Article

Isolated native *Bacillus thuringiensis* from southeastern Mexico

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Abstract

In order to find native isolates of *Bacillus thuringiensis*, in the present work isolations were made from different materials obtained from the southeast of Mexico, in the period from March 2014 to July 2015. Bacterial colonies were selected based on the typical characteristics of the *Bacillus* genus and were identified as *Bacillus thuringiensis*, based on the presence of parasporal crystals, *cry* gene, *hag* gene, protein profile and insecticidal activity. An isolate of soil samples (ITCBT34) and two larvae of *Chlosyne lacinia* (ITCBT61, ITCBT62) was obtained. These isolates presented amplification of the *cry* and *hag* genes. Isolate ITCBT34 presented crystals with oval morphology, while isolates ITCBT61 and ITCCBT62 presented dipyramidal crystals. Differences were observed in the size of the fragment produced by the amplification of the hag gene and in the protein profile of the different isolates. The insecticidal activity was determined by crude bioassay with a concentration of 10 μg cm^{-2}. The three isolates caused 100% mortality of *Manduca sexta* larvae at 96 h of exposure. Isolates ITCBT61 and ITCBT62 showed morphological characteristics and size of the hag gene similar to the reference strain *B. thuringiensis* svar. *kurstaki* HD1, the isolate ITCBT34 showed no similarity, so it could be an isolate not related to *serovar kurstaki* HD1.

Keywords: *Chlosyne lacinia*, *Manduca sexta*, gene *cry*, gene *hag*.

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Introduction

Bacillus thuringiensis is the most used entomopathogen in the biological control of agricultural pests due to its high insecticidal specificity, which is conferred by the ability to form parasporal inclusions of a protein nature, called Cry proteins (Schnepf *et al*., 1998). The nomenclature of Cry proteins is based on the amino acid sequence, this allows to classify the closely related toxins (Crickmore *et al*., 1998), Cry proteins have insecticidal activity against insect larvae of the orders Lepidoptera, Diptera and Coleoptera (Burges, 1982) and in some cases also against hemiptera (Torres-Quintero *et al*., 2015), hymenoptera (van Frankenhuyzen and Tonon, 2013), mites (Erban *et al*., 2009) and nematodes of agricultural importance (Li *et al*., 2008).

There are reports of *B. thuringiensis* isolates from diseased or dead insect larvae due to pathogenesis, phylloplane or internal parts of plants (Monnerat *et al*., 2009), in this case it has the property of using *B. Thuringiensis* against insects difficult to control for their feeding habits, such as suckers or borers. Therefore, there is an interest in the search for new isolates, with the aim of finding strains that show novel insecticidal activity or with greater activity than isolates and strains of known references (Saadaoui *et al*., 2010). *B. thuringiensis* isolated from soil were significantly more toxic against *Pieris brassicae*, *Ephestia kuehniella* (Senfi *et al*., 2012), *Heliothis armigera* and *Plutella xylostella* (Xavier *et al*., 2007), than the reference strains.

Strains of *B. thuringiensis* native to Mexico showed high toxicity towards *Spodoptera frugiperda* when compared with the commercial standard strain HD1 (Vázquez-Ramírez *et al*., 2015). Strain LBIT-1200, isolated from soil showed greater toxicity activity against *Manduca sexta* and *Trichoplusia ni* compared to strain HD-73 (Reinoso-Pozo *et al*., 2016). The characterization of isolates is important to determine if they are known varieties or to determine new strains of *B. thuringiensis* with their own characteristics. In this regard, several reports present characterization of *B. thuringiensis* isolates that include the morphology of the parasporal body (Azizoglu *et al*., 2011), insecticidal activity (Alper *et al*., 2014) and molecular characterization (Sauka *et al*., 2010; Patel *et al*., 2012).

Recently, the sequencing of the *hag* genes that encode the proteins responsible for the formation of flagellin, which is an alternative to the serotyping technique and that allows the relationship between serotypes of *B. thuringiensis* (Reinoso-Pozo *et al*., 2016). Based on previous studies, the objective of this work was to isolate native strains of *B. thuringiensis* from different types of samples, which have potential to be used in the biological control of agricultural pests of economic importance in the southeast region of Mexico.

Materials and methods

Isolation of *B. thuringiensis*

Samples obtained from soil, larvae of *Spodoptera frugiperda* J.E.Smith (Lepidoptera: Noctuidae), larvae of *Chlosyne lacinia* ssp. *lacinia* Geyer (Lepidoptera: Nymphalidae), adults of *Aidemona azteca* Saussure (Orthoptera: Acrididae), leaves of *Nerium oleander* L. and

stubble of *Zea mays* L. These materials were obtained from different regions of the Mexican Southeast the soil samples were collected eight in the state of Tabasco, eight in Yucatan, one in Campeche and one in Quintana Roo. Regarding the other materials were obtained, 23 samples of larvae of *C. lacinia*, two samples of adults of *A. azteca* and four samples of leaves of *N. oleander* in the municipality of Conkal, six samples of larvae of *S. frugiperda* and five samples of corn stubble in the municipality of Tekax, both in the state of Yucatán (Table 1). For the isolation of bacteria from soil, 5 g of the sample were weighed and placed in a 15 mL Falcon tube, which was calibrated with sterile distilled water until said volume and the mixture was resuspended by vigorous vortexing for 1 min. It was pasteurized in a water bath at 80 °C for 15 min and cooled immediately on ice.

Table 1. Description of the type and number of samples collected, Municipality and State, climate and soil characteristics.

Samples (number)	Collection place	Climate characteristics*	Type of soil*
Soil (2)	Huimanguillo, Tabasco	Warm humid with rain all year	Plintosol
Soil (1)	Jalapa, Tabasco	Warm humid with rain all year	Leptocol
Soil (1)	Teapa, Tabasco	Warm humid with rain all year	Luvisol
Soil (1)	Cunduacán, Tabasco	Warm humid with rains in summer	Phaeosem
Soil (1)	Cárdenas, Tabasco	Warm humid with rains in summer	Vertisol
Soil (1)	Paraíso, Tabasco	Warm humid with rains in summer	Gleysol
Soil (1)	Centro, Tabasco	Warm humid with rains in summer	Solonchak
Soil (1)	Baca, Yucatán	Warm subhumid with rain in summer	Phaeosem
Soil (2)	Conkal, Yucatán	Warm subhumid with rain in summer	Histosol
Soil (1)	Dzidzantun, Yucatán	Very warm and warm semi-dry	Histosol
Soil (1)	Acanceh, Yucatán	Warm subhumid with rain in summer	Histosol
Soil (1)	Chabihau, Yucatán	Very warm and warm semi-dry	Lixisol
Soil (1)	Tekax, Yucatán	Warm subhumid with rain in summer	Solonchak
Soil (1)	DB, Yucatán	Very warm and warm semi-dry	Lixisol
Soil (1)	BJ, Quintana Roo	Warm subhumid with rain in summer	Leptosol
Soil (1)	Calakmul, Campeche	Warm subhumid with rain in summer	Phaeosem
Corn stubble (5)	Tekax, Yucatán	Warm subhumid with rain in summer	Solonchak
N. oleander (4)	Conkal, Yucatán	Warm subhumid with rain in summer	Histosol
S. frugiperda (10)	Tekax, Yucatán	Warm subhumid with rain in summer	Solonchak
C. lacinia (23)	Conkal, Yucatán	Warm subhumid with rain in summer	Histosol
A. azteca (2)	Conkal, Yucatán	Warm subhumid with rain in summer	Histosol

*= INEGI (2017a, b, c, d); DB= Dzilam de Bravo; BJ= Benito Juárez.

The 5 mL of the suspension was taken and added to a flask containing 50 mL of nutrient broth (BD Bioxon®) and 0.25 M sodium acetate (Fermont) (to inhibit the germination of *B. thuringiensis* spores), incubated for 4 h at 200 rpm and at 29 °C. Then 1 mL of the culture was taken and diluted in 1 mL of sterile distilled water. This sample was again subjected to the

pasteurization process, which is recommended to inactivate the growth of other microorganisms in the culture medium (Travers *et al*., 1987). At the end of the second pasteurization process, an aliquot of 100 µL was taken and evenly distributed in a Petri dish with nutritive agar. The Petri dishes were incubated at 30 °C for 24 h to favor the germination of *B. thuringiensis* spores and the development of bacterial colonies.

For the bacterial isolation from larvae and adult insects, 3 to 5 individuals were processed per sample depending on the size of these, which were macerated directly in the tube with the help of a glass rod and then hardened with sterile distilled water until 15 mL, vigorously shaken (Aldebis *et al*., 1994). In the case of isolates from plant leaves, 2 g of the sample was weighed, disinfected in 2% sodium hypochlorite solution, for 10 min and double washing was applied with distilled water, the sample was finely macerated in a mortar and 15 mL of sterile distilled water were added, the solution was transferred to a 15 mL Falcon tube (Monnerat *et al*., 2009). In both cases, a pasteurization process was carried out and the described process was continued to obtain isolates from soil samples.

Individual colonies were selected according to the typical characteristics reported for *Bacillus* (Sneath, 1986). Subsequently tests were performed to verify positive Gram stain and positive catalase reaction. Selected isolates were individually cultured on nutrient agar (Bioxon®) and stored at 4 °C for further studies.

Morphological characterization of the parasporal body

The presence and parasporal morphology of the selected isolates was carried out by direct observation of the crystals under DM500 optical microscope (Leica Microsystems, Switzerland) (1000X), morphology was confirmed by direct smear in phase contrast and staining with Coomassie blue (Sharif and Alaeddinoğlu, 1988).

Extraction of genomic DNA

Total DNA extraction was performed according to the modified method reported by Rosso and Delécluse (1997). A bacterial culture of 2 mL was obtained in Luria-Bertani® medium (Invitrogen) at 28 °C with constant agitation at 250 rpm overnight, centrifuged at 8 000 rpm for 5 min, the supernatant and the cell pack were removed it was resuspended in 500 µL of buffer J (1.0 M TrisHCl, 0.1 M EDTA and 0.15 M NaCl, pH 8). Again, it was centrifuged under the same conditions as above the cell pack was resuspended in 300 µL of buffer J containing 40 mg mL of lysozyme (Sigma Aldrich Quimica) and incubated at 37 °C for 1 h. Subsequently, 20 µL of 10% SDS was added, it was homogenized gently and incubated at 70 °C for 20 min.

The 5 µL of RNAse (Promega) (10 mg mL), 10 µL of proteinase K (10 mg mL) (Thermo Fisher Scientific) were added and incubated at 60 °C for 90 min. 50 µL of 5 M NaCl (Baker, J. T.) was added and incubated on ice while stirring constantly. It was centrifuged at 13 000 rpm for 20 min, the supernatant was recovered, precipitated with the same volume of isopropanol (Sigma) and incubated at -70 °C for 30 min. Subsequently, it was centrifuged under the above conditions and the supernatant was removed, the pellet was washed with 200 µL of 70% ethanol (Fermont) and allowed to dry at room temperature, the DNA was resuspended in 20 µL of sterile distilled water and preserved at -20 °C until use.

Amplification of *cry* and *hag* genes

To determine the presence of *cry* genes in the isolates, PCR amplification was performed with the universal primers of block 1 (5' TATGCWCAAGCWGCCAATYTWCATYT3') and block 5 (5' GGRATAAATTCAATTYKRTCWA 3') (Sigma Aldrich Química) according to Noguera and Ibarra (2010).

The presence of the *hag* gene of flagellin in the native isolates was determined by PCR with the specific primers Bthag-F1 (5'-AGTACATGCGCCAAAACCAAG) and Bthag-R1 (5'-GTTTGCTTGAGAAAGCATGCT) (Sigma Aldrich Chemistry) according to Xu and Cote (2006). The PCR products were verified by 0.8% agarose gel electrophoresis (Invitrogen) and visualized in a Biorad Molecular Imager Gel Doc XR photodocument (Bio-Rad®).

Protein profile

The protein profile was obtained by SDS-PAGE electrophoresis of the spore-crystal complex of the isolates according to the reported methodology (Laemmli 1970). For this, each isolate was inoculated in 5 mL of LB medium and incubated in an orbital shaker at 30 °C with 200 rpm for 72 h. The spore-crystal complex was centrifuged at 10 000 rpm for 10 min and washed with sterile distilled water, this operation was repeated three times. The tablet was recovered and stored at 4 °C. A mixture was prepared with 5 µL of loading buffer with 5% β-mercaptoethanol (Sigma Aldrich Química), 3 µL of the spore-crystal complex sample and 2 µL of water, incubated at 95 °C for 10 min.

For the electrophoresis a 12% separating gel and the 4% polyacrylamide compactor (Sigma Aldrich Química) were prepared, the components used were 30% polyacrylamide, Tris-HCL buffer pH 8.8 (Sigma Aldrich Química), Tris-HCL buffer pH 6.8, 10% SDS, sterile distilled water, TEMED (Invitrogen®) and 10% ammonium persulphate (Sigma Aldrich Química), a volume of 5 mL was prepared for each gel and the concentrations were calculated according to the instructions in the manufacturer's manual. Electrophoresis was performed in a modular vertical electrophoresis chamber Enduro™ (Bio-Rad), using the Page Ruler™ Plus Prestained Protein Ladder molecular weight marker (Bio-Rad®).

The electrophoresis was carried out in two phases, that of compaction at 40 V and 100 A for 1 h and the separation phase was carried out at 90 V and 100 A for 3 h. Subsequently the gel was stained with a solution of Coomassie blue dye G-250 0.1% for 1 h and stained in methanol/acetic acid. The molecular weight of the protein bands present in each sample was estimated according to the molecular weight marker used. The protein profiles observed were compared with the profile of the reference strain *B. thuringiensis* subsp. *kurstaki* HD1.

Detection of insecticide activity

A preliminary test was carried out at a high concentration with the isolates obtained to determine the insecticidal activity against *Manduca sexta*, since several studies report low lethal doses for several lepidopteran insects ranging from 1 to 164 ng cm^{-2} (Uribe *et al*., 2003; Sharma *et al*., 2010). The lyophilisate of the spore-crystal complex was obtained in a Labconco Lyph-Lock 4.5 lyophilizer (Labconco®), a final concentration of 10 µg cm^{-2} was used. The sample was weighed

and diluted in 200 µL of Tween 80 (Sigma Aldrich Chemistry) (0.02%), added to the Petri dish with the artificial diet (Yamamoto, 1969) for the insect and distributed evenly over the entire surface of the diet, the surface was allowed to dry for 45 min and 20 neonatal larvae were placed for each isolate with two repetitions and the mortality was recorded at 96 h, as a negative control, 0.02% Tween 80 was used.

Results and discussion

Isolation of *B. thuringiensis*

Sixty-two samples of various collected materials were processed, including 18 soil samples, 10 of *Spodoptera frigiperda*, 23 of *Chlosyne lacinia* ssp. *lacinia*, two of *Aidemona azteca*, four of *Nerium oleander* leaves and five samples of stubble of *Zea mays*. A total of 210 colonies were selected, of which only 27 were observed in the presence of structures in the sporangia, among which were observed spherical, amorphous, bipyramidal, cubic, ovoid and some bodies adhered to the spores. The Coomassie blue staining test (Figure 1) confirmed the presence of parasporal crystals in only three isolates, which were designated with the ITCBT34, ITCBT61 and ITCBT62 keys (Table 2). These selected isolates were used in subsequent tests.

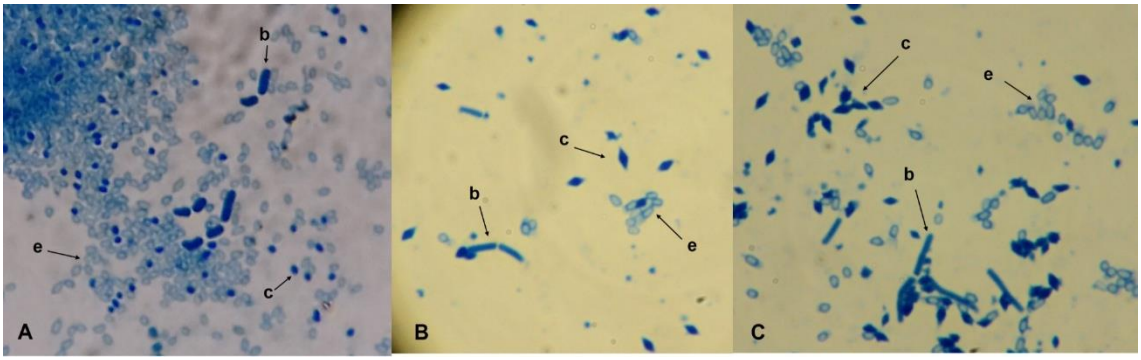

Figure 1. Morphology of the parasporal body by staining with Coomassie blue. A= ITCBT34. B= ITCBT61. C= ITCBT62; b) vegetative cell; c) parasporal crystal; and e) spore.

Previous studies have reported obtaining native *B. thuringiensis* isolates from habitats such as soil (Adbullah *et al*., 2014), plants (Alper *et al*., 2014) and insects (Alquisira-Ramírez *et al*., 2014). In this diversity of environments, it is possible to find isolates that have dipyramidal, spherical, cubic crystals (Cicero *et al*., 2009, Azizoglu *et al*., 2011) and irregular ones (Assaeedi *et al*., 2011). In relation to southeastern Mexico, there are reports of *B. thuringiensis* isolates only from soils in the states of Tabasco (Bravo *et al*., 1998) and Yucatán (Ornelas-Pérez *et al*., 2016).

In the present work the selected isolates that were confirmed as *B. thuringiensis* presented oval crystals, sample obtained from soil, and dipyramidal samples obtained from larvae of *Chlosyne lacinia* (Table 2). The isolation index of *B. thuringiensis* obtained was 0.11. This result is within the values previously reported, since it is possible to find reports with indexes of 0.04 to 1 (Rosas-García *et al*., 2008; Alper *et al*., 2014).

Table 2. Characteristics of the native isolates of *Bacillus thuringiensis*.

Isolated	Habitat	Collection site	Location of the site (GPS)	Parasporal morphology	Genes *cry*	Genes *hag*
ITCBT34	Soil	Huimanguillo, Tabasco	17°53'26.31" 93°26'32.48"	Ovoid	+	+
ITCBT61	*Chlosyne lacinia* ssp. *lacinia*	Conkal, Yucatán	21°4'45.85" 89°29'59.46"	Dipyramidal	+	+
ITCBT62	*Chlosyne lacinia* ssp. *lacinia*	Conkal Yucatán	21°4'16.55" 89°30'24.16"	Dipyramidal	+	+

Cry gene amplification

The three isolates showed the presence of cry genes, presented amplification bands with approximate sizes of 1400 bp (ITCBT61), 1500 bp (ITCBT62), 1650 bp (ITCBT34) and 1300 bp in the reference strain (HD1) (Figure 2a). The use of universal primers is common to determine the presence of *cry* genes (Patel *et al*., 2012; Reinoso-Pozo *et al*., 2016). According to the dipyramidal morphology of the parasporal crystals of the ITCBT61 and ITCBT62 isolates, it is likely that the amplification bands are correlated with cry1 and cry2 genes, which encode toxic proteins mainly towards Lepidoptera larvae (Sun *et al*., 2007; Sauka and Benintende, 2008). On the other hand, the fragment amplified in the ITCBT34 isolate shows a different band size (Figure 2a), so it could be another type of *cry* gene.

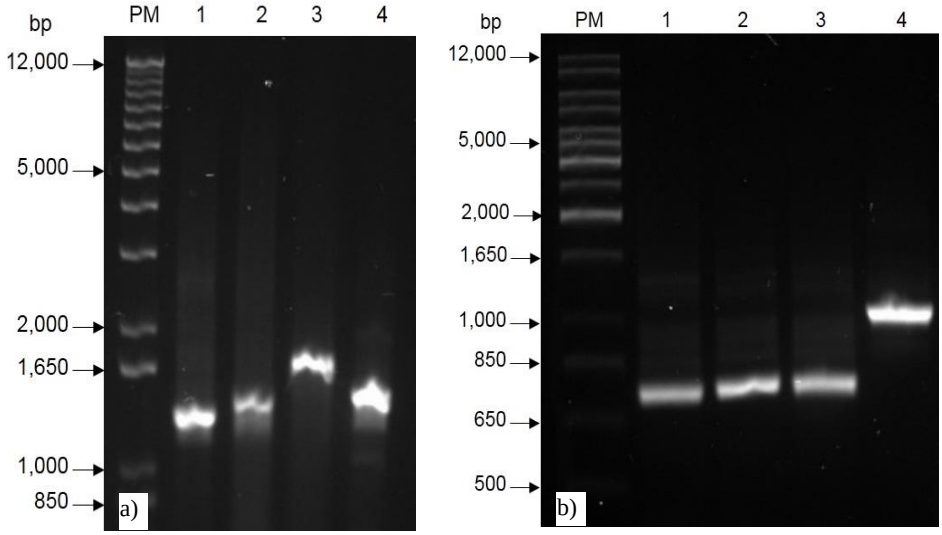

Figure 2. Amplification of *cry* (a) genes and *hag* (b) genes in the isolates of *B. thuringiensis*. PM: molecular marker 1 Kb Plus DNA Ladder (Invitrogen, USA), Lane 1: HD1, Lane 2: ITCBT61, Lane 3: ITCBT62, Lane 4: ITCBT34.

Determination of *hag* genes

The three isolates presented *hag* gene amplification products by PCR (Figure 2b). Isolates ITCBT61 and ITCBT62 presented a band of approximately 700 bp in size, similar to the band that showed the reference strain HD1. The isolate ITCBT34 presented a band of different size of approximately 1000 bp. The amplified fragments of the *hag* genes reported in *B. thuringiensis* show polymorphism with bands with sizes from 700 bp up to 1.9 kb (Xu and Cote, 2006). The fragment size of the *hag* gene of the isolate ITCBT34 reinforces that it is an isolate different from the other two (ITCBT61 and ITCBT62) and to the reference strain HD1. The determination of the presence of flagellin genes is a method that is gaining use as an alternative to serotyping in *B. thuringiensis* (Hendriksen and Hansen, 2006; Reinoso-Pozo *et al*., 2016).

Protein profile

By means of the SDS-PAGE analysis, the protein profile of the three isolates under study was determined. The profiles observed were different among the isolates and only the isolate ITCBT61 coincided with the profile of the reference strain HD1. In the case of the isolate ITCBT62, the presence of a band of 45 kDa was observed and the absence of a band of 130 kDa that is present in the isolate ITCBT61 and the strain HD1, the ITCBT34 presented a profile of bands different from 100, 85, 70 and 50 kDa (Figure 3).

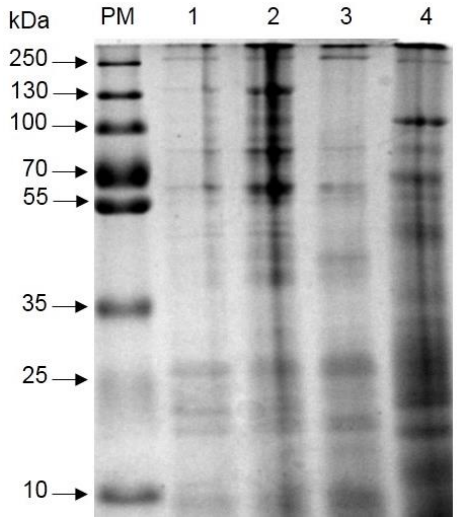

Figure 3. SDS-PAGE of the protein profile presented by the native isolates of *B. thuringiensis*. PM= Molecular weight marker PageRuler ™ Plus Prestained Protein Ladder (Thermo Scientific), lane 1: HD1, lane 2: ITCBT61, lane 3: ITCBT62, lane 4 = ITCBT34.

We report mainly bands of 130 and 65 kDa for the strain HD1, which are product of proteins encoded by the *cry1* and *cry2* genes (Alper *et al*., 2014), so that the bands of 130 and 65 kDa of molecular weight that presented the native isolates, they can belong to the group of proteins Cry1 and Cry2. The protein profile that has been observed in other works varies, but the molecular weight range of the bands is 130 to 44 kDa (Silva *et al*., 2010; Barathi *et al*., 2012).

Previous studies have reported the presence of proteins of sizes similar to those obtained in the present work, which showed toxicity mainly against pests of the order Lepidoptera (Valicente *et al*., 2010; Li and Bouwer, 2012).

Insecticidal activity

The test of insecticidal activity was carried out with a concentration of 10 µg cm^{-2}, the three isolates showed insecticidal activity against *Manduca sexta* larvae. At 96 h, 100% mortality of larvae was observed (Table 3). The larvae exposed to the isolates showed symptoms of general necrotic and internal liquefied, characteristics typical of the toxicity caused by the Cry proteins of *B. thuringiensis* (Silva *et al*., 2010) (Figure 4).

Table 3. Qualitative bioassay of insecticidal activity of the native isolates of *Bacillus thuringiensis* against *Manduca sexta* at 96 h, with a concentration of 10 µg cm^{-2}.

Isolated	Average*	Mortality (%)
ITCBT34	20	100 ±0
ITCBT61	20	100 ±0
ITCBT62	20	100 ±0
Witness**	20	0 ±0

*= average number of dead larvae of two replicates, with 20 larvae each; **= Tween 80 (0.02%).

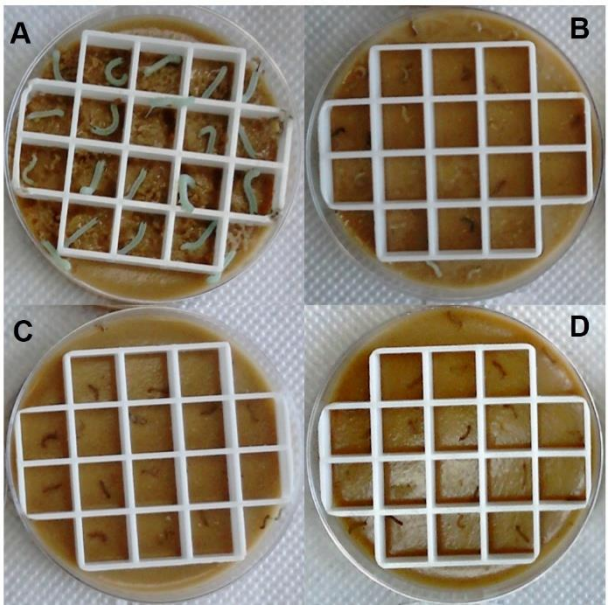

Figure 4. Qualitative test of native isolates of *B. thuringiensis* against newborn larvae of *Manduca sexta* at a concentration of 10 µg cm^{-2}. A) negative control (Tween 80), B)= ITCBT34, C)= ITCBT61 and D)= ITCBT62.

The activity observed in the ITCBT61 and ITCBT62 isolates responds to expectations, since both isolates have a dipyramidal parasporal morphology and protein profile typical of the Cry1 and Cry2 proteins, which have insecticidal activity against Lepidoptera larvae. Isolate ITCBT34 also had activity towards these larvae. This result is not common, since this isolate presented parasporal body morphology and protein profile very different from the type of proteins that act on Lepidoptera larvae. This activity may be related to the presence of *cry2* genes, which were reported in *B. thuringiensis* isolates with only oval morphology crystals (Arrieta and Espinoza, 2006).

The presence of *cry1* and *cry2* genes and activity against Lepidoptera larvae is characteristic of *B. thuringiensis*, and is frequently reported in studies on the search and characterization of native isolates. Were used in these works, several species of Lepidoptera such as *Cadra cautella* and *Thaumetopoea wilkinsoni* (Yilmaz *et al*., 2013), *Spodoptera littoralis* (Assaeedi *et al*., 2011), *Heliothis armígera* and *Plutella xylostella* (Xavier *et al*., 2007) and even against Coleoptera larvae *Carpophilus hemipterus* (Alper *et al*., 2014).

Conclusions

In general, similarity was observed in the tests between the native isolates of the present work and the reference strain *B. thuringiensis* svar. *kurstaki* HD1, as dipyramidal morphology and size in the amplification of the *hag* genes in the ITCBT61 and ITCBT62 isolates, in addition to the protein profile and amplification of *cry* genes in the ITCBT61 isolate. This shows that isolates ITCBT61 and ITCBT62 are highly related to strain *B. thuringiensis* svar. *kurstaki* HD1. Regarding the isolate ITCBT34, in the results of all the tests carried out, no similarity was observed with the HD1 strain, indicating that this isolate belongs to a different variety. Finally, the result of the toxicity test shows that the three isolates have potential for the control of pests of the order Lepidoptera.

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