *E. lignosellus.* For the most toxic bacterial strains, the lethal concentration (LC_{50}) was estimated and morphological, biochemical and molecular methods were used to characterize the isolates. Among the 47 isolates tested, 12 caused mortality above 85% and showed LC_{50} values from 0.038E+8 to 0.855E+8 spores mL^{-1}. Isolates BR83, BR145, BR09, BR78, S1534, and S1302 had the lowest LC_{50} values and did not differ from the standard HD-1 strain; the exception was BR83.The protein profiles produced bands with molecular masses of 60-130 kDa. The genes *cry1, cry2, cry3,* and *cry11* were identified in the molecular characterization. The morphological analysis identified three different crystal inclusions: bipyramidal, spherical and cuboidal. Among the tested isolates, 12 isolates have potential for biotechnological control of *E. Lignosellus* by development of new biopesticides or genetically modified plants.

Keywords: biological control, *cry* genes, entomopathogenic bacteria, lesser cornstalk borer.

Seleção e caracterização de linhagens de *Bacillus thuringiensis* ativas contra *Elasmopalpus lignosellus* (Zeller, 1848) (Lepidoptera, Pyralidae)

RESUMO. *Elasmopalpus lignosellus* (Zeller, 1848) (Lepidoptera, Pyralidae) é considerada praga para mais de 60 espécies de plantas cultivadas como o milho, cana-de-açúcar, trigo, soja, arroz, feijão, sorgo, amendoim e algodão. O objetivo deste trabalho foi selecionar e caracterizar isolados de *Bacillus thuringiensis* virulentos a *E. lignosellus* atuando como método alternativo de controle. Assim, 47 isolados foram avaliados em bioensaios seletivos contra lagartas de 1º ínstar de *E. lignosellus*. Para os isolados mais tóxicos, a concentração letal (CL_{50}) foi estimada e a sua caracterização foi realizada por métodos morfológicos, bioquímicos e moleculares. Dos 47 isolados testados, 12 causaram mortalidade acima 85% e apresentaram CL_{50} entre 0.038E+8 a 0.855E+8 esporos mL^{-1}. Os menores valores de CL_{50} foram obtidos pelos isolados BR83, BR145, BR09, BR78, S1534 e S1302, os quais não diferiram da linhagem padrão HD-1, com exceção do BR83. O perfil proteico revelou proteínas Cry entre 60 e 130 kDa, e a caracterização molecular mostrou a presença dos genes *cry1, cry2, cry3* e *cry11.* A análise morfológica identificou três diferentes inclusões cristalinas: bipiramidais, esféricas e cuboides. Entre os isolados avaliados, 12 apresentam potencial biotecnológico para controle de *E. lignosellus* via formulação de novos bioinseticida ou produção de plantas transgênicas.

Palavras-chave: controle biológico, genes *cry,* bactéria entomopatogênica, broca-do-colo.

Introduction

The lesser cornstalk borer *Elasmopalpus lignosellus* (Zeller, 1848) (Lepidoptera, Pyralidae) is a polyphagous pest, and larvae feed on more than 60 species of cultivated plants (Viana, 2004). These plants include crops of high economic value, such as corn, beans, wheat, soy, peanuts, and sugarcane, which suffer extensive losses by attack from this pest. The larvae damage newly germinated plants and reduces the number of seedlings per planting area. Larvae penetrate the stalk of a recently sprouted plant, make galleries toward the central core and then feed inside the stem causing new leaves to dry up and die, resulting in the so-called "dead heart" (Gallo et al., 2002).

The lesser cornstalk borer is a difficult pest to control because it can remain close to the plant stem, within the stem, or in silken web habitats and land shelters they build on the soil surface. In experiments conducted with different control methods, pest management using pheromone and light traps and

soil cover with *Crotalaria jucea* resulted in a small reduction in the pest population (Jham, Silva, Lima, & Viana, 2007; Gill, McSorley, Goyal, & Webb, 2010). Thus, preventive chemical control and seed treatment remain the most widely used methods to control *E. lignosellus*. However, when chemicals are indiscriminately applied, human contamination and environmental imbalance can result, leading to an increase in the pest population.

Entomopathogenic bacteria, such as *Bacillus thuringiensis*, are among the alternatives to reduce the use of insecticides for pest control. The insecticidal characteristics of these bacteria are caused by the formation of parasporal crystals in the early sporulation phase. These crystals are composed of Cry proteins, which are toxic to a variety of insects that attack crops of high economic value (Vilas-Bôas, Peruca, & Arantes, 2007; Vidal-Quist, Castañera, & González-Cabrera, 2009).

The toxic activity of these proteins against insect pests led to the formulation of bioinsecticides and the selection of genes encoding insecticidal proteins to produce transgenic plants resistant to different species of insects. Several *Bacillus thuringiensis* isolates specific to insects of the orders Lepidoptera, Coleoptera, and Diptera have been investigated (Pardo-López, Soberón, & Bravo, 2013). These isolates typically harbour one or more *cry* genes, and the isolates containing a wider range of genes are the most targeted. Thus, further studies are required to select these isolates, identify the *cry* genes, and assess isolate toxicity (Sun, Fu, Ding, & Xia, 2008).

Although the search for isolates of *B. thuringiensis* that are effective against *E. lignosellus* is of great significance for the management of this insect pest, studies remain limited. Therefore, the aim of this work was to select and characterize native isolates of *B. thuringiensis* toxic to *E. lignosellus*, with the goal to conduct further studies focused on new formulations of bioinsecticides and development of genetically modified plants.

Material and methods

Insect rearing

Larvae of *E. lignosellus* were reared on an artificial diet according to the methodology described by Greene, Leppla, and Dickerson (1976). The adults were maintained at 27 ± 2ºC and 60 ± 10% RH with a 14h photoperiod in plastic cages (10 cm diameter, 20 cm height) coated with filter paper and closed on the upper end with tissue and on the lower end with a petri dish (14.3 cm diameter) and fed a 10% aqueous honey solution. The eggs obtained were transferred to petri dishes at 25°C for incubation; the first instar larvae were used in bioassays.

Bacterial isolates

Forty-seven native isolates of *B. thuringiensis* were examined from the Collection of Entomopathogenic Microorganisms of Londrina State University (Universidade Estadual de Londrina, UEL) and the Brazilian Agricultural Research Corporation, Embrapa Genetic Resources and Biotechnology (Empresa Brasileira de Pesquisa Agropecuária – Embrapa Recursos Genéticos e Biotecnologia). The HD-1 strain of *B. thuringiensis* subsp. *kurstaki* was obtained from the Collection of *B. thuringiensis* at the Institut Pasteur, Paris, France.

Selective bioassay to choose the most toxic isolates

Suspensions of each *B. thuringiensis* isolate were prepared by adding 1.0 mg of lyophilized material to 1.0 mL of sterile distilled water. The artificial diet was prepared according to Greene et al. (1976) and distributed (3 mL) when still liquid into glass tubes (2 cm diameter x 3 cm height). After the diet solidified, 50 μL of a mixture of spores and crystals were applied on the diet surface. The glass tubes were kept in a laminar flow hood until the complete absorption of the suspension by the diet. Subsequently, five first-instar larvae were released inside each glass tube, which were sealed with a plastic lid. The bioassay consisted of three replicates with four glass tubes for each *B. thuringiensis* isolate. The standard strain *B. thuringiensis* subsp. *kurstaki* HD-1 (Btk) and water were used as positive and negative controls, respectively. The insects were maintained in an incubator (27 ± 2ºC, 60 ± 10% RH and a 14h photoperiod) for six days after which mortality was assessed. The corrected mortality was calculated using Abbott's control adjusted mortality (Abbott, 1925). The data were subjected to analysis of variance (ANOVA), and the means were compared using Tukey's test at 5% probability. The most toxic isolates, those that caused a reliable mortality (above 85%) and therefore had potential for further testing, were used in dose-response bioassays and evaluated according to their molecular, protein and morphological profiles. The mortality rate was selected based on the minimum efficacy threshold (80% efficacy) required for pesticide registration in Brazil (MAPA, 1995).

Estimation of the lethal concentration (LC_{50}) of *B. thuringiensis* isolates

Bioassays for dose estimation were performed with the 12 isolates that showed the greatest toxicity

in the selective bioassays and with the HD-1 standard strain. Seven suspensions of spores and crystals of *B. thuringiensis* were prepared to estimate the concentration of each isolate that would cause 50% mortality in *E. lignosellus* larvae (LC_{50}). The suspensions were prepared with 5.0 mg of lyophilized material that was diluted in 5 mL of sterile distilled water. Dilutions were performed using the initial suspension to obtain the seven concentrations used in the study (1.0, 0.2, 0.1, 0.05, 0.025, 0.008, and 0.0025 mg mL^{-1} in sterile distilled water), and the number of spores per mL of water in each dilution was counted using a Neubauer chamber. The bioassay was conducted in the same way as previously described. For each concentration evaluated, three replicates with four tubes were used, for a total of 20 larvae per replicate and 60 per concentration. The mortality data were subjected to Probit analysis (Finney, 1971) to determine the lethal concentration. The LC_{50} bioassay results were analysed by checking for the overlap of the 95% confidence intervals according to Probit analysis.

Protein and molecular characterization of *B. thuringiensis* isolates pathogenic to *E. lignosellus*

Genomic DNA samples of the *B. thuringiensis* strains were isolated according to the method described by Ricieto, Fazion, Carvalho Filho, Vilas-Boas, and Vilas-Bôas (2013). The isolates were cultivated for 15h at 30°C on plates containing Luria-Bertani (LB) medium (Bertani, 1951). With the aid of a sterile toothpick, a colony of approximately 2 mm in diameter was transferred to microtubes containing 200 μL of TE (10 mMTris; 1 mM EDTA; pH 8.0). The suspension was homogenized and incubated in boiling water for 10 min. Then, the suspension was centrifuged at 12,000 x*g* for 3 min, and the supernatant was transferred to a new tube and used as DNA template in the PCR reactions. The presence of the genes *cry1*, *cry2*, *cry3*, *cry4A, cry4B, cry10,* and *cry11* was analysed using specific primers and amplification conditions (Céron, Ortí, Quintero, Güereca, & Bravo, 1995, Bravo et al., 1998; Ibarra et al., 2003; Vidal-Quist et al., 2009). DNA amplification was performed using an Endurance TC-412 thermocycler. For each amplification reaction, a total reaction volume of 20 μL was prepared that contained 1 U *Taq* DNA polymerase (Invitrogen, Brazil), 2.0 μL of Buffer 10 (200 mM Tris-HCl, pH 8.0, 500 mM KCl), 1.5 mM $MgCl_2$, 0.25 mM dNTP, 0.5 μM each primer, 2 μL of DNA and sterile Milli-Q water. The same reaction was used for all the primers. The PCR products were visualized by electrophoresis on 1.2% agarose gel in TBE buffer (89 mM Tris Borate, 2 mM EDTA, pH 8.0) stained with Syber Safe (Invitrogen, UK) using a 100 bp DNA ladder (Invitrogen, UK). After electrophoresis, the gel images were captured using a Sony Cyber-shot 8.1 digital camera.

The *B. thuringiensis* isolates were characterized by the protein profile of their crystals using protein electrophoresis on 10% polyacrylamide gel (SDS-PAGE). Initially, the crystals were obtained according to the protocol described by Lecadet, Chaufaux, Ribier, and Lereclus. (1992). Each isolate was cultivated in NB medium (Downes & Ito, 2001) at 30°C for 72h at 200 rpm, until complete sporulation. The *B. thuringiensis* subsp. *kurstaki* HD-1 standard strain was used as the reference.

Morphological characterization of *B. thuringiensis* isolates

The morphological characterization of the isolates was initially performed by optical microscopy using a microscope (Model CHS; Olympus Optical Co. Ltd., Tokyo, Japan) with a 100x phase contrast lens. For electron microscopy, the lyophilized material of each isolate used in the previous bioassays was directly deposited over metal supports and coated with gold for 180 s, using a 40 mA current under vacuum (10-1 mbar) in a BAL-TEC model SCD–050 Sputter Coater (Santos et al., 2009). Subsequently, the material was analysed using a scanning electron microscope Philips QUANTA 200 (FEI) in high vacuum under 20 kV tension with a working distance of 10.2 mm. The selected images were captured and stored for later analysis.

Results

Selective bioassay and determination of the Median Lethal Concentration (LC_{50}) of *Bacillus thuringiensis* isolates

Among the 47 isolates of *B. thuringiensis* tested, 12 isolates (25.53%), in addition to *B. thuringiensis* subsp. *kurstaki* (Btk) HD-1 strain, caused mortality of *E. lignosellus* above 85%, for a total of 13 isolates, which were selected for all other conclusive tests. Dose-response bioassays were used to evaluate these 13 isolates. The X^2 values related to the LC_{50} were no significant for 12 of the isolates, which indicated that the data were homogeneous for those strains and fit the Probit analysis model (Table 1) (Finney, 1971).

Table 1. Mortality (%) and LC_{50} of *Bacillus thuringiensis* isolates against first instar larvae of *Elasmopalpus lignosellus* (Zeller, 1848) (Lepidoptera, Pyralidae) (n = 60) on the sixth day of the dose-response bioassay.

Isolate	[a]Mortality (%)	[b]LC_{50} (Spores mL^{-1})	CI (95%)		Slope ± SE	X^2
			Lower	Upper		
BR83	100.00 a	0.038E+8 a	0.025E+8	0.053E+8	1.92 ± 0.26	2.004 ns
BR145	100.00 a	0.059E+8 ab	0.020E+8	0.122E+8	1.25 ± 0.13	11.009 ns
BR09	100.00a	0.063E+8 ab	0.037E+8	0.102E+8	1.47 ± 0.13	11.070 ns
BR78	100.00 a	0.086E+8 ab	0.030E+8	0.179E+8	1.19 ± 0.14	10.806 ns
S1534	100.00 a	0.090E+8 ab	0.043E+8	0.189E+8	1.46 ± 0.18	7.098 ns
S1302	93.33 ab	0.097E+8 b	0.071E+8	0.134E+8	1.30 ± 0.13	3.724 ns
HD1	100.00 a	0.114E+8 b	0.084E+8	0.151E+8	1.38 ± 0.15	3.384 ns
BR52	100.00 a	0.141E+8 bc	0.098E+8	0.250E+8	1.38 ± 0.24	6.702 ns
BR38	100.00 a	0.346E+8 c	0.233E+8	0.445E+8	2.17 ± 0.31	1.637 ns
BR53	100.00 a	0.378E+8 c	0.239E+8	0.593E+8	1.69 ± 0.23	5.969 ns
S545	91.23 b	0.392E+8 c	0.218E+8	0.767E+8	1.39 ± 0.20	8.546 ns
S1269	94.92 ab	0.855E+8 d	1.39E+8	0.543E+8	1.43 ± 0.30	3.978 ns
S1450	95.00 ab	(-)	(-)	(-)	0.96 ± 0.16	15.880 s★

[a]Mortality (%) from selective bioassay. Means followed by the same letter in the column are not significantly different (Tukey's test) at $p< 0.05$; [b]Means followed by the same letter in the column are not different from one another based on the overlap of 95% confidence intervals, according to Probit analysis; (-)significant X^2: Estimate of the LC_{50} was not possible. s★significant based on the Chi-squared test ($p < 0.05$). ns- not significant based on the Chi-squared test ($p < 0.05$).

The LC_{50} values of the selected isolates varied from 0.038E+8 to 0.855E+8 spores mL^{-1}. The lowest LC_{50} values were obtained for the group consisting of BR83, BR145, BR09, BR78, and S1534. The LC_{50} values within that group did not differ statistically according to the Probit analysis, as shown by the overlap of the 95% confidence intervals (Table 1). Only theLC_{50} value of isolate BR83 was significantly lower than that of the HD-1 standard strain, with a toxicity that was three-fold greater than that of the standard. Additionally, the BR 83 isolate was approximately 22-fold more toxic than the S1269 isolate, which had the highest LC_{50}. Only the S1450 isolate presented a significant X^2; thus, the LC_{50} could not be estimated (Table 1).

Protein and molecular characterization of *B. thuringiensis* isolates pathogenic to *E. lignosellus*

The PCR technique using total DNA of isolates and specific primers for the detection of *cry1, cry2, cry3, cry4A, cry 4B, cry10,* and *cry11* resulted in the amplification of fragments of the expected sizes (Bravo et al., 1998; Céron et al., 1995; Ibarra et al., 2003; Vidal-Quist et al., 2009) and consequently, the determination of which *cry* genes were in the isolates of *B. thuringiensis*.

The amplicons produced with the greatest frequency corresponded to *cry1, cry2*, and *cry3* genes. The *cry1* gene was detected in all isolates, except BR52 and BR53, which also did not exhibit the *cry2* gene. The expected fragment for the *cry3* gene appeared in BR145, S1534, and S1302 isolates, whereas the fragment for the *cry11* gene occurred only in the BR53 isolate. Only the BR52 isolate did not show a PCR product consistent with the selected *primers.* The fragments of the expected size for the other *primers* used were not observed in all isolates, indicating the absence of *cry4A, cry4B*, and *cry10* genes in some isolates (Table 2). The protein profile analysis of the spore-crystal mixtures of the isolates from the selective bioassays revealed bands of 60, 65, 70, 80, and 130 kDa. The isolate used as the standard, *B. thuringiensis* subsp. k*urstaki*, had a protein profile of 65 and 130 kDa (Höfte & Whiteley, 1989; Lereclus, Delécluse, & Lecadet, 1993). All data related to the protein and molecular characterization of *B. thuringiensis* isolates are presented in Table 2. An image of protein electrophoresis on 10% polyacrylamide gel (SDS-PAGE) illustrating the protein profile of some isolates is shown in Figure 1.

Table 2. Genetic protein profiles and morphological characterization of crystals of *Bacillus thuringiensis* isolates.

Isolate	Genetic profile	Protein profile (kDa)	Morphological analysis		
			Bipyramidal	Spherical	Cuboidal
BR83	*cry1, cry2*	130/70	+	+	+
BR145[a]	*cry1, cry2,cry3*	130/65-70	+	+	-
BR09[b]	*cry1, cry2*	130/65	+	+	+
BR78	*cry1, cry2*	65/80	+	+	+
S1534	*cry1, cry2, cry3*	130/70	+	+	-
S1302	*cry1, cry2, cry3*	70	+	+	-
HD1	*cry1, cry2*	65/130	+	-	+
BR52	-	70	+	-	-
BR38	*cry1, cry2*	130/70	+	+	-
BR53	*cry11*	70	+	-	-
S545	*cry1*	130	+	-	-
S1269	*cry1*	130	+	-	-
S1450	*cry1, cry2*	130/65	+	+	+

[a]Data from Ricieto et al. (2013); [b]Data from Santos et al. (2009).

Morphological characterization of *B. thuringiensis* isolates

Morphological analysis using optical and scanning electron microscopy showed protein inclusions of different forms. The BR83, BR09, BR78, and S1450 isolates showed three different crystalline protein inclusions: bipyramidal, spherical, and cuboidal. Isolates BR145, S1534, S1302, and BR38 exhibited bipyramidal and spherical crystals, whereas isolates S545, S1269, BR52, and BR53 contained only bipyramidal crystals (Table 2).

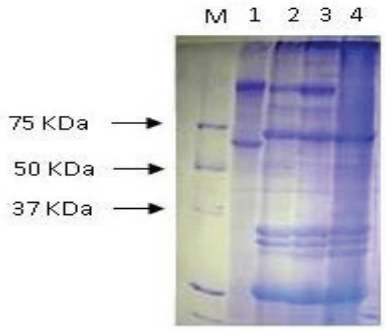

Figure 1. Protein profile produced by isolates toxic to *Elasmopalpus lignosellus* (Zeller, 1848) (Lepidoptera, Pyralidae). M, Rainbow molecular weight marker (GE); 1 - BR09; 2 - BR38; 3 - BR83; 4 - BR52.

Discussion

The selective and dose bioassays revealed that *B. thuringiensis* isolates were active against *E. lignosellus.* Of the isolates tested, 25.53% caused mortality above 85%; therefore, these isolates can be further tested as a possible alternative method for the management of populations of *E. lignosellus*. Based on dose bioassays, isolate BR83 had the lowest lethal concentration. Furthermore, BR83 had superior activity compared with that of the standard *B. thuringiensis* subsp. *kurstaki* HD-1 strain, although the activity was not significantly different from that of the BR145, BR09, BR78, and S1534 isolates. Those four isolates carried *cry1* and *cry2* genes, which are toxic to insects of the order Lepidoptera (Ricieto et al., 2013). In addition to these genes, BR145 and S1302 isolates also carried the *cry3* gene, with reported toxicity to Lepidoptera and Coleoptera (Brizzard & Whitley, 1988).

The *cry1* and *cry2* genes were found in almost all selected isolates, except for BR52, which did not yield an amplification product for any of the used primers, and BR53, which amplified only for the *cry11* gene. The genes in the Cry1 subfamily are the most abundant and occur in approximately half of the isolates identified to date. The *cry2* gene is also very common, particularly among isolates harbouring the *cry1* gene (Porcar & Juárez-Perez, 2003; Arrieta, Hernández, & Espinoza, 2004).

Isolates containing *cry1* and/or *cry2* genes were also the most abundant in the collection studied by Vidal-Quist et al. (2009), representing over 45% of the total. In studies with the BR37 isolate, Santos et al. (2009) identified eight genes of which seven were in the Cry1 group, showing the frequent occurrence of *cry1* genes in isolates of *B. thuringiensis* (Bravo et al., 1998).

Genes in the Cry1 and Cry2 protein families have been reported in the isolates used for the control of *E. lignosellus*, and the effects of Cry1 proteins in transgenic peanut and soybeans plants have been examined under field conditions. In peanuts, various levels of resistance to the lesser cornstalk borer were provided by the introduction of the *cry1Ac* gene, from complete larval mortality to 66% reduction in larval weight (Singsit et al., 1997). For soybean, plants expressing the *cry1Ac* gene had four-fold more resistance to *E. lignosellus* than that of the wild-type isolate (Walker, All, Mcpherson, Boerma, & Parrott, 2000).

Assessments of genetically modified corn crops revealed that hybrids containing *cry9C, cry1F,* and *cry1Ab* genes did not differ in resistance to *E. lignosellus*; however, transgenic plants were superior to non-transgenic hybrids (Vilella, Waquil, Vilela, Siegfried, & Foster, 2002). Additionally, in laboratory bioassays, isolates containing *cry2A* gene and the HD-1 standard isolate proved effective against *E. lignosellus* (Moar, Pusztai-Carey, & Mack, 1995).

These examples demonstrate the different methodologies used for the control of *E. lignosellus*, but the mortality rates are different between isolates harbouring many genes and those caused by genetically modified plants carrying only the primary gene responsible for the toxicity. However, to select new isolates that carry toxic genes to be tested individually against the pest, the bioassays used in this study must be conducted. Thereby, the selected gene, after many tests, can be inserted into plants for pest control and increase the possibilities for pest management.

The protein profiles of most of the tested isolates that amplified with the *cry1* and *cry2* genes (BR83, BR145, BR09, BR38, S1534, and S1450) showed bands of 130 and 65/70 kDa, which confirmed their specificity for Lepidoptera. Cry1 class proteins have a molecular weight of approximately 130-140 kDa (Höfte & Whiteley, 1989), whereas proteins of the Cry2 and Cry3 groups, which are active against Lepidoptera and Coleoptera, have values of 65-70 kDa (Bravo et al., 2004).

Although the BR09 isolate did not differ from the most toxic isolate (BR83) or the HD-1 standard, the LC_{50} of the BR09 isolate was approximately 1.8-fold lower than that of HD-1. Santos et al. (2009) obtained a similar value when testing the same isolate against *Spodoptera eridania*. Nevertheless, LC_{50} values may vary among species; for example, in studies against *Spodoptera cosmioides*, the LC_{50} of the HD-1 standard strain was two-fold lower than that of the BR09 isolate (Santos et al., 2009). Moreover,

in a selection performed by Constanski et al., 2015, no differences were detected between the LC_{50} of the HD-1 standard strain and that of the other isolates tested against *S. eridania* and *S. cosmioides.*

The toxicological profile of the BR09 isolate that was effective against *E. lignosellus* can be explained by the expression of *cry1* and *cry2* genes, similar to the HD-1 strain, which has the *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2A*, and *cry2B* genes in its genome (Li et al., 2005). Furthermore, SDS-PAGE protein analysis of the spore and crystal mixture revealed two polypeptides with approximate molecular weights of 70-130 kDa. Santos et al. (2009) also tested the BR09 isolate and showed a gene profile with *cry1Aa*, *cry1Ab*, *cry1Ac,* and *cry2Aa* genes.

Although the S1450 isolate caused mortality of *E. lignosellus* larvae above 85%, in the selective bioassays, the X^2 value was significant, i.e., the data did not fit the Probit model. Thus, the LC_{50} could not be estimated for isolate S1450. This isolate is in the *kurstaki* serotype described in the literature as toxic to insects of the order Lepidoptera (Monnerat et al., 2007) and causing 100% mortality of *Agrotis ipsilon* (Lepidoptera: Noctuidae). Additionally, *cry1Aa, cry1Ab, cry1Ac, cry1,* and *cry2* genes were revealed by the molecular analysis, which confirmed the activity of the isolate against Lepidoptera (Menezes, Fiuza, Martins, Praça, & Monnerat, 2010).

The BR78 isolate, with an LC_{50} that did not differ from that of the most toxic isolates, also carried *cry1* and *cry2* genes. However, only two primary polypeptides of approximately 65 and 80 kDa were associated with this isolate, which are related only to the *cry2* gene. The result was similar for the S1302 isolate, which despite amplifying with *cry1, cry2,* and *cry3* genes, revealed only a band of 70 kDa. As a possible explanation, a poor performance or even the absence of the promoter led to low expression of the *cry1* gene, which prevented the display of a 130 kDa band.

According to Alper et al. (2014), some strains that harbour the same genes may not be as effective as other toxic strains that have those same genes, indicating that these genes could be poorly expressed because of a weak promoter in the strains. Thus, poor expression of the *cry* genes in the BR78 and S1302 isolates could explain the *cry* genes in the genetic profile but not in the protein profile.

Armengol, Escobar, Maldonado, and Orduz (2007), identified isolates toxic to *S. frugiperda* containing *cry1Aa, cry1Ab, cry1Ac, cry1B,* and *cry1D* genes, with protein profiles that revealed bands only at 60 kDa. According to these authors, the correlation between the identified protein profiles and the *cry* genes cannot occur when the *cry* proteins are encoded by unknown genes or have not been amplified by the *primers* used. Additionally, the identified genes may encode proteins with low-level or inactive expression.

Although the LC_{50} did not differ from that of the HD-1 standard isolate, only the BR52 isolate failed to obtain amplification of the expected fragment sizes with the specific primers employed. Nevertheless, the optical and electron microscope observations revealed the production of protein inclusions, suggesting that the isolate might contain genes not covered by the set of primers used in the PCR analysis or *cry* genes not yet described. Additionally, the analysis of the protein profile showed a polypeptide of approximately 70 kDa that corresponded to the Cry2 class, possibly explaining the insecticidal activity of isolate BR52 against Lepidoptera.

Only isolate BR53 expressed the *cry11* gene. However, this gene was most likely not the cause of the toxicity of this isolate against *E. lignosellus*, because the gene is usually associated with activity against larvae of Diptera, such as *Simulium* spp., *Culex* spp. and *Aedes aegypti* (Vidal-Quist et al., 2009). Thus, isolate BR53 might contain some other gene toxic to *E. lignosellus* not disclosed by the set of primers used, requiring further investigation to search for new *cry* genes.

Based on the different shapes of protein crystals detected by optical and electron microscopy, the types of Cry proteins that composed the crystal could be inferred, which provided information on the insecticidal activity of the isolates (Lereclus, Delécluse, & Lecadet, 1993; Vilas-Boas et al., 2007).

The similarity between the morphological and molecular analyses was notable in this study. Isolates that expressed *cry1* and *cry2* genes contained primarily bipyramidal, cuboidal, and spherical shaped crystals. These three different shapes were observed in the BR09, BR78, and BR83 isolates, which contained the *cry1* and *cry2* genes, and in the S1450 isolate that harboured *cry1, cry2,* and *cry3* genes. The BR38 isolate with *cry1* and *cry2* genes and the S1302, S1534, and BR145 isolates with *cry1, cry2* and *cry3* genes all contained bipyramidal and spherical crystals. The crystal protein composition was studied in some of these isolates previously, and those studies confirm the shapes observed in this study (Praça et. al., 2004; Santos et al., 2009; Ricieto et al., 2013). Only bipyramidal crystals were produced by the isolates S545 and S1269, which contained only the *cry1* gene, and BR52 and BR53.

The selection of isolates containing Cry1 and Cry2 proteins and harbouring more than one Cry

protein is essential in the prospecting for new virulent *cry* genes against lepidopterans such as *E. lignosellus*. Once identified, these are the isolates that can be used for pest management either through bioinsecticide formulations or as a source for transgenic plant manipulation.

*Bacillus thuringiensis*is the most successful pathogen agent used for insect control, representing almost 2% of the total insecticide market and up to 90% of bioinsecticide formulations. Currently, formulations based on *B. thuringiensis* are increasing, and this market is expected to continue to grow. Most of these products are based on spore-crystal preparations derived from a few strains such as *B. thuringiensis* var. *kurstaki* HD1. Thus, the detection of *B. thuringiensis* strains with new genes is extremely important to develop more efficient bioinsecticides (Bravo, Likitvivatanavong, Gill, & Soberón, 2011; Lemes et al., 2015).

For some products formulated with *B. thuringiensis*, their use in agriculture is limited, because Cry toxins are more specific for first larval instars, are sensitive to sun radiation and have limited activity against borer insects (Bravo et al., 2011). Therefore, the internal feeding behaviour of *E. lignosellus* inside stalks and stems makes management with pesticide and biologic product applications alone difficult. Thus, to more effectively *E. lignosellus* control, plants must be genetically modified with *B. thuringiensis* genes.

The discovery of these *cry* genes is of great interest for the production of new transgenic plants and also for genes tacking or pyramidalization. For example, insertion of different *cry* genes into genetically modified plants that use different receptors in the insect midgut membrane can help to extend the protection against more insect pests. Additionally, the beginning of resistance can be delayed or prevented, because more than one toxic protein would be acting against the same insect species (Sanahuja, Banakar, Twyman, Capell, & Christou, 2011; Hernández-Rodríguez, Hernández-Martínez, Van Rie, Escriche, & Ferré, 2013).

Researchers and commercial companies have recently used the technique of gene pyramidalization. As an example, the SmartStax® corn developed in cooperation between Monsanto and Dow Agro Sciences companies showed that the pyramidalization of *cry1A.105, cry2Ab, cry3Bb1, cry34Ab1, cry35Ab1,* and *cry1Fa2* genes was essential for managing pesticide-resistant pests, in addition to providing effective control against a long list of pests in Coleoptera and Lepidoptera, including *E. lignosellus* (Marra, Piggott, & Goodwin, 2010).

The isolates evaluated in this study have potential for biotechnological control of *E. lignosellus.* Additionally, the isolates can be a gene source for the production of new crops or the management of currently insect-resistant plant cultivars. The genome sequencing of the studied isolates ensured accurate quantification of *cry* genes for further selection and use against *E. lignosellus* through insertion into the genome of economically relevant crops attacked by this pest.

Conclusion

Among the 47 isolates studied, 12 caused mortality above 85%. Isolates BR145, BR09, BR78, S1534, and S1302 had the lowest LC_{50} values and did not differ from the standard HD-1. The protein profiles produced bands with molecular masses of 60-130 kDa. The molecular characterization showed the presence of *cry1*, *cry2*, *cry3*, and *cry11* genes.

The morphological analysis identified three different crystal inclusions: bipyramidal, spherical and cuboidal. As a result of these characterizations, these isolates have potential for biotechnological control of *E. lignosellus* and should be important candidates for more studies and the development of new biopesticides or genetically modified plants.

Acknowledgements

The authors wish to thank the National Council for Scientific and Technological Development (CNPq) for financial support.

References

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, *18*(2), 265-266.

Alper, M., Günes, H., Tatlipina, A., Çöl, B., Civelek, H. S., Özkan, C., & Poyraz, B. (2014). Distribution, occurrence of *cry* genes, and lepidopteran toxicity of native *Bacillus thuringiensis* isolated from fig tree environments in Aydın Province. *Turkish Journal of Agriculture and Forestry, 38*(6), 898-907.

Armengol, G., Escobar, M. C., Maldonado, M. E., & Orduz, S. (2007). Diversity of Colombian strains of *Bacillus thuringiensis* with insecticidal activity against dipteran and lepidopteran insects. *Journal of Applied Microbiology*, *102*(1), 77-88.

Arrieta, G., Hernández, A., & Espinoza, A. M. (2004). Diversity of *Bacillus thuringiensis* strains isolated from coffee plantations infested with the coffee berry borer *Hypothenemus hampei* Ferrari. *Revista de Biología Tropical, 52*(3), 757-764.

Bertani, G. (1951). Studies on lysogenesis I. The mode of phage liberation by lysogenic *Escherichia coli. Journal of Bacteriology, 62*(3), 293-300.

Bravo, A., Gómez, I., Conde, J., Muñoz-Garay, C., Sánchez, J., Miranda, R., ... Soberón, M. (2004). Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. *Biochimica et Biophysica Acta*, *1667*(3), 38-46.

Bravo, A., Likitvivatanavong, S., Gill, S. S., & Soberón, M. (2011). *Bacillus thuringiensis*: a story of a successful bioinsecticide. *Insect Biochemistry and Molecular Biology, 41*(7), 423-431.

Bravo, A., Sarabia, S., Lopez, L., Ontiveros, H., Abarca, C, Ortiz, A., ... Quintero, R. (1998). Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Applied and Environmental Microbiology*, *64*(12), 4965-4972.

Brizzard, B. L., & Whiteley, H. R. (1988). Nucleotide sequence of an additional crystal protein gene cloned from *Bacillus thuringiensis* subsp. *thuringiensis*. *Nucleic Acids Research, 16*(6), 2723-2724.

Céron, J., Ortíz, A., Quintero, R., Güereca, L., & Bravo, A. (1995). Specific PCR primers directed to identify *cry*1 and *cry3* genes within a *Bacillus thuringiensis* strains collection. *Applied and Environmental Microbiology*, *61*(11), 3826-3831.

Constanski, K. C., Zorzetti, J., Vilas Bôas, G. T., Ricieto, A. P. S., Fazion, F. A. P., Vilas Boas, L. A., ... Neves, P. M. O, J. (2015). Seleção e caracterização molecular de isolados de *Bacillus thuringiensis* para o controle de *Spodoptera* spp. *Pesquisa Agropecuária Brasileira*, *50*(8), 730-733.

Downes, F. P., & Ito, K. (2001). *Compendium of methods for the microbiological examination of foods* (4th ed.). Washington, DC: American Public Health Association.

Finney, D. J. (1971). *Probit analysis* (3rd ed.). Cambridge, UK: Cambridge University Press.

Gallo, D., Nakano, O., Neto, S. S., Carvalho, R. P. L., Batista, G. C., Filho, E. B., ... Omoto, C. (2002). *Entomologia agrícola*. Piracicaba, SP: Fealq.

Gill, H. K., McSorley, R., Goyal, G., & Webb, S. E. (2010). Mulch as a potential management strategy for lesser cornstalk borer, *Elasmopalpus lignosellus* (Insecta: Lepidoptera: Pyralidae), in bush bean (*Phaseolus vulgaris*). *Florida Entomologist*, *93*(2), 183-190.

Greene, G. L., Leppla, N. C., & Dickerson, W. A. (1976). Velvetbean caterpillar: a rearing procedure and artificial medium. *Journal of Economic Entomology, 69*(4), 487-488.

Hernández-Rodríguez, C. S., Hernández-Martínez, P., Van Rie, J., Escriche, B., & Ferré, J. (2013). Shared midgut binding sites for Cry1A, Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa proteins from *Bacillus thuringiensis* in two important corn pests, *Ostrinian ubilalis* and *Spodoptera frugiperda*. *PLoS ONE*, *8*(7), e68164.

Höfte, H., & Whiteley, H. R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis. Microbiology and Molecular Biology Reviews*, *53*(2), 242-255.

Ibarra, J. E., Rincón, M. C. D., Ordúz, S., Noriega, D., Benintende, G., Monnerat, R., ... Bravo, A. (2003). Diversity of *Bacillus thuringiensis* strains from Latin America with insecticidal activity against different mosquito species. *Applied and Environmental Microbiology*, *69*(9), 5269-5274.

Jham, G. N., Silva, A. A., Lima, E. R., & Viana, P. A. (2007). Identification of acetates in *Elasmopalpulus lignosellus* pheromone glands using a newly created mass spectral database and Kóvats retention indices. *Química Nova*, *30*(4), 916-919.

Lecadet, M. M., Chaufaux, J., Ribier, J., & Lereclus, D. (1992). Construction of novel *Bacillus thuringiensis* strain with different insecticidal activities by transduction and transformation. *Applied and Environmental Microbiology*, *58*(3), 840-849.

Lemes, A. R. N., Marucci, S. C., Costa, J. R. V., Alves, E. C. C., Fernandes, O. A., Lemos, M. V. F., & Desidério, J. A. (2015). Selection of strains from *B. thuringiensis* genes containing effective in the control of *Spodoptera frugiperda. Bt Research*, *6*(1), 1-8.

Lereclus, D., Delécluse, A., & Lecadet, M. M. (1993). Diversity of *Bacillus thuringiensis* toxins and genes. In: P. F. Enwistle, J. Cory, M. Bailey, S, Higgs (Eds.), *Bacillus thuringiensis*, *an environmental biopesticide: Theory and practice* (p. 37-69). Chichester, UK: John Wiley & Son Ltd.

Li, H., Oppert, B., Higgins, R. A., Huang, F., Buschman, L. L., & Zhu, K. Y. (2005). Susceptibility of Dipel-resistant and -susceptible *Ostrinia nubilalis* (Lepidoptera: Crambidae) to individual *Bacillus thuringiensis* protoxins. *Journal of Economic Entomology*, *98*(4), 1333-1340.

Ministério da Agricultura, Pecuária e Abastecimento [MAPA]. (1995). *Normas e exigências para execução de testes de produtos químicos para fins de registro no MAPA*. Brasília, DF: Ministério da Agricultura e Reforma Agrária.

Marra, M. C., Piggott, N. E., & Goodwin, B. K. (2010). The anticipated value of Smart Stax™ for US corn growers. *AgBio Forum*, *13*(1), 1-12.

Menezes, R. S., Fiuza, V. D., Martins, E. S.,Praça, L. B., & Monnerat, R. G. (2010). Seleção e caracterização de estirpes de *Bacillus thuringiensis* tóxicas a *Agrotisipsilon*. *Universitas Ciências da Saúde*, *8*(1), 1-13.

Moar, W. J., Pusztai-Carey, M., & Mack, T. P. (1995). Toxicity of purified proteins and the HD-1 strain from *Bacillus thuringiensis* against lesser cornstalk borer (Lepidoptera: Pyralidae). *Journal of Economic Entomology*, *88*(3), 606-609.

Monnerat, R. G., Batista, A. C., Medeiros, P. T., Martins, E., Melatti, V., Praça, L., Dumas, V., … Berry C. (2007). Screening of Brazilian *Bacilus thuringiensis* isolates active against *Spodoptera frugiperda, Plutella xylostella* and *Anticarsia gemmatalis*. *Biological Control*, *41*(3), 291–295.

Pardo-López, L., Soberón, M., & Bravo, A. (2013). *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. *FEMS Microbiology Reviews*, *37*(1), 3-22.

Porcar, M., & Juárez-Pérez, V. (2003). PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. *FEMS Microbiology Reviews*, *26*(5), 419-432.

Praça, L. B., Batista, A. C., Martins, E. S., Siqueira, C. B., Dias, D. G. S., Gomes, A. C. M. M., ... Monnerat, R. G. (2004). Estirpes de *Bacillus thuringiensis* efetivas contra insetos das ordens Lepidoptera, Coleoptera e Diptera. *Pesquisa Agropecuária Brasileira*, *39*(1), 11-16.

Ricieto, A. P. S., Fazion, F. A. P., Carvalho Filho, C. D., Vilas-Boas, L. A., & Vilas-Bôas, G. T. (2013). Effect of vegetation on the presence and genetic diversity of *Bacillus thuringiensis* in soil. *Canadian Journal of Microbiology*, *59*(1), 28-33.

Sanahuja, G., Banakar, R., Twyman, R. M., Capell, T., & Christou, P. (2011). *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnology Journal*, *9*(3), 283-300.

Santos, K., Neves, P. M. O. J., Meneguim, A. M., Santos, R. B., Santos, W. J., Vilas-Bôas, G. T., ... Monnerat, R. (2009). Selection and characterization of the *Bacillus thuringiensis* strains toxic to *Spodoptera eridania* (Cramer), *Spodoptera cosmioides* (Walker) and *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae). *Biological Control*, *50*(2), 157-163.

Singsit, C., Adang, M. J., Lynch, R. E., Anderson, W. F., Wang, A., Cardineau, G., & Ozias-Akins, P. (1997). Expression of a *Bacillus thuringiensis* cryA(c) gene in transgenic peanut and its efficacy against lesser cornstalk borer. *Transgenic Research*, *6*(2), 169-176.

Sun, Y., Fu, Z., Ding, X., & Xia, L. (2008). Evaluating the insecticidal genes and their expressed products in *Bacillus thuringiensis* strains by combining PCR with Mass Spectrometry. *Appliedand Environmental Microbiology*, *74*(21), 6811-6813.

Viana, P. A. (2004). Lagarta-elasmo. In J. R. Salvadori, C. J. Ávila, M. T. B. Silva. (Ed.), *Pragas de solo no Brasil* (p. 379-408). Passo Fundo, RS: Embrapa Trigo; Dourados, MS: Embrapa Agropecuária Oeste; Cruz Alta, RS: Fundacep Fecotrigo.

Vidal-Quist, J. C., Castañera, P., & González-Cabrera, J. (2009). Diversity of *Bacillus thuringiensis* strains isolated from citrus orchards in Spain and evaluation of their insecticidal activity against *Ceratitis capitata*. *Journal of Microbiology and Biotechnology*, *19*(8), 749-759.

Vilas-Bôas, G. T., Peruca, A. P. S., & Arantes, O. M. N. (2007). Biology and taxonomy of *Bacillus cereus, Bacillus anthracis* and *Bacillus thuringiensis*. *Canadian Journal of Microbiology*, *53*(6), 673- 687.

Vilella, F. M. F., Waquil, J. M., Vilela, E. F., Siegfried, B. D., & Foster, J. E. (2002). Selection of the fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) for survival on Cry 1A(b)*Bt*. toxin. *Revista Brasileira de Milho e Sorgo*, *1*(1), 12-17.

Walker, D. R., All, J. N., Mcpherson, R. M., Boerma, H. R., & Parrott, W. A. (2000). Field evaluation of soybean engineered with a synthetic *crylAc* transgene for resistance to corn earworm, soybean looper, velvetbean caterpillar (Lepidoptera: Noctuidae), and lesser cornstalk borer (Lepidoptera: Pyralidae). *Journal of Economic Entomology*, *93*(3), 613-622.

Received on July 13, 2016.
Accepted on December 9, 2016.