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Bacillus thuringiensis: molecular characterization, ultrastructural and nematoxicity to *Meloidogyne* sp..

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ABSTRACT

Nematodes are important pests in agriculture, causing losses that reach $ 125 million annually. In addition to chemical control, biological control using strains of the bacterium *Bacillus thuringiensis* has been studied. The aim of this study was to evaluate the toxicity of new strain of *B. thuringiensis* to *Meloidogyne* sp., in laboratory and in greenhouse, as well as the content of *cry* genes and structural and ultrastructural analyzes. The MTox 1886-2 isolate was used, at different concentrations, to evaluate the toxicity to juveniles (J2) of *Meloidogyne* in laboratory, and to eggs and juveniles in greenhouse. Differential Interference Contrast Microscopy, Scanning Electron Microscopy and molecular characterization by PCR were performed. Laboratory results showed 96% of corrected mortality of nematodes treated with 1 x 10^{10}cells/mL, getting a LC_{50} of 2.6x10^{7}cells/mL. In greenhouse, MTox 1886-2 showed 36% reduction in the number of nematodes, compared to control, and presented better results in the weight of the aerial part and the roots of lettuce plants. Structural and ultrastructural analysis of the spore-crystal mixture of the strain revealed the presence of a bipyramidal crystal protein. The analysis of the content of *cry* genes by PCR amplification resulted in a fragment of approximately 1000 bp which was sequenced revealing a target gene of the *cry1D* subfamily. Thus, the prospects for this study is the use of this gene in the development of genetically modified plants, granting resistance to nematodes, or the use in new commercial formulations.

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INTRODUCTION

Losses in agricultural production worldwide due to nematodes have been reported long ago, showing a 12.3% drop in productivity (Freckman and Sasser, 1986; Oka *et al.*, 2010). Other estimates indicate that damage could exceed $ 125 billion annually (Chitwood, 2002; Oka *et al.*, 2010). In the United States, the incidence of nematodes generates an annual loss in soybean culture of $ 500 million (Acrissul, 2013), while in India the annual losses in 24 different crops in monetary terms has been worked out to the tune of $ 21 million (Rao, 2013). In Brazil the estimated loss is $ 400 million annually in different target cultures, such as soybean, bean, cotton and corn.

Among the major nematode pests, the genus *Meloidogyne* stands out as the most important in worldwide agriculture due to their adaptation to parasitism of almost all crops that produce food and fiber in the world (El-Hadad *et al.*, 2010). The species of this genus form galls on the roots of host plants, partially or completely inhibiting the absorption of water and nutrients, debilitating the plant development and its production (Abo-Hashem and Elyousr, 2011). The use of synthetic nematicides is one of the main methods used to minimize the damage caused by these nematodes. However, techniques such as fumigation are being withdrawn from the market due to its high toxicity, especially the use of methyl bromide (Li *et al.*,

2012). Furthermore, this method has high cost, low efficiency and is environmentally harmful, making it necessary to develop new technologies in controlling these pests. Among new techniques adopted, stands out the use of natural nematicides, the introduction of resistance into plants by transgenic and the biological control with the use of antagonist microorganisms (Radwan *et al.,* 2012).

The bacterium *Bacillus thuringiensis* is widely used to control various pests of agriculture worldwide, accounting for about 53% of the global market for biopesticides, generating annual revenues of $ 210 million (CAB International Centre, 2010). Their insecticidal activity is due to the production of proteinaceous inclusions in the form of crystals formed during the process of sporulation (Höfte and Whiteley, 1989; Crickmore *et al.,* 1998; Crickmore, 2005; Fiuza *et al.,* 2012). Genes that express these proteins are designated as *cry* genes due to crystalline phenotype and are usually located on plasmids with a large molecular weight (Schnepf *et al.,* 1998; Stopdan *et al.,* 2004; Walnut and Ibarra, 2010).

The cry proteins are solubilized in the gut of the target organism and activated proteolytically, generating a fragment with toxic activity capable of entering and forming pores in the membrane of the gut of the target pest (Aranda *et al.,* 1996; Vachon *et al.,* 2012). The most active forms of the cry proteins form a molecule with three globular domains that generally consists of 600 amino acids. The first domain is considered the most conserved and participates in the insertion in the cell membrane of the target organism and the pore formation. Domains II and III are related to receptor recognition and binding, which determine the specificity of the toxin (Maagd *et al.,* 2001; Schawartz *et al.,* 2001; Davolos *et al.,* 2009; Federici *et al.,* 2010). Due to the absence of toxicity to vertebrates, the toxins of *B. thuringiensis* have been safely used for the control of insects of various orders, such as: Lepidoptera, Coleoptera, Diptera, Homoptera, Orthoptera, Hymenoptera (Schnepf *et al.,* 1998; Wei *et al.,* 2003) and also other organisms such as mites, protozoa, flatworms and nematodes (Porcar *et al.,* 2003; Tian *et al.,* 2007; Jouzani *et al.,* 2008).

Although a few studies on the toxicity of cry proteins in nematodes have been reported in the literature, some authors have identified six cry proteins (Cry 5, Cry 6, Cry 12, Cry 13, Cry 14 and Cry 21) with toxicity to larvae of a large number of parasite and free-living nematodes (Wei *et al.,* 2003; Sato *et al.,* 2004; Jouzani *et al.,* 2008). Thus, the *cry* genes of these proteins potentially toxic to nematodes may be introduced into plants to create a resistance to these parasites (Sato *et al.,* 2004; Tabashnik *et al.,* 2011). In this context, the objective of this research was to test a new strain of *B. thuringiensis* against *Meloidogyne* sp. nematodes, evaluating the toxicity in laboratory and in greenhouse as well as the content of *cry* genes and structural and ultrastructural analyzes.

MATERIAL AND METHODS

Bacteria

The strain MTox 1886-2 used in this study was isolated from the soil of the South Coast Region of Rio Grande do Sul state, in the Laboratory of Microbiology and Toxicology, Unisinos (29º47’32” S, 51º09’07” W). The bacterial culture was performed in glucose usual medium for 48h at 30 °C and under 180 rpm agitation. The culture was centrifuged and the bacterial pellet diluted in water. The cell counts were performed in a Neubauer chamber in Phase-Contrast Microscopy (400x). A series of decreasing decimal concentrations was adjusted from 1 x 10^{10} cells/mL to 1 x 10^{6} cells/mL.

Microscopy

The culture of MTox 1886-2 was assessed in Differential Interference Contrast Microscopy (DICM) in the Laboratory of Microbiology and Toxicology of Unisinos using a Zeiss microscope and the Axio Vision software. The ultra structural analysis of *B. thuringiensis* was performed in a Scanning Electron Microscope (SEM)(JEOL JSM-7001F). Both preparations were performed with standard protocols of these laboratories.

Phytonematodes

The nematodes of the genus *Meloidogyne* were provided by the company "ICB BIOAGRITEC LTDA." The roots of lettuce plants, infected and containing galls, were crushed and the material was filtered. The nematode suspension was prepared at a

concentration of 2000 eggs and juveniles (J2)/mL, with Peters chamber and microscopy (400x). For the tests in laboratory, only nematodes in the juvenile stage were used.

Bioassay in Laboratory

Ten *Meloidogyne* juveniles were applied in Elisa plate, in triplicate, with 80µL of each bacterial suspension: 1 x 10^{10}, 1 x 10^{9}, 1 x 10^{8}, 1 x 10^{7} and 1 x 10^{6} cells/mL. In the control, the bacterial suspension was replaced with sterile distilled water. The experiment was maintained at 28°C, 75% Relative Humidity and Scotophase. The mortality analysis was performed 24 hours after treatment application. The corrected mortality (CM) was calculated using Abbott's formula and the Medium Lethal Concentration (LC_{50}) was measured in Probit Analyses (Haddad, 1998).

Bioassay in Greenhouse

The bioassay was established in a greenhouse using lettuce, *Lactuca sativa*, cultivar Vera, and sterile soil as substrate. In each treatment, 5mL of 1 x 10^{9} cells/mL of bacterial inoculate were applied to 5mL of suspension of nematodes (2000 eggs and J2/mL). Treatments were: (A): MTox 1886-2 at 1 x 10^{9} cells/mL with nematodes; (B): control with only nematodes; (C): control without Bt and without nematodes. Each treatment consisted of five replicates, and the evaluation was done 60 days after the application.

The evaluation was done comparing the fresh weight of the aerial part and the roots, and the number of galls per root system. In laboratory, the eggs and J2 were extracted from the plant roots by the Hussey and Barker (1973) technique and the counting was performed with Peters Chamber and optical microscopy (400x). For the determination of the nematode reproduction factor, eggs (mature and immature) and juveniles were quantified according to Miranda *et al.* (2012).

Extraction of Bacterial DNA

The extraction was performed according to the protocol "Max-prep" described in Delécluse *et al.* (1991). The initial culture was incubated overnight at 30°C under 180rpm. Then, the Optical Density (O.D.) was checked at 650nm by spectrophotometry, which was monitored until it reached a value between 0.95 and 1. Total DNA was obtained from the centrifugation of the bacterial culture, washing the pellet in lysis buffer (0.1 M TrisHCl, 0.1 M EDTA, 0.15 M NaCl), lysis buffer and lysozyme (40mg/mL), incubated at 37°C for 1 hour. RNase was then added at 10 mg/mL (50°C for 15 minutes), 20% SDS (20 minutes at 70°C) and 10mg/mL proteinase K at 55°C (overnight). 6M NaCl was added to the culture and centrifuged. The supernatant was precipitated with isopropanol, the DNA was collected, washed with 70% ethanol and resuspended in TE Buffer (10mM Tris HCl, 1mM EDTA pH 8.0).

DNA Amplification

For molecular characterization, two pairs of universal primers (degenerate) were used, capable of amplifying the active part of most of the *cry* genes currently known. These primers were designed by Nogueira and Ibarra (2010), based on 5 blocks originally preserved located by Höffe and Whiteley in 1989. The first pair of primers was designed to amplify sequence between the 1st and the 5th block, while the second primer amplifies sequences of blocks 2 to 5. Together, *in silico*, they have the ability to amplify 98% of known *cry* genes.

The reactions followed a protocol adapted from Nogueira and Ibarra (2010). PCR reactions were carried out separately for each primer pair. The final reaction volume was 25µL containing 1µL of DNA (10pmol), 0.4µL of each primer (20pmol), 2.5µL of 10x buffer, 5 mM of $MgCl_2$, 0.2 mMof each dNTP and 2.5U of Taq DNA polymerase (Ludwig Biotectnolgy). Amplifications were performed in Eppendorf thermocycler (PTC-100, MJ Research, Inc.) with the following conditions: 5 min denaturation at 94°C followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 2 min at 56°C, and polymerization at 72°C for 1 min and 30 seconds for each cycle. The amplification ended with an extended step at 72°C for 7 minutes. The negative control was prepared under the same conditions without the addition of DNA. The amplification results were analyzed on agarose gel at 0.7% stained with ethidium bromide,

photographed under UV and compared with the molecular weight marker (100pb, Sigma).

Alignments and Phylogenetic Analysis

The sequence of the amplified fragment obtained by PCR with the primer pair 1 was compared with the GenBank database using the BLAST online service (http://www.ncbi.nlm.nih.gov/BLAST) of NCBI for locating sequences with the highest similarity. The alignments of amino acid and DNA sequences were constructed using Clustal X2 software and then transferred to the software MEGA 5.1 to phylogenetic analysis using the Neighbor-Joining method (Crickmore *et al.*, 1998; Mailund *et al.*, 2006).

RESULTS

Microscopic Analysis

The DICM results revealed the presence of a bacterial spore and an intracellular protein crystal (Figure 1A). In the SEM analysis (Figure 1B), the MTox 1886-2 isolate revealed the presence of a bipyramidal crystal, characteristic of this species.

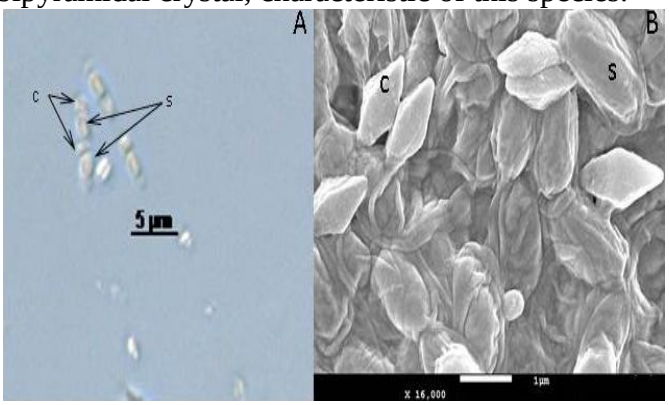

Figure 1. Cytological and ultrastructural characteristics of the strain *Bacillus thuringiensis* MTox 1886-2 in Differential Interference Contrast Microscopy (A) and Scanning Electron Microscopy (B). c = crystal, s = spore.

Bioassay in Laboratory

The average Corrected Mortality for the highest concentration, in the three replicates, was 96% which decreased to 26% at a concentration of 1 x 10^6cells/mL (Figure 2). These data were subjected to Probit Analysis Program Polo-PC (Haddad, 1998) to determine the Median Lethal Concentration, which was equivalent to 2.6 x 10^7cells/mL.

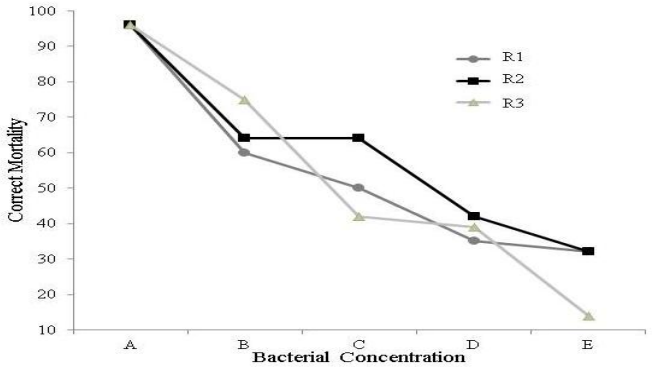

Figure 2. Corrected Mortality of *Meloidogyne*sp. treated with *Bacillus thuringiensis* MTox 1886-2. A= 1 x 10^{10}cells/mL; B= 1 x 10^9 cells/mL; C=1 x 10^8 cells/mL; D=1 x 10^7 cells/mL; E=1 x 10^6 cells/mL; R= replicates.

Toxicity in Greenhouse

Data from the toxic activity of MTox 1886-2 in greenhouse showed that there was a decrease in the number of nematodes, in accordance with the methodology by Coolen and D'Herde (1971), and in the reproduction factor (RF) of *Meloidogyne*, when plants were treated with *B. thuringiensis*. The weight of the aerial part and roots of plants also showed a statistical difference when compared to the control group (Table 1).

Table 1. Data from the toxicity bioassays of *Bacillus thuringiensis* (MTox 1886-2) against *Meloidogyne* sp., in lettuce, after 60 days of the treatment.

Treatment	Aerial Part Weight	Root Weight	Root System Galls	Mean J2/ Root (90g)	Reproduction Factor	Decrease J2 Over Control
MTox 1886-2	55,6[a]	18,00[a]	5[a]	459[a]	0,22[a]	36%
Control B	10,00[b]	1,86[b]	4,33[a]	1.271,63[b]	0,6358[b]	
Control C	40,66[a]	15,19[a]				

Same letters are not statistically different by the LSD test (P= 0,05). Dosage in cells/mL; weight in g; Decrease in %. Control B: only with nematodes, Control C: non-Bt and without nematodes

PCR Amplification

The PCR analysis of MTox 1886-2 using the 1st pair of primers showed no amplification of fragments. On the other hand, the 2nd amplification with primer pair showed a fragment, with approximately 1100 bp, in agarose gel (Figure 2). The PCR product obtained from MTox 1886-2 DNA was sequenced (Macrogen, Korea).

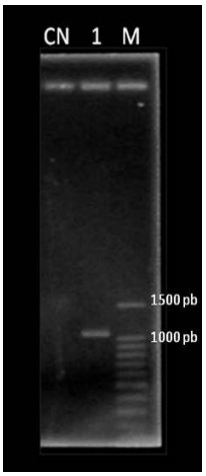

Figure 2. Analysis of the content of *cry* genes from MTox strain 1886-2. Agarose gel (0.7%) of PCR amplified products. (M) Molecular Marker 100bp (Promega®) the molecular mass of two fragments are indicated; (CN) Negative Control (water): (1) DNA from *Bacillus thuringiensis* MTox 1886-2 amplified with the second pair of primers.

Phylogenetic Analysis of MTox 1886 Strain

The results of the sequencing revealed a fragment with 990 base pairs which, compared to the GenBank database using the BLAST program, showed high identity with some *cry1D* gene sequences, and was called *cry1Dlike*. The sequence with highest similarity was then selected (*cry1Da*) for alignment of DNA sequences and their amino acid using the software Clustal X2. The alignment of the DNA sequences revealed a similarity of 98% and differed only in 14 of these regions. The alignment of amino acid sequences showed that the primers annealed the correct blocks, there by indicating which domains were amplified. The result, as compared with the locations of conserved blocks, described by Whiteley and Höffe in 1989 and Crickmore *et al.*,(1998), showed that the second and third domains of this protein were amplified according to the diagram below (Figure 4).

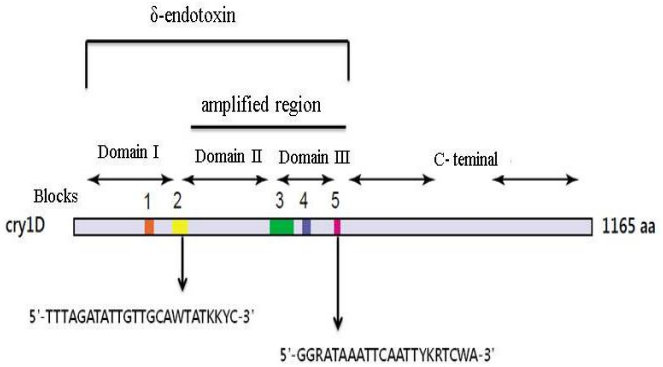

Figure 4. Diagram showing the structure of the protein encoded by the *cry1D* gene and the regions amplified by the second primer pair. The sets of areas forming the δ-endotoxin part, the location of 5 conserved blocks and the C-terminal part (only involved in the formation of the crystalline structure). Source: the autors based on: De Maagd *et al*. (2003), De Maagd *et al*., 2001, Crickmore *et al* (1998).

For the construction of the phylogeny by neighbor joining, amino acid sequences were selected, in the website of Neil Crickmore (www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/), from some known δ-endotoxins, active against different types of hosts, and then aligned with the amino acid sequence of *cry1Dlike* in the software Clustal X2.

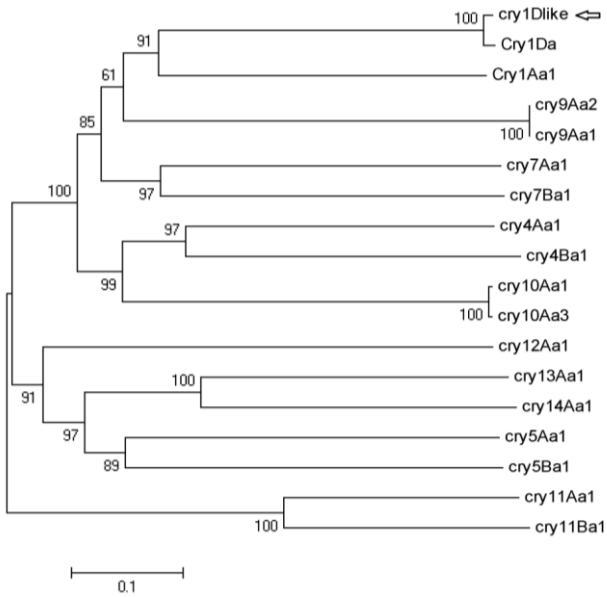

Figure 5. Phylogenetic analysis of MTox 1886-2 by the neighbor joining method in the MEGA 5.1 software after aligning the with known amino acid sequences selected with the *cry1Dlike* sequence using the software Clustal X2. The arrow indicates the position of the sequence

*cry1Dlike*of MTox 1886-2. The numbers of the branches indicate the amount of bootstrap based on 1000 replicates.

Then, these data were transferred to the program MEGA 5.1, where the neighbor joining analysis was performed using 1000 replicates. In the results of the phylogeny (Figure 5), it can be observed that, in most cases, each subfamily is located in a group with a high similarity value between their amino acid sequences. The sequence *cry1Dlike* grouped with *cry1Da* within *cry1* subfamily with a high value of bootstrap, indicating that it probably evolved from a common ancestor.

DISCUSSION

Data lethality of nematodes subjected to treatment with *B. thuringiensis* are scarce, especially when values of Median Lethal Concentration are determined in the laboratory. The authors Ashoub and Amara (2010) evaluated three isolates of *B. thuringiensis* against *M. incognita* and observed mortality of 90, 88 and 70% respectively, of juveniles after 24h treatment. In the present work, the juvenile mortality reached 96% after 24 hours of the treatment application with the MTox 1886-2 strain.

El-Moneim and Massoud (2009) incubated the purified proteins of four isolates of *B. thuringiensis* with 500 juvenile *M. incognita* for 30 minutes. The results showed that all treatments showed a reduction in infection caused by nematodes, compared to the control group. These data corroborate the results of this study, which showed a decrease in the number of nematodes and the reduction of harmful effects on plants treated with *B. thuringiensis* MTox 1886-2 in greenhouse relative to the control group, without the presence of the MTox 1886-2 strain. Additionally, the bacterial treatment reduced by 36% the infection caused by parasitic nematodes in plants of lettuce.

Isolates of *B. thuringiensis* were also used in the experiments of Joo *et al.* (2012), in greenhouse, significantly reducing the egg mass of *Meloidogyne*, besides obtaining a better result from the dry weight of the aerial part and the roots of the plants. Other studies also using isolates of *B. thuringiensis* showed good results in relation to plant growth and decreased infection caused by nematodes (Chen *et al.*, 2000; Dawar *et al.*, 2008; Khan *et al.*, 2010; Khan *et al.*, 2011; Khalil *et al.*, 2012).

Regarding the content of *cry* genes of MTox 1886-2, a DNA fragment which partially encodes a protein with 98% similarity to the protein Cry1Da was amplified. Hooss *et al.* (2008) analyzed the growth and reproduction of *Caenorhabditis elegans* using transgenic corn plants containing the *cry1Ab* gene and the activated Cry1Ab protein, in soil. The reproduction of the nematodes was reduced significantly when transgenic maize plants were used, compared with non-transgenic maize plants. The amount of *cry* genes identified as being toxic to nematodes is very small compared with the subfamily of*cry1* genes. A study with 95 isolates of *B. thuringiensis* by Ejiofor and Johnson (2002) showed a frequency of 62% of *cry1* genes, followed by 30% *cry2* genes and only 8% of other *cry* genes, demonstrating that the family of *cry1* genes is very abundant.

Therefore it can be concluded that this novel strain of *B. thuringiensis*, MTox 1886-2, obtained from soil samples from southern Brazil, has high toxicity to nematodes of the genus *Meloidogyne*, characterized by the presence of a *cry1Dlike* gene. This strain can serve as a source of *cry* genes for the construction of transgenic plants resistant to nematodes. It is also important to note that data from bioassays in greenhouse also showed high mortality of nematodes, whose biological material, gene or strain, may be indicated for future application in control of the studied target species.

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