Note

Characterization and selection of *Bacillus thuringiensis* isolates effective against *Sitophilus oryzae*

Najara da Silva[1]; Ana Maria Guidelli Thuler[1]; Irlan Leite de Abreu[1]; Camila Chiaradia Davolos[1]; Ricardo Antonio Polanczyk[2]; Manoel Victor Franco Lemos[1]*

[1]*UNESP/FCAV – Depto. de Biologia Aplicada à Agropecuária – Via de Acesso Prof. Paulo Donato Castellane, s/n – 14884-900 – Jaboticabal, SP – Brasil.*
[2]*UFES/CCA – Depto. de Produção Vegetal – Alto Universitário s/n. – 29500-000 – Alegre, ES – Brasil.*
**Corresponding author <mvictor@fcav.unesp.br>*

ABSTRACT: The entomopathogenic bacterium *Bacillus thuringiensis* is a control agent with toxic and environmental characteristics that allows the control of pest insects according to the Integrate Pest Management (IPM) precepts. In order to find new strains, potentially toxic to *Sitophilus oryzae* L. 1763 (Coleoptera: Curculinidae), 1.073 strains of *B. thuringiensis* from parts of Brazil were used. Genetic material was extracted with InstaGene Matrix kit, used for the amplification of sequences in Polymerase chain reaction (PCR), and viewed in 1.5% agarose gel. The gene *cry35Ba* class was represented by 60 *B. thuringiensis* isolates (5.6%), which were then subjected to bioassays with *S. oryzae* larvae. Among the isolates studied, four caused more than 50% mortality in pathogenicity tests, and the isolates 544 and 622 were the most virulent, as determined by CL_{50} estimates. The four toxic isolates had spherical, bi-pyramidal and cuboid crystals, and a 44-kDa protein was found in sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), which coded for the product of *cry35Ba* genes. These data demonstrate the potential of *B. thuringiensis* for the management of *S. oryzae* larvae.
Key words: Coleoptera, Curculionidade, crystal protein, entomopathogenic bacterium

Caracterização e seleção de isolados de *Bacillus thuringiensis* efetivos contra *Sitophilus oryzae*

RESUMO: A bactéria entomopatogênica *Bacillus thuringiensis* (*Bt*) é um agente de controle com características tóxicas e ambientais que permitem o controle de insetos-praga de acordo com as premissas do Manejo integrado de pragas (MIP). Com o objetivo de buscar novas linhagens potencialmente tóxicas para *Sitophilus oryzae* L. 1763 (Coleoptera: Curculinidae), caracterizaram-se molecularmente 1,073 isolados de *B. thuringiensis* de regiões do Brasil. O material genético foi extraído através do kit InstaGene Matrix, utilizado para a amplificação das seqüências através da técnica de Polymerase chain reaction PCR, sendo os resultados visualizados em gel de agarose 1,5%. A classe do gene *cry35Ba* foi representada por 60 isolados (5,6%) de *Bt*, os quais foram submetidos a bioensaio com larvas de *S. oryzae.* Quatro causaram mortalidade acima de 50% nos testes de patogenicidade e os isolados 544 e 622 foram os mais virulentos, conforme determinado pela estimativa da CL_{50}. Nos quatro isolados que demonstraram toxicidade, foram detectados cristais esféricos, bipiramidais e cubóides, além de proteínas com 44 kDa, referentes aos genes *cry35Ba* por Sodium dodecil sulphate - polyacrilamide gel electrophoresis (SDS-PAGE). Estes dados demonstram o potencial de *Bt* no manejo de *S. oryzae.*
Palavras-chave: bactéria entomopatogênica, Coleoptera, Curculionidae, proteína cristal

Introduction

Among the factors that affect grain cereals during storage periods is the attack by the pest insect *Sitophilus oryzae* L. 1763 (Coleoptera: Corculinidae) larvae, which is particularly important, under tropical conditions, when the grains become an ideal habitat for its development (Athié and Paula, 2002). This pest also presents the characteristic of cross infestation, i.e., it can migrate from the field to the warehouse, which significantly increases its damage potential (Gallo et al., 2002).

The control of this pest is currently carried out with chemical insecticides that can be toxic also to man and to the environment. The entomopathogenic bacterium *Bacillus thuringiensis*, applied as a bioinsecticide, or its genetic material inserted into plants, known as genetically modified organisms, is today one of the major alternatives to chemical control (Federici, 2005; Glare and O'Callagham, 2000; Praça et al., 2004). This bacterium is noteworthy for producing, during the sporulation process, one or more toxic protein inclusions called Cry proteins (Bravo et al., 2005), and its pathogenicity and specificity are determined by the functional *cry* gene types that an isolate possess (Crickmore, 2008).

The toxic activity of a binary group of insecticide proteins (Moellenbeck et al., 2001) belonging to the

classes Cry34 and Cry35, toxic for *Diabrotica virgifera* larvae was reported by Ellis et al. (2002). This new mode of action was observed in pathogenicity and virulence bioassays, and also in genetically modified maize (Schnepf et al., 2005; Storer et al., 2006).

This study characterized several *B. thuringiensis* isolates that contain the *cry35Ba* gene among others and show insecticidal effect against *S. oryzae* larvae and determined the morphological a total protein profiles of the isolates.

Material and Methods

Isolate's cultivation: One thousand seventy three isolates of *B. thuringiensis* collected from several locations in Brazil, and also belonging to the collection of the Laboratório de Genética de Bactérias e Biotecnologia Aplicada (LGBBA) from the Department of Applied Biology and Agriculture, Faculdade de Ciências Agrárias Veterinárias, UNESP - Campus Jaboticabal, were analyzed. These isolates were stored in the form of spores, adsorbed in sterile filter paper strips, and maintained in plastic tubes, with ultrapure sterile water, in a refrigerator, kept at 10°C. An aliquot of each of the bacterial suspension was removed and spread on Nutrient Agar (3 g L^{-1} meat extract, 5 g L^{-1} bacteriological peptone and 15 g L^{-1} agar), with the aid of a platinum loop in disposable Petri plates. The plates were incubated at 30°C for 12 h in an incubator.

Molecula characterization of the *cry 35* gene: Total DNA samples of the 1,073 isolates were extracted using the ionic exchange resin of Insta-Gene Matrix kit (Bio-Rad®), according to the manufacturer's instructions; the supernatants containing the genetic material were transferred to a set of polypropylene plates with 96 wells. The DNA samples were stored at -20°C in a freezer until their use.

Primers were designed for the sequence of *cry35Ba* gene deposited on special data banks such as http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html, using the software Gene Runner 3.0 (Hastings Software, Inc.) and *Clustal W* for sequence alignment and identification of conserved regions (Thompson et al., 1994). They are listed in Table 1 and were used for the identification of the *cry35Ba* gene.

The bacterial isolates DNA samples were subjected to PCR in reaction batches and in all it was used a negative control, substituting the DNA templates for water. These reactions included buffer (200 mmol L^{-1} Tris, 500 mmol L^{-1} KCl, pH 8.4) 1 mmol L^{-1} magnesium chloride, 250 µmol L^{-1} dNTPs solution, 10 pmol μL^{-1} of each primer for gene *cry35Ba*, 1 U *Taq* DNA *polymerase* (INVITROGEM), 30 ng de DNA template and Milli-Q filtered and sterilized water (enough amount to complete 20 µL).

These reactions were placed in a thermocycler (PTC-100 MJ Research, Inc®) programmed with a first five min denaturation step at 94°C, followed by 35 cycles consisting of three steps: denaturation at 94°C for 30 s; primer annealing at 40°C for 45 s and an extension period of one minute at 72°C; and a last extension step at 72°C for seven min. It is important to point out that primer lower annealing temperatures need to be used since the nucleotide sequence difference between the *cry* genes detected on Lepidoptera and Coleoptera are quite similar and higher annealing temperatures would not allow the detection of PCR indicative bands. The final choice of which isolates really act on Coleoptera larvae was determined by the bioassays carried out in this study. Besides this, other authors have described the use of lower annealing temperatures Davolos et al. (2009) and Nazarian et al. (2009).

After amplification, 3 µL buffer ("loading buffer" – 0.5% bromophenol blue in glycerol 50%) were added to each sample, and an aliquot of each amplified product was analyzed by electrophoresis in 1.5% agarose gel (Sambrook and Russel, 2001). Horizontal trays model Sunrise™ Life Technologies™ and FB–SB2025 Fisher Scientific were used for the electrophoresis, in TBE buffer (Tris 89 mmol L^{-1}, EDTA 2.5 mmol L^{-1}, Boric acid 89 mmol L^{-1}, enough amount to complete 1000 mL; pH 8.2), with 0.5 µg mL^{-1} ethidium bromide. The amplicons still in the agarose gels were observed under UV light and registered in appropriate equipment, and evaluated using a negative image, with the use of the software Quantity-One (GEL DOC 2000 - Bio-Rad®).

Insect rearing: S. oryzae maintenance and reproduction was carried out in four 8-liter plastic recipients. Approximately 2.5 kg corn (Zea mays L.) were placed in each recipient and infested with 100 non sexed insects. The recipients were closed and population development monitoring was carried out through the clear sides of the recipients, without moving the kernel mass. The insects were used in the bioassays six months after the start of mass rearing.

Bioassays: Pathogenicity was evaluated in 60 *B. thuringiensis* isolates, identified by the amplification of the *cry35Ba* gene, from all 1,073 isolates screened by molecular techniques. The isolates were grown in nutrient broth (3 g L^{-1} meat extract, 5 g L^{-1} bacteriological peptone) at 28°C, 180 rpm, for 76 h, for the production of the protein crystals. Subsequently the suspensions were subjected to three consecutive centrifugations at 2,655 χ g for 20 min to wash the sediments and remove the

Table 1 – Oligonucleotide sequences of primers.

Denomination	Primer sequence	Size
cry35Ba	F 5'AACTGATGAAATACCTGAAG3′	535pb
	R'5'TCAACAATAAATCCTACAGC 3′	

growth medium excess. A 1 ml aliquot of the resulting suspension was diluted 1,000 times in sterile distilled water, and the spore concentration was determined as described by Alves and Moraes (1998).

The sixty *B.thuringiensis* isolates were tested in six bioassays containing ten isolates one at a time. Each treatment consisted of seven repetitions of 20 g corn kernels. Six days before applying the treatments, the kernels were made available for 20 non sexed adults for oviposition during three days. After this process, the kernels were immersed, for 1 min, in a suspension containing $3 \chi 10^8$ spores mL^{-1} of the *B. thuringiensis* isolate being tested. Subsequently, the kernels were placed in sterile air flow hood to remove the excess moisture.

Mortality was calculated by the difference between the number of emerged insects in the treatments and those emerged from the control between 25 and 60 days after applying the treatments. Data correction was made using the formula proposed by Abbot (1925), and submitted to the Tukey test ($p < 0.05$).

Estimation of the average lethal concentration (LC_{50}) was carried out for the isolates that caused more than 50% mortality in the pathogenicity test. The conditions for running the bioassays, its evaluation, and the media for *B. thuringiensis* isolates suspension preparation were the same as those described for the pathogenicity test, with counts of $3 \chi 10^3$, $3 \chi 10^4$, $3 \chi 10^5$, $3 \chi 10^6$, $3 \chi 10^7$ and $3 \chi 10^8$ spores mL^{-1}.

Protein purification: was performed for the isolates that presented toxic activity against *S. oryzae* in the bioassays.The isolates were grown as described in item 2.1. Subsequently the bacterial mass was collected and transferred to nutrient broth (3 g L^{-1} meat extract, 5 g L^{-1} bacteriological peptone), and shacked in a rotating incubator at 200 rpm, and 30°C for 96 h until complete sporulation. Purifications were conducted by ultracentrifugation in sucrose gradient, according to Juárez-Perez et al. (2002).

Scanning electron microscopy: Isolate morphology was analyzed by scanning electron microscopy. A purified protein aliquot of each sample (spore/crystal) was spread over a glass cover slip with the aid of a bacteriological loop, and dried in a laminar flow hood. Subsequently the sample was fixed in a metal stub and covered with gold for 180 s using a metalizer, model K550, and analyzed in a Zeiss scanning electron microscope, model DSM 962.

Protein electrophoresis in polyacrylamide-SDS gel: The composition of the spore-crystal complex was analyzed by electrophoresis in polyacrylamide gel containing sodium dodecyl sulphate at 8 and 12%. The samples containing spores-crystal were prepared with 62 mmol L^{-1} Tris-HCl pH 6.8; 4% SDS; 20% Glycerol; 5% β-mercaptoethanol and 0.02% bromophenol blue, heated at 100°C for five min and centrifuged at 10,621 χ g for 30 s. SDS-PAGE electrophoresis was developed according to the method described by Laemmili (1970).

Results and Discussion

Pinto and Fiúza (2003) and Praça et al. (2004) have reported the characterization of *B. thuringiensis* isolates for the products of *cry* genes with activity against insects of the order Coleoptera, as well as for other ones, such as Lepidoptera and Diptera. The present study analyzed 1,073 isolates of *B. thuringiensis*, demonstrating that 60 isolates (5.6%) contained the gene *cry35Ba*, and were selected for the bioassays, revealing that only four isolates (6.6%) caused more than 50% mortality of *S. oryzae* larvae (Figure 1).

The lack of efficacy against *S. oryzae* and the specificity of *B. thuringiensis* strains for different insect orders was confirmed by the use of the variety *var. kurstaki* HD-1 as a negative control, since it does not present the gene *cry35Ba*, and caused only 4% mortality of *S. oryzae* lavae. The positive control had 26.30% efficacy against *S. oryzae*, represented by the variety var. *tolworth*i, which harbors the gene *cry35Ba*. It is relevant to highlight the importance of the present study, since new *B. thuringiensis* isolates are reported with efficacy above 50%, thus surpassing the positive control.

The results obtained in the bioassays denoted differences in toxic activity presented by the isolates. The isolate 544 was the most active against *S. oryzae* caused 60% mortality in the two bioassays. In the third bioassay, isolate 573 caused 56%, mortality, different from all others. In the fourth bioassay two isolates were noteworthy, 622 causing 78% mortality, and isolate 629 causing 51% mortality. All other isolates presenting the gene for protein Cry35Ba, had toxic activity against *S. oryzae* larvae below 50%.

The low frequency with which effective isolates against the order Coleoptera is found has been reported by other authors, using different Cry protein classes, active against the order Coleoptera. Pinto and Fiúza (2003) collected soil samples from Rio Grande do Sul state, Brazil, and determined the genetic profile of 46 isolates, finding five with gene profile for the order Coleoptera. Similarly, Praça et al. (2004) detected only two isolates (0.7%) effective against coleoptera among 300 isolates analyzed. In contrast Bravo et al. (1998) identified several *cry* genes in a collection of 496 isolates from soil samples collected in different regions of Mexico, and found 16 isolates presenting active crystal proteins against insects of the order Coleoptera. In the same direction, Ben-Dov et al. (1997) analyzed 215 isolates from the Asian continent and demonstrated the lack of isolates containing the *cry3*. Similar situation was reported by Chak et al. (1994), from a collection composed of 225 isolates obtained from soil samples in Taiwan, where different gene profiles were found, all with the lack of gene *cry3*. Thus few are the gene products with toxic potential for Coleoptera and in some cases they are lacking.

Among the diversity of known Cry proteins, new proteins have been reported with entomocide activity against the order Coleoptera, such as the protein class Cry35 (Ellis et al., 2002; Herman et al., 2002; Schenef et al., 2004), espe-

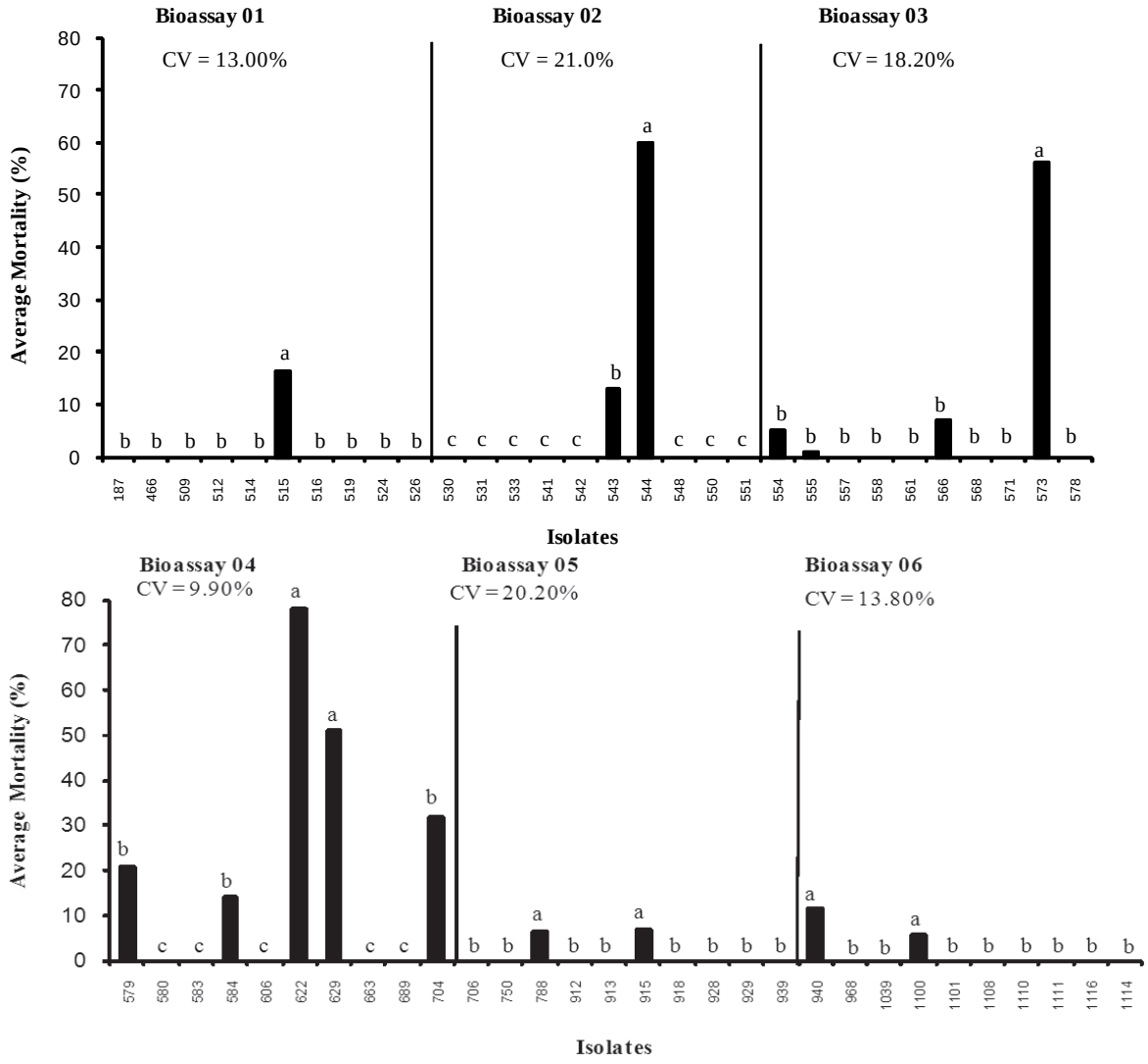

Figure 1 – Mortality caused by *Bacillus thuringiensis* isolates on *Sitophilus oryzae* larvae. Averages followed by the same letter do not differ (Tukey's test, $p < 0.05$).

cially for insects of the family Chrysomelidade. Susceptibility of species in other families, such as Bostrichidae and Curculionidae has been studied for the other Cry proteins (Beegle, 1996; Saade et al., 1996), demonstrating that the bacterium *B. thuringiensis* can be an important source of different toxins, many of which with insecticide activity against species in the order Coleoptera.

Despite apparently simple, the study of the toxic activity of *B. thuringiensis* has some specific characteristics that must be considered. In this study, there were variations on the efficacy of the tested isolates against *S. oryzae*, of the order Coleoptera, that could be explained by a series of factors related to the mode of action of this pathogen, such as pro-toxin activation, toxin solubilization, binding of the activated protein to the larvae gut epithelium receptors, and insertion of the toxin in the membrane. This last factor is the one with the most complexity and, in general, is determinant for toxicity development in insect larvae gut (Bravo et al. 2007).

Although membrane insertion is the major determining factor for the susceptibility of a given species to the toxin Cry, Forcada et al (1996), observed that for *H. virescens* (Lepidoptera) digestive enzymes of resistant insect populations are capable of degrading the toxins in such a way that significantly the amount of active toxin in the gut in a given moment, reducing toxicity. Moreover, some toxins can bind to the receptors and this binding would not be sufficient to cause death of the insect larvae. The reason for the high binding affinity with little or no toxicity is still unknown, suggesting the initial binding of toxin-receptor could be a poor indicator of the receptor function in toxicity and that membrane insertion could be best correlated to toxicity (Aronson and Shay, 2001).

Isolates 544, 573, 622 and 629 of *B. thuringiensis*, presenting more than 50% efficacy against *S. oryzae* larvae, were selected for estimation of lethal concentration (Table 2). There were no differences between isolates

544 and 622, indicating that they were the most virulent, since they presented the lowest CL_{50}. However, between the two isolates, isolate 544 caused insect deaths with the lowest lethal concentration, followed by isolates 622, 573 and 629. Better identification of these isolates was carried out by the morphological characterization, by scanning electron microscopy, of the crystals produced. This analysis highlighted three types of crystals for isolate 544: spherical, bi-pyramidal and cuboid, while isolates 573, 622 and 629 had spherical crystals (Figure 2).

The formation of crystals can be associated to the type of Cry protein and, consequently, to its *cry* gene (Höfte et al., 1989), providing indication about the insecticide activity of the crystals of an isolate (Taylor et al., 1992; Lereclus et al., 1993; Habib and Andrade, 1998). Thus, isolate 544 had in its morphological assembly a 130 kDa bi-pyramidal protein (Figure 3) described as active against the order Lepidoptera (Schenepf et al., 1998). These results were confirmed by PCR analysis, using the primer gral-cry1 described by Bravo et al. (1998) (data not shown). Cuboid crystals could be associated to protein type Cry2, also present in this isolate and confirmed by PCR (data not shown), presenting toxicity against Lepidoptera and Diptera (Höfte et al., 1988); however, this protein was not seen in SDS-PAGE, with a 70 kDa size, according to Crickmore (2008).

The spherical formations, common to the studied isolates, suggest that this crystal morphology could be associated to toxicity of this bacterium to Coleoptera, since they also present a 44 kDa protein profile (Figure 2) referring to protein Cry35Ba, active against Coleoptera (Ellis et al., 2002; Schnepf et al., 2005; Storer et al., 2006). These authors have described the binary action of 14 kDa protein Cry34 with Cry35; however this study did not confirm their presence in the proteic and genetic profiles.

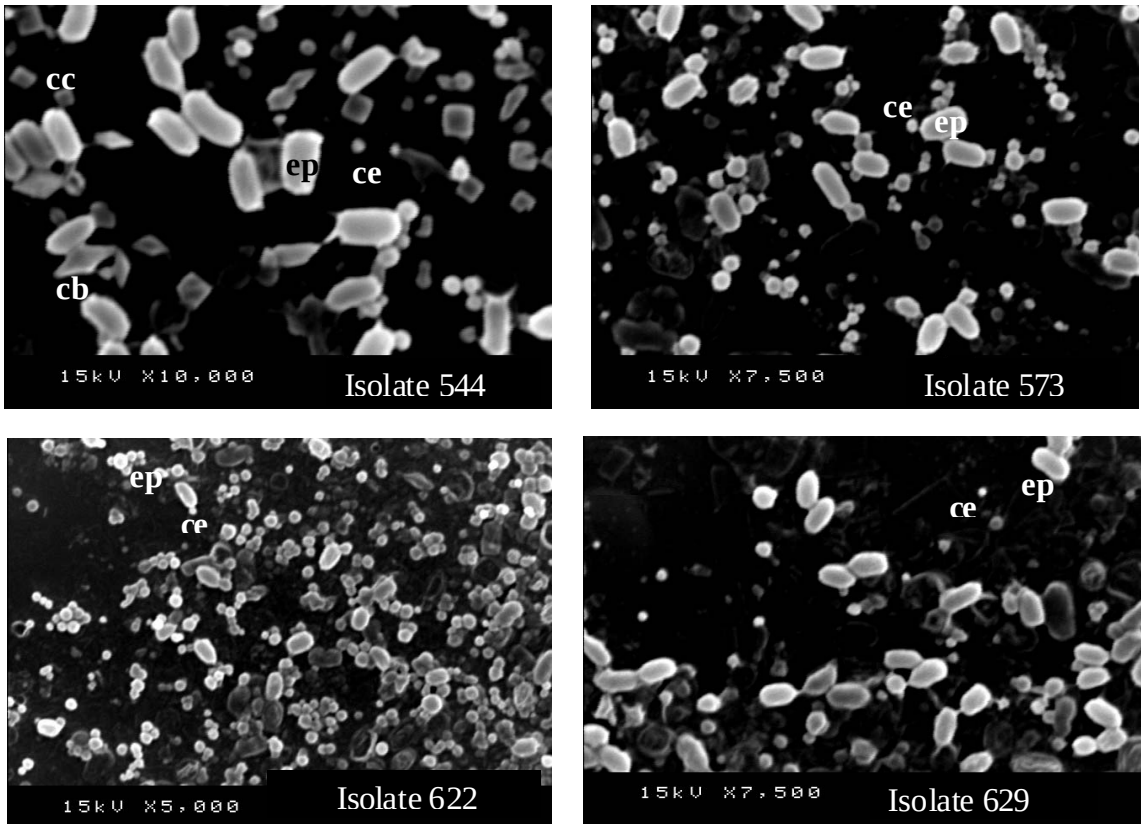

Figure 2 – Scanning electron micrography of the mixture spore-crystal; cb: bi-pyramidal crystal; cc: cuboid crystal; ce: spherical crystal; ep: spore.

Table 2 – Virulence test of *Bacillus thuringiensis* isolates against *Sitophilus oryzae*.

Isolates	Regression equation	LC_{50} ($IC_{0.05}$)	nº	χ^2
544	y = 7.08 + 0.61*log	4.70×10^3 (4.31×10^3 - 6.1 χ 10^3)	120	6.79 n.s
573	y = 3.19 + 0.70*log	3.55×10^5 (3.10×10^5 - 4.11 χ 10^5)	120	8.60 n.s
622	y = 3.42 + 0.12*log	9.14×10^3 (5.1×10^3 - 6.40 χ 10^4)	120	6.49 n.s
629	y = 4.71 + 0.11*log	7.03×10^5 (4.05×10^5 - 5.90 χ 10^6)	120	7.01 n.s

n.s.: non significant, *significant at 5%, nº: number of tested insects/dose, tabulated χ^2 = 10.30

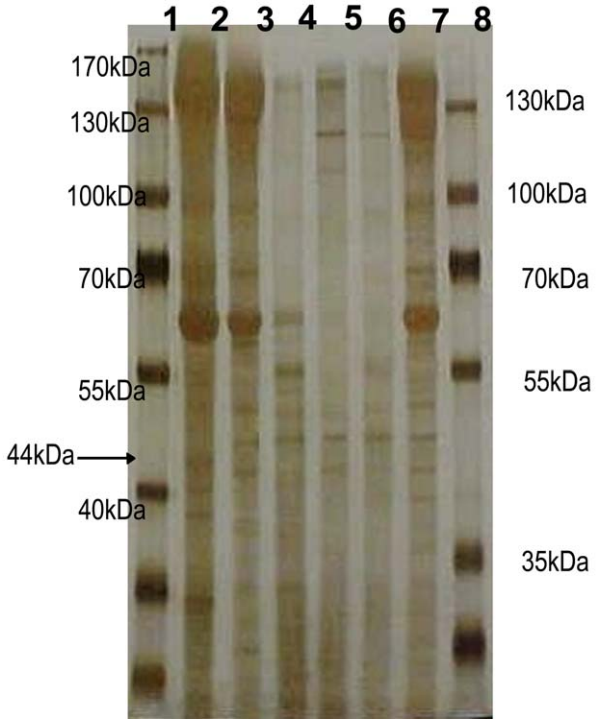

Figure 3 – Analysis of the mixture spore-crystal of *B. thuringiensis* by SDS-PAGE in 8% polyacrylamide gel.1: Molecular marker Fermentas Page Ruler™ Prestained protein Ladder Plus #SM0671 2: var. *toworthi*, 3: isolate 544, 4: isolate 573, 5: isolate 622, 6: isolate 629, 7: var. *kurstaki* HD-1, 8: Molecular marker Fermentas Page Ruler™ Prestained protein Ladder Plus # SM1811.

Other proteins were identified by SDS-PAGE analysis, demonstrating a 55 kDa protein profile for isolates 573 and 629, indicating a new profile for them. A 120 kDa protein band was found for isolate 622 (Figure 2), which can indicate a new Cry protein, since this isolate did not present in its morphological profile the 120 kDa bi-pyramidal crystal, described by Bravo et al. (1998). Moreover, this study highlights that the relations between the genetic profile for gene *cry35Ba*, the 44 kDa proteins and the spherical morphology are present in all isolates effective against *S. oryzae*. No active isolate against *S. oryzae* larvae was found containing simultaneously the genes for the proteins Cry34 and Cry35Ba, and the lack of gene *cry34*, as proposed by the binary mode of action of Ellis et al. (2002) and Schnepf et al. (2005).

Several *B. thuringiensis* subspecies produce a series of other toxins besides the Cry proteins (Caballero and Ferré, 2001). Aranda et al. (1997) have used purified Cry proteins to detect their toxic activity each at a time. However this was not the aim of the present work that was carried out using spore/crystal suspension in the bioassays, with the possibility of one or more Cry proteins being expressed at one time as described by Fatoretto et al. (2007) and Álvarez et al. (2009).

Laboratory results are not always confirmed by field experiments, where there is some difficulty in evaluating some biological parameters (Polanczyk and Alves, 2005), thus reinforcing the need of control measures for *S. oryzae* in stored corn. An alternative would be the use of genetically modified corn, expressing the protein Cry35Ba of *B. thuringiensis* in the kernels, protecting them against *S. oryzae* larvae.

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