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Screening of Brazilian *Bacillus thuringiensis* isolates active against *Spodoptera frugiperda*, *Plutella xylostella* and *Anticarsia gemmatalis*

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Abstract

The toxicity of a collection of 1400 isolates of *Bacillus thuringiensis* was assessed against the Lepidoptera *Spodoptera frugiperda, Anticarsia gemmantalis* and *Plutella xylostella*. Twenty seven isolates showed toxicity to the larvae of these insects with three isolates demonstrating significantly greater potency than the standard strain against Lepidoptera, *B. thuringiensis* serovar *kurstaki* HD1. These isolates were all found to produce bipyramidal crystals and major spore-associated protein bands of approximately 130 and 65 kDa, consistent with the detection of at least one *cry1* and one *cry2* family gene in each. The high level of insecticidal activity of these isolates makes them excellent candidates for further development for use in the field.

Keywords: *Bacillus thuringiensis*; *Spodoptera frugiperda*; *Anticarsia gemmantalis*; *Plutella xylostella*; Biological control; Crystal protein; *cry* genes

1. Introduction

The extensive monoculture of crops like soybean, maize, cotton and cabbage requires a high utilization of agrochemicals to counter the attack of insect pests, especially Lepidoptera such as the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae), the velvet caterpillar *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) and the diamondback moth *Plutella xylostella* (Lepidoptera: Yponomeutidae) (Moscardi and Souza, 2002; Cruz et al., 1999; Monnerat et al., 1999). The costs for controlling these insects is sometimes very high so that the cultivation of these crops may not be financial viable. In addition an awareness of the environmental impact of pesticide use requires the development of safe and non polluting products. In this context, the utilization of entomopathogenic agents such as *Bacillus thuringiensis* (Bt) is an attractive alternative.

This bacterium has worldwide distribution and individual strains produce potent protein toxins, each of which is specific to a small group of insect targets without effects on higher animals or on the environment (Schnepf et al., 1998). The extensive variety of Bt strains and the toxins they produce permit the production of bioinsecticides using the bacteria themselves and also allows use of the toxin genes in the development of transgenic plants (Romeis et al., 2006). Crucial to this development is the identification of the most active strains with respect to a given target insect. The collection of *B. thuringiensis* held by Embrapa Genetic Resources and Biotechnology (Monnerat et al., 2001) was screened for the presence of isolates that are able to generate spore/crystal products on fermentation, with higher potency against *S. frugiperda, A. gemmatalis* and

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P. xylostella than those in current use. These approaches are essential to identify the most potent strains and to allow the exploitation of local biodiversity for the optimization of biological control programs.

2. Materials and methods

2.1. Isolates and culture conditions

One thousand four hundred isolates of *B. thuringiensis* were used from a collection of entomopathogenic *Bacillus* curated by Embrapa Genetic Resources and Biotechnology. These isolates were obtained from soil and water samples from different parts of Brazil (Monnerat et al., 2001). The standard lepidopteran-active *B. thuringiensis kurstaki* (Btk) HD-1, obtained from the Collection of *Bacillus thuringiensis* and *Bacillus sphaericus* at the Institut Pasteur, Paris, was also included in all tests as a reference strain.

All isolates and HD-1 were grown in Embrapa medium (8 g/l nutrient broth; 1 g/l yeast extract; 1 g/l KH_2PO_4; 1 mg/l $CaCO_3$; 1 mg/l $MgSO_4{\cdot}7H_2O$; 0.1 mg/l $FeSO_4{\cdot}7H_2O$; 0.1 mg/l $MnSO_4{\cdot}7H_2O$; 0.1 mg/l $ZnSO_4{\cdot}7H_2O$; pH adjusted to 7.0 by addition of NaOH), for 72 h., 200 rpm, 30 °C. Cultures (600 ml) were centrifuged at 12,800*g* for 30 min, at 4 °C (BR4i centrifuge Jouan), the cell pellets were frozen for 16 h and lyophilized for 18 h in Labconco model Lyphlock 18 freeze-dryer. Dried powders were resuspended in water to 10% (w/v) for use in bioassays.

2.2. Bioassays

Bioassays were carried out using insects reared in the laboratory, at 26 ± 2 °C, 70 ± 10% RH, and a photoperiod of 14:10 (L:D) (Schmidt et al., 2001). Different methods were used for each insect. Two kinds of bioassays were carried out: (i) a selective or excluding one, the aim of which was to identify those isolates presenting toxic activity to the insect, that is, any which was capable of killing 100% of the larvae tested at a single dose; and (ii) the dose dependent assay, the purpose of which was to quantify the toxicity of each isolate.

2.2.1. Spodoptera frugiperda

The selective bioassays were performed on fresh artificial diet comprising 17 g agar (Biobrás, Brazil), 42.9 g brewer's yeast, 67.4 g wheat germ, 140.4 g minced beans, 4.3 g ascorbic acid in a total volume of 1.0 l, poured into a 24 well cell culture plate (1.5 ml per well). After solidification, 35 μl of the resuspended spore/crystal powder was applied onto the diet surface in each well and allowed to dry. Subsequently, one 1-day-old second instar larva of *S. frugiperda* was added to each well. Twenty-four larvae for each isolate and a control (*Bacillus*-free diet) were tested. The plates were covered with acrylic lids and incubated under the same conditions used for rearing the insects. Five cell culture plates per isolate were used. After 48 h, the surviving larvae were individually transferred to 50 ml cups containing rearing diet and the mortality was assessed. Larval mortality was assessed again at day 5 (Silva et al., 2004). Dose dependent bioassays were performed in the same way, using final concentrations of dry spores and crystals in a range between 2 and 2000 ng/cm^2.

2.2.2. Anticarsia gemmatalis

The selective bioassays were performed on fresh artificial diet, comprising 10 g agar (Biobrás, Brazil), 15.6 g brewer's yeast, 25 g wheat germ, 25 g soy protein 31.2 g minced beans, 2.2 g ascorbic acid, 12.5 g casein, 2.5 ml vitamin solution (1.2% ascorbic acid, 0.03% calcium pantothenate, 0.015% niacin, 0.008% riboflavin, 0.004% Thiamin HCl, 0.004% pyridoxine HCl) in a total volume of 1.0 l poured into 50 ml plastic cups (5 ml per cup). After solidification, 150 μl of the resuspended spore/crystal powder was applied onto the diet surface in each cup and allowed to dry. Subsequently, ten 1-day-old second instar larvae of *A. gemmatalis* were added to each cup. The cups were covered with acrylic lids and incubated under the same conditions used for rearing the insects. Five cups for each isolate were used. After 48 h the surviving larvae were transferred to 50 ml cups containing rearing diet and the mortality was assessed. Larval mortality was assessed again at day 5 as described above (Silva et al., 2004). Dose dependent bioassays were performed in the same way, using final concentrations of dry spores and crystals in a range between 0.02 and 200 ng/cm^2.

2.2.3. Plutella xylostella

Plutella xylostella selective bioassays were conducted by a dip-assay procedure derived from that described by Tabashnik et al. (1990). Instead of using leaf disks 3 cm in diameter, the dip assay was conducted on young cabbage leaves of the same size. The leaves were dipped vertically for 5 min in resuspended spore/crystal powder. Leaves were then air dried vertically for 1 h at 25 °C then placed in a Petri dish with 10 s-instar larvae. Five repetitions were prepared. After 48 h the surviving larvae were transferred to a new leaf and the mortality was assessed. Larval mortality was assessed again at day 5 (Monnerat et al., 1999). Dose dependent bioassays were performed in the same way, using final concentrations of spores and crystals in a range between 0.020 and 200 μg/ml.

All bioassays were repeated three times. Mortality data were analyzed by Probit analyses (Finney, 1971) and the lethal concentration for 50% of larvae (LC_{50}) was determined.

2.3. DNA sample preparation and PCR

Bacillus thuringiensis isolates that killed 100% in selective bioassays after 5 days were characterized by PCR. They were grown on NYSM agar (Yousten, 1984) for 14–15 h, at 30 °C and DNA was extracted as described by Sambrook et al. (1989). Molecular characterization

through PCR was performed to identify the toxin-coding genes, by using a variety oligonucleotide pairs specific for the following genes/gene families: *cry1*, *cry2*, *cry3*, *cry4*, *cry5*, *cry7*, *cry8*, *cry9*, *cry10*, *cry11*, *cry12*, *cry13*, *cry14*, *cry17*, *cry19*, *cry21*, *cry24*, *cry25*, *cry27*, *cry29*, *cry30*, *cry32*, *cry39*, *cry40*, *cyt1* and *cyt2* (Ceron et al., 1995; Bravo et al., 1998; Ibarra et al., 2003).

2.4. SDS–PAGE (Polyacrylamide gel electrophoresis)

The protein analysis of the isolates that showed 100% mortality at day 5, was achieved through protein electrophoresis in polyacrylamide gels (SDS–PAGE 10%). The proteins were extracted from the culture according to Lecadet et al., 1991. The gel received 15 µl of each sample. Afterwards, the gel was stained and fixed in 40% methanol; 10% acetic acid and Coomassie blue (0.1%) for about 16 h, under slight shaking and it was destained in 40% methanol and 10% acetic acid for 2 h, with agitation.

2.5. Scanning electron microscopy

The crystalline inclusions of the most toxic isolates were prepared by centrifugation in discontinuous sucrose gradients (Silva et al., 2004). These preparations were washed and lyophilized before being deposited on a metallic support. The samples were covered with gold for 180 s, using a sputter coater (EMITECH model K550) and observed in a ZEISS model DSM 962 scanning microscope.

3. Results and discussion

Twenty seven isolates killed 100% of tested larvae of *S. frugiperda*, *A. gemmatalis* and *P. xylostella* after 5 days

Table 1
Toxicity of *B. thuringiensis* isolates against *S. frugiperda*, *A. gemmatalis* and *P. xylostella*

Estirpes	*S. frugiperda* LC_{50} (ng/cm^2)	*A. gemmatalis* LC_{50} (ng/cm^2)	*P. xylostella.* LC_{50} (µg/ml)
S0550	27 (20–33)	5.1 (4.0–8.1)	1.56 (0.41–5.57)
S0845	24 (10–46)	0.21 (0.1–0.3)	1.29 (0.39–5.03)
S1905	18 (03–43)	3.3 (0.9–7.0)	1.46 (0.51–3.94)
Btk[a]	285 (201–418)	13.7 (9.0–20.0)	2.82 (0.95–7.12)

LC_{50} values at day 5 are shown (with confidence limits at a level of 95%).
[a] Standard strain for lepidoteran toxicity.

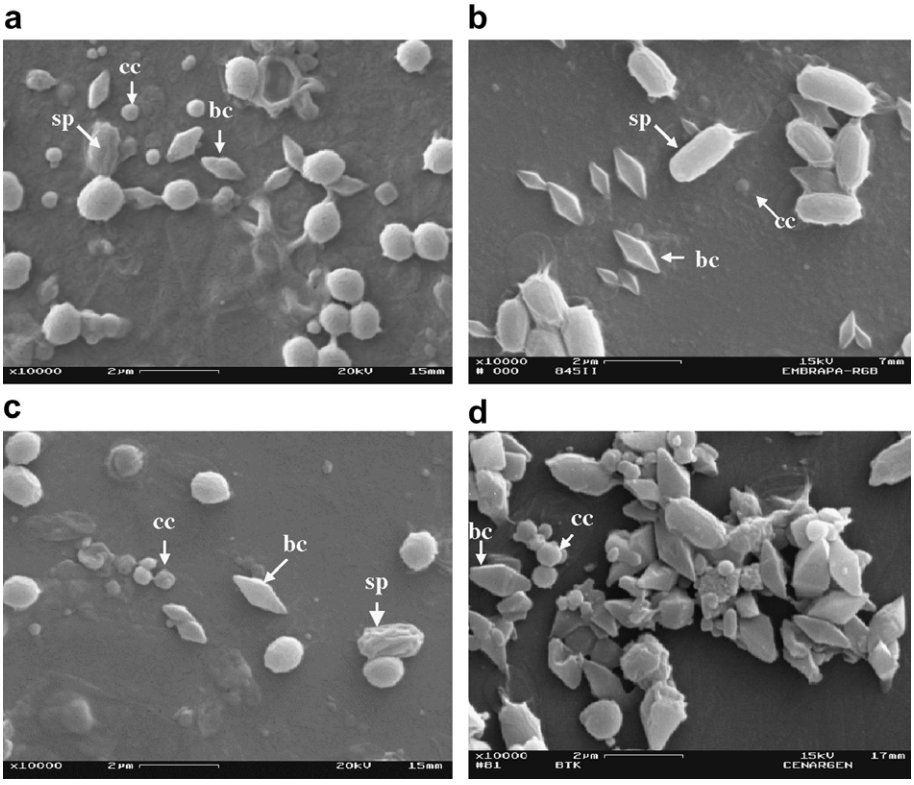

Fig. 1. Scanning electron microscopy of crystals and spores of *Bacillus thuringiensis* S0550 (a), S0845 (b), S1905 (c) and *B. thuringiensis* subsp. *kurstaki* HD-1 (d). bc, bipyramidal crystal; cc, cuboidal crystal; sp, spore.

in selective bioassays and were submitted to dose response bioassays. Of the isolates, 19 exhibited LC_{50} values that were higher than that of the standard strain Btk HD-1, against all the insect tested (95% confidence) (isolates S0093, S0102, S0166, S0392, S0764, S0811, S0844, S0906, S0907, S0908, S0997, S1533, S1537, S1538, S1539, S1540, S1548, S1549, S1876). Five isolates were no better than Btk but had similar LC_{50} values against at least one insect (S0234, S0711, S1269, S1551, S2003). However three isolates S0550, S0845 and S1905 exhibited a significantly greater potency than the standard strain against two of the three insects tested, with indications of somewhat lower LC_{50} against *P. xylostella* (Table 1) and were selected for further characterization. The isolates showed the lowest LC_{50} among all isolates. S0845 was the most toxic isolate against *A. gemmatalis* and *P. xylostella* resulting in LC_{50} values of 0.21 ng/cm^2 and 1.29 μg/ml, respectively, and S1905 was the most toxic against *S. frugiperda* (LC_{50} 18 ng/cm^2).

Like the standard strain Btk HD-1, all these three isolates produced bipyramidal crystals (Fig. 1) and major 130 and 65 kDa protein bands (Fig. 2). All three isolates produced PCR amplicons for *cry* genes (Table 2), with isolate S1905 producing the same profile as Btk, while in isolates S0550 and S0845, fewer genes were detected. The presence of at least one *cry1* gene and a *cry2* gene in each isolate is consistent with their production of the above proteins of 130 and 65 kDa, respectively. Cry1 toxins are associated with the production of bipyramidal crystals and with toxicity against lepidopteran insects including *S. frugiperda* (Bravo et al., 1998) while Cry2 produces cuboidal crystals that also show lepidopteran toxicity (Dankocsik et al., 1990; Wu et al., 1991; Höfte and Whiteley, 1989; Lereclus et al., 1989). Cuboidal crystals appear to be produced by isolates S0550, S1905 and S845 (Fig. 1). The presence of the *cry1* and *cry2* genes detected, may account for the toxicity of the three isolates against Lepidoptera. The greater potency of these isolates than Btk against the target insects may be due to: minor variations in the sequences of the Cry1 and/or Cry2 proteins; higher levels of accumulation of the toxins; the presence of other toxin genes that are not detected by the primers used in the screening; or a combination of these factors. The high-level toxicity of isolates S0550 and S0845 to the Noctuidae *S. frugiperda, A. gemmatalis* and *P. xylostella* compared to Btk, suggests that the *cry1Ac* and *cry1B* genes, present in Btk but not in S0550 or S0845, may contribute little to toxicity against these insects. Whatever the cause, the key property of these three isolates is that the products of their fermentation yield a more biologically active biomass than the standard strain Btk. This has obvious efficiency implications for the industrial production of cheap and effective bioinsecticides for use in Brazilian agriculture. for the control of important lepidopteran pests and their further development should be pursued.

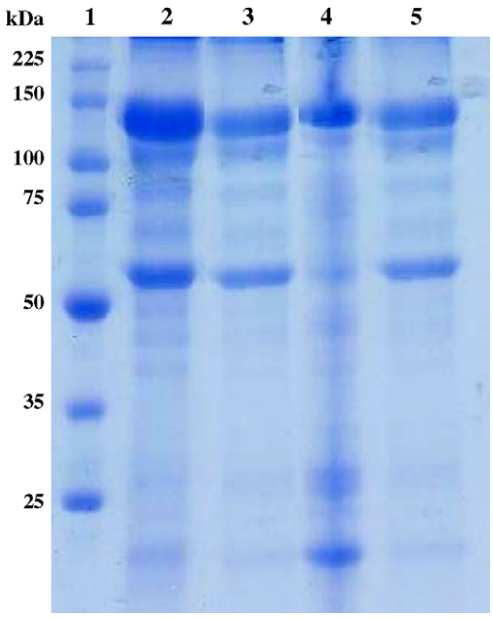

Fig. 2. SDS–PAGE of spore–crystal from *B. thuringiensis* isolates. 1, Molecular marker Gibco BRL; 2, Btk; 3, S0550; 4, S0845; 5, S1905.

Table 2
Molecular profile of the selected isolates of *B. thuringiensis*

Isolates	*cry* genes
S0550	*cry1Ab* and *cry2*
S0845	*cry1Aa, cry1B* and *cry2*
S1905	*cry1Aa, cry1Ab, cry1Ac, cry1B* and *cry2*
Btk	*Cry1Aa, cry1Ab, cry1Ac, cry1B* and *cry2*

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