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ORIGINAL ARTICLE

Larvicidal activity of *Bacillus thuringiensis* Colombian native strains against *Bemisia tabaci* (Hemiptera: Aleyrodidae)

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Abstract

The whitefly, *Bemisia tabaci,* an insect of the order Hemiptera which attacks more than 600 species of plants, is one of the most important agricultural pests around the world. The insecticidal Cry proteins from *Bacillus thuringiensis* (Bt) are useful biological pesticides, and some are toxic to Hemipteran insects. In this study, Colombian native isolates of Bt were functionally characterized at molecular and biological levels. The strains contained between one and five different crystal shapes: round, triangular, amorphous, bipyramidal and squared. The strains presented between three to seven bands of proteins in their electrophoretic pattern that were organized into six groups according to their possible biological activity on insect pests. *Cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1B and *cry*1C genes were identified for PCR in the different Bt isolates. Bioassays were performed on tomato leaves whose surface was spread with 3 μg · ml^{-1} crude extract of Bt toxins. Second instar larvae of whitefly, which were placed on top of leaves and exposed to the toxins for 7 days, exhibited mortalities from 18 to 69%. The lethal concentration 50 (LC_{50}) of ZBUJTL39, *Bt kurstaki* HD1 and ZCUJTL9 strains were 1.83, 1.85 and 2.16 μg · ml^{-1}, respectively ($p < 0.05$). These results show that the native Bt strain ZBUJTL39, which contained the genes *cry*1Aa, *cry*1Ab, *cry*Ca and *cry*Ba could eventually be used for the development of an integrated management program together with other tools for the control of *B. tabaci*.

Key words: biological control, genes *cry*, insect pest, molecular characterization

Introduction

The whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae) is one of the most important agricultural pests around the world. This insect easily adapts to new environments and different geographical regions, using more than 600 species of plants as hosts (Oliveira *et al.* 2003).

In tomato crops, *B. tabaci* is a potential vector of more than 200 viruses (Vázquez *et al.* 2007), mainly from the *Begomovirus* genus, family Geminiviridae (Lapidot and Friedmann 2002). The nymphs of *B. tabaci* cause damage by sap extraction, produce foliage fall and slow down the ripening of fruits (ICA 2004). In addition, they excrete a sweet substance (sooty mold) ideal for the development of microorganisms (Bargen *et al.* 1998; Leroy *et al.* 2011).

Control of *B. tabaci* is carried out with chemical pesticides, a practice which has generated resistance (Oliveira *et al.* 2003; Vassiliou *et al.* 2011; Basit *et al.* 2013), environmental contamination, toxicity to beneficial insects and farmers, and loss of efficacy (Hernández 2016; Hernández and López 2011). For these reasons, biological control is an alternative against the whitefly. *Bacillus thuringiensis* (Bt) is the most promising bacteria for the production of bio-pesticides and insect-resistant plants, associated with environmental preservation (Van Frankenhuyzen 2009; Sansinenea

2012). Nowadays, there is no commercial formulation based on Bt for controlling *B. tabaci* (Salazar-Magallon *et al.* 2015).

Bt is an entomopathogenic spore-forming bacterium which synthesizes insecticide proteins called δ-endotoxins (Crickmore *et al.* 1998). Currently there is a great number of diverse insecticidal proteins. More than 500 proteins, 78 families and 778 holotypes are known (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/; May 2019). Their broad genetic diversity has been the basis of many programs focused on the isolation and characterization of strains that present novel biological activities (Van Frankenhuyzen 2009). Its importance lies in its toxicity against larvae of insect pests of the orders: Lepidoptera, Coleoptera, Diptera, Homoptera, Himenoptera, Ortoptera and Malofaga, and in other organisms such as nematodes, mites, protozoa and flatworms (Höfte and Whiteley 1989; Feitelson *et al.* 1992; Bravo *et al.* 1992, 1998; García-Robles *et al.* 2001; Xu *et al.* 2004).

Schnepf *et al.* (1998) noticed that pesticides based on Bt and transgenic crops containing *cry* genes of Bt for the control of Hemipteran insects are limited. However, Bt toxins (Cyt1Aa, Cry1Ac and others) have been evaluated on Hemipteran species such as aphids (*Acyrthosiphom pisum*) (Porcar *et al.* 2009; Li *et al.* 2011; Chougule *et al.* 2013) and bedbugs (*Lygus herperus* y *L. lineolaris*) (Baum *et al.* 2012). For unknown reasons the toxic effect has been low to moderate (Shao *et al.* 2013).

Davidson *et al.* (1996) evaluated the Bt strain on silver whitefly adults (*Bemisia tabaci*). Their study showed that Cry proteins had no effect on *B. argentifolii* due to the fact that this insect has no digestive proteases which are needed for the cleavage and activation of these proteins. Nevertheless, Salazar-Magallon *et al.* (2015) evaluated the toxicity of Bt which has produced three industrial by-products, at a concentration of 40 mg · ml^{-1} on whitefly through bioassays under controlled greenhouse conditions. They registered mortality rates of up to 90% against third and fourth instars of the insect. González (2011) evaluated Bt at a concentration of 1 × 10^{8} conidia · g^{-1} CFU achieving control of up to 80% of the population of *B. tabaci*. Al-Shayji and Shaheen (2008) isolated strains of Bt from Kuwaiti soil, which encoded effective toxins against nymphs of whitefly in a concentration of 500 μg · ml^{-1}, with mortality rates of up to 68.2%. El-Assal *et al.* (2013) isolated strains of Bt from Egyptian soil, which proved to be effective against second instar whitefly nymphs in tomato crops.

Based on these previous results, the objective of this study was to characterize ten native Bt isolates from Colombian tomato-producing soils to determine their insecticidal activity on tomato whitefly.

Materials and Methods

Obtaining samples of Bt and bacterial isolation

Ten native strains of Bt were selected from the Bank of Native Strains at Jorge Tadeo Lozano University and functionally characterized at molecular and biological levels (Hernández *et al.* 2011). These strains were isolated from the soil of five tomato-producing Colombian locations: 1 – Susa (3 strains), Department of Cundinamarca; 2 – Ráquira (2), 3 – Santa Sofia (1), 4 – Villa de Leyva (3) and 5 – Sutamarchán (1), Department of Boyacá. Each soil sample (20 g) was collected by scraping surface soil to a depth of 5–10 cm, using a sterile spatula, and then stored at room temperature in a labeled sterile plastic bag. Bt strains were recovered according to a method described by Travers *et al.* (1987).

As positive controls for the standardization of the methods, reference strains Bt var. *kurstaki* HD1 and Bt var. *aizawai* HD137 were obtained from the commercial products Dipel® and Xentari® and used in the different evaluations (Valent Bioscience Corporation, USA).

Crystal microscopic characterization

The strains were incubated on Luria Bertani (LB) agar for 7 days at 30°C until at least 90% of the cells had lysed. Two-milliliter samples of lysed cultures were washed by centrifugation twice in cold sterile water and resuspended once in 2 ml of 0.1 M NaCl. To evaluate the presence and shape of spores and crystal inclusions, a phase-contrast microscope was used (Nikon Eclipse Ciat 100X magnification) and then photographed with a Nikon DS-L3 camera. Crystal inclusions of each isolate were classified according to the following types: amorphous, bi-pyramidal, square, spherical and triangular (Uribe *et al.* 2003; Seifinejad *et al.* 2008).

Biochemical characterization

An electrophoresis profile of total protein in crude extracts was determined using the method described by Laemmli (1970). To characterize the diversity of Bt proteins, native isolates and reference strains were incubated in a modified LB culture medium (Triptone, 10 g · l^{-1} NaCl, 10 g · l^{-1}, yeast extract 5 g · l^{-1}, 4 g · l^{-1} ammonium sulfate 4 g · l^{-1}) (Pitre *et al.* 2008) at 30°C for 7 days. All the cell biomass of native isolates was utilized for analysis of total proteins for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Hernández *et al.* (2011). The entire cell biomass was resuspended in sterile water and washed three times; 4 μl of 100 mM of phenylmethanesulfonyl

fluoride (PMSF) and 5 ml of sterile water were added. The suspension was then vortexed for 10 s and centrifuged at 10,000 rpm for 10 min (EBA 21, Hettich Zentrifugen, Germany). Centrifugation was repeated three times, washing only with sterile water to eliminate possible residues of the culture medium. Then, 2.36 µl of $CaCO_3$ 100 mM, 60 µl of dithiothreitol (DTT) 10 mM and 4 µl of phenylmethanesulfonyl fluoride (PMSF) 100 mM were added and the samples were shaken at 200 rpm at room temperature overnight. Finally, the supernatant was placed in microtubes of 1.5-ml and centrifuged at 14,000 rpm for 15 min at 4°C, transferred to another microtube and kept at 4°C for subsequent analysis. The crude extracts were sonicated using a Sonics Vibra Cell, 750 W sonicator probe (Newtown, CT, USA) at 36% amplitude with 12 pulses for 120 s. Aliquots of these samples were incubated in boiling water for 10 min and separated in SDS-PAGE at 10% (Laemmli 1970). Gels were stained with Comassie brilliant blue R250 (0.1%) for 40 min.

Cry genes identification by PCR y M-PCR

Plasmid DNA was obtained by alkaline lysis (Sambrook and Rusell 2001). The DNAs were analyzed on a 1% agarose gel, visualized and photo-analyzed with UVP Gel-Doc-IT TM imaging system (UVP LSC, USA).

For the amplification of *cry*1 family genes by PCR, a pair of general, previously reported primers were used (Bravo *et al.* 1998). It was possible to recognize 25 different *cry*1 genes in one single reaction using the general primer: forward 5'CTGGATTTACAGGTGGGGATAT3' and reverse 5'TGAGTCGCTTCGCATATTTGACT3'. Subsequently, positive strains for the presence of *cry*1 genes were subjected to two rounds of multiplex PCR (M-PCR) from two mixes of three pairs of primers each, recognizing then three specific genes in each PCR reaction. The primers used in the M-PCR had been designed previously (Cerón *et al.* 1994). The first mixture (A) contained primers which detect specific genes *cry*1Aa, *cry*1Ab and *cry*1Ac, and for the second mixture (B), the primers detect genes *cry*1B, *cry*1C and *cry*1D. DNAs Bt var. *kurstaki* HD1, containing specific genes *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1D, *cry*2Aa, *cry*2Ab and *cry*9D (Schünemann *et al.* 2014). Bt var. *aizawai* HD137, containing genes *cry*1Aa, *cry*1Ba, *cry*1Ca and *cry*1Da (Hernández *et al.* 2011), were used as reference strains. Reaction conditions corresponded to procedures reported by Bravo *et al.* (1998) and Cerón *et al.* (1994, 1995).

Obtaining crude extracts and quantification of total proteins

In order to obtain crude extracts of spores and crystals, bacilli of ten native isolates were cultivated in LB medium with stirring at 340 rpm (Lab – Line® Orbit Environ – shaker, Lab – line Instruments, Melrose Park, IL, USA) and incubated at 28°C for 7 days.

All cell biomass was resuspended in distilled deionized water in 15 ml falcon tubes with an addition of 4 ml of $NaHCO_3$ according to previous studies (Bravo and Cerón 1998). The quantification of total protein in crude extracts was carried out using the methodology described by Bradford (1976).

Bioassays

Whitefly breeding was carried out in the Laboratory of Plant Physiology at the Juan de Castellanos University (FUJC located in the city of Tunja, Boyacá, Colombia) under controlled greenhouse conditions. Tomato seedlings (*Solanum lycopersicum* L.) of the Milano Tropic variety, 10 cm in height, were used. The plants were grown at 27 ± 13°C with 40–90% relative humidity. To establish the reproduction of *B. tabaci*, adult individuals were collected in a tomato crop located in Nobsa (Boyacá, Colombia) (5°46'50, 75 "N and 72°59'17, 25" W). Reproduction was done in cages (80 × 60 × 60 cm) covered with Swiss veil. A pot with a tomato plant and approximately 10 adult individuals were placed in a cage. Once the seedlings were infested, the different stages of larval development were monitored daily, using a binocular stereomicroscope (Motic SMZ-168, Hong Kong, China). The phases were differentiated according to larval size: larva of 1st instar (L1: 0.26 mm length, 0.15 mm width), which were the smallest, 2nd instar (L2: 0.38 mm, 0.24 mm), 3rd instar (L3: 0.44 mm, 0.35 mm) and 4th instar (L4: 0.86 mm, 0.63 mm), which were the largest (Hill 1969; González and Gallardo 1999). Since the first instar larvae have functional legs and are able to actively crawl on the leaves, while the other instars are sessile (Hill 1969), larvae were chosen for the bioassay as soon as they stopped moving at the start of the second instar. Second instar larvae have a translucent, oval appearance, with wavy and sessile edges (Espinel *et al.* 2008). Second instar nymphs were chosen for the bioassay according to Espinel *et al.* (2008) who found that this instar is the most susceptible. When applying a biopreparation of the fungus *Isaria fumosorosea*, this instar presented the highest larval mortality (94.6%), which was significantly greater than that obtained in the other larval instars of *B. tabaci*.

A completely random design with five repetitions was used for the experiments. In each bioassay, the absolute control (leaves and nymphs), a positive control (leaves treated with the reference strain – HD1), and treatments with sporulated native bacilli were included.

To identify the sporulated native bacilli with the highest pathogenic potential, bioassays were performed using jars with leaflets technique (Ramírez *et al.* 2010).

For this process, phenologically similar tomato leaves (leaves composed of 7 folioles), 10 cm long, were used. They were washed with water and dried with absorbent paper. Then, each leaf was infested with nine second instar nymphs (*B. tabaci*), which were carefully detached using a brush of marten hair and placed individually on the leaflets. Although nymphs are very susceptible to manipulation, in this study mortality due to manipulation was less than 12%. Discriminative bioassays (screening) were performed from an initial concentration of 3 μg · ml^{-1}. The application of the crude extract of spores and crystals was carried out by aspersion, using a 25 ml atomizer. Two ml of the dilution was applied on the upper and the undersides of the leaves, at a distance of 15 cm, allowing it to dry at room temperature.

The mortality of nymphs was evaluated every 24 h for 8 days using a binocular stereomicroscope (Motic SMZ-168, Hong Kong, China) and the percentage of mortality was corrected both in the controls and in the treatments with the formula of Henderson and Tilton (1955). An ANOVA and a Tukey comparison test to compare treatments were performed, and a correlation analysis was made between the variables (crystal forms, *cry* genes and biocontrol activity).

The LC_{50} was determined for two native strains of Bt in dose-response experiments, which had the highest mortality in the preliminary assessment. There were five serial dilutions containing crude extract of the two native strains (1, 3, 5, 7 and 9 μg · ml^{-1}). Mortality was registered as described above and PROBIT regression analysis was performed using Biostat Program (2009).

Results

Isolation and microscopic characterization of *Bacillus*

The ten native strains of Bt presented colonies characteristic of the genus *Bacillus* sp., with circular morphology, irregular edges and cream color. Microscopic characterization of the native strains corresponded to five different crystal shapes: round, triangular, amorphous, bipyramidal and squared. Strains showed between one and four different crystal shapes (Fig. 1, Table 1), with a round shape being the most frequent (8 strains).

Parasporal crystal proteins

The parasporal crystal proteins from the ten native Bt strains revealed protein bands that ranged from 15–120 kDa. The strains presented from three to seven bands of proteins in their electrophoretic pattern (Table 1). The native strains were organized into six groups (I to VI) (Quesada-Moraga *et al.* 2004), according to the weight of the observed proteins and their possible biological activity on insect pests (Table 2) (Barloy *et al.* 1996; Zhang *et al.* 1997; Van Frankenhuyzen 2009). The ten strains evidenced a higher frequency among groups II, III, IV, V, and VI, which would have possible biological activity against insects of the orders Lepidoptera, Coleoptera, Diptera, Hymenoptera, Hemiptera and organisms such as nematodes and mites.

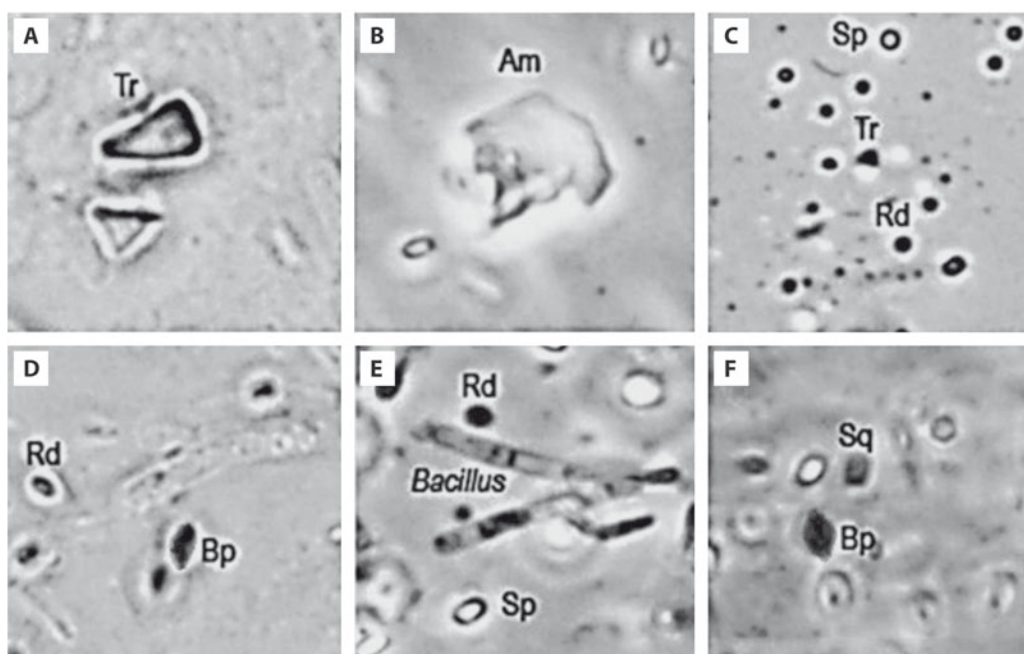

Fig. 1. Different crystal shapes observed in native Bt strains evaluated in this study. Strains with one crystal shape are displayed (A, B) two shapes (C, D, E, F). Determined with phase-contrast microscope (100×). Sp – spores; Am – amorphous; Bp – bi-pyramidal; Rd – round; Sq – square; Tr – triangular round

Table 1. General characteristics and toxicity of 10 native *Bacillus thuringiensis* strains selected for toxicity against the second instar of *Bemisia tabaci*

Colombian locations	Strain	Crystal shape	Proteins profile [kDa]	Specific *cry*1 gene profile	Mortality* [3 μg · ml^{-1}]
Susa	zcujtl9	Am, Rd	120, 110, 100, 80, 60, 40, 30	aa, ab, ac, ba	68,767 a
	zcujtl11	Bp, Rd	100, 80, 65, 40, 30, 20	aa, ab, ac, ba, ca	62,283 a
	zcujtl14	Rd, Bp, Am	100, 80, 65, 40, 25	aa, ab, ba, ca	54,774 ba
Ráquira	zbujtl21	Tr	100, 70, 50, 25	aa	54,09b a
	zbujtl23	Rd, Am	100, 50, 25	aa, ab, ac, ba, ca	64,139 a
Santa sofía	zbujtl24	Rd, Bp, Am	100, 80, 60, 40, 15	aa	18,755 dc
Villa de leyva	zbujtl33	Bp, Rd	80, 40, 30, 15	aa	54,09 ba
	zbujtl34	Tr, Rd, Am	120, 85, 80 ,15	aa	27,802 bdc
	zbujtl35	Bp, Rd	80, 70, 65, 50, 30	ab	49,646 bac
Sutamarchán	zbujtl39	Tr, Am, Bp	80, 70, 65, 30	aa, ab, ba, ca	66,886 a
Reference strain	btk hd1	Am, Bp	180, 216, 246	aa, ab, ac	49,853 bac

*Tukey hypothesis test, mortalities (dose: 3 μg · ml^{-1}). Data with the same letter are not different statistically, $\alpha \leq 0.05$; $df = 12$; $F = 9.08$; $p = 0.00$)
Sp – spores; Am – amorphous; Bp – bi-pyramidal; Rd – round; sq – square; TR – triangular round

Amplification of genes *cry*1 in Bt

The presence of *cry*1 genes was identified in all ten native Bt strains in the first reaction using general primers. The M-PCR amplification allowed for the identification of six *cry*1 specific genes in the second reaction. The strains had between one and five *cry*1 genes. The most common gene was *cry*1Aa present in nine of the strains. Five of the strains showed a single gene, three presented four genes, and two of the strains showed five genes (*cry1*Aa, *cry1*Ab, *cry1*Ac, *cry1*Ba, *cry1*Ca) corresponding to the strains ZCUJTL11 and ZBUJTL23 (Table 1).

Insecticidal activity of the native Bt strains

Evaluation of crude extracts of the crystal-spore complex from the ten strains of Bt produced a rise in mortality of second-instar whitefly nymphs as days after the application (DAA) increased. Greater mortality after 7 DAA was recorded in the native strain ZCUJTL9 (69%), followed by the native strain ZBUJTL39 (67%). Strain ZBUJTL24 produced lower mortality (18%) (Table 1). The Anova and Tukey tests did not detect statistically significant differences ($p < 0.05$) between mortalities of second-instar whitefly nymphs produced by the two native Bt strains and the reference HD1 strain.

Strains that had the greatest mortality (ZCUJTL9 and ZBUJTL39) were chosen to determine the LC_{50} with HD1 as the reference strain. There is a linear correlation between dose and response determined by the mortality of instars ($p < 0.05$). The native strain ZBUJTL39 at 1.83 μg · ml^{-1} concentration generated a reduction of 50% of the whitefly population, followed by the HD1 reference strain (1.85 μg · ml^{-1}) and ZCUJTL9 (2.16 μg · ml^{-1}).

Corrected mortality increased proportionally, according to the concentration of the doses used in the bioassay. Evaluation with 9 μg · ml^{-1} of crude extract of strain ZCUJTL9 obtained a mortality of 73%. With the same dose, the crude extract of strain ZBUJTL39 presented 76% of mortality and HD1 reference strain, 71%. Anova and Tukey tests showed no statistically significant differences ($p < 0.05$) between the corrected mortality obtained with doses of 5, 7 and 9 μg · ml^{-1} in the three strains analyzed (Fig. 2).

Discussion

Ten native Bt strains isolated from soil samples of tomato crops in open field and greenhouse in five municipalities belonging to two regions in Colombia were characterized. This study is of great importance and relevance since it identified two native strains of Bt which represent an alternative for the control of insect pest *B. tabaci*. This study represents the first characterization of native Bt strains diversity from Colombia, in terms of crystal shape, protein profile, *cry*1 gene content and toxicity against *B. tabaci*.

The ten native strains of Bt contained between one and four different crystal shapes showing a high diversity. The most frequently observed shapes were: round (eight strains), bipyramidal (six strains) and amorphous (five strains). The morphology, size and number of crystals vary between different strains of Bt (López-Meza and Ibarra 1996; Lonc *et al.* 2001). The

Table 2. The relationship between molecular protein weights revealed in each native Bt strain characterized and groups of possible biological activity

Group	No. native Bt strains	Ranges protein weight [kDa]	Possible biological activity	References
I	24, 33, 34	0–20	Protein without nonprotein associated cry or cyt	
II	9, 11, 14, 21, 23, 33, 35, 39	25–30	Cyt, Lepidoptera, Diptera	Gough *et al.* 2005
III	9, 11, 14, 21, 23, 24, 33, 35	35–50	Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Nematodes, Acari	Barloy *et al.* 1996; Zhang *et al.* 1997; Van Frankenhuyzen 2009
IV	9, 11, 14, 21, 23, 24, 39	60–75	Lepidoptera, Diptera, Coleoptera, Hemiptera	Zhang *et al.* 1997; Arango *et al.* 2002; Van Frankenhuyzen 2009
V	9, 11, 14, 24, 33, 34, 35, 39	80–85	Lepidoptera, Diptera	Arango *et al.* 2002
VI	9, 11, 14, 21, 23, 24, 34	100–150	Lepidoptera, Coleoptera, Diptera	Höfte and Whiteley 1989; Barloy *et al.* 1996; Zhang *et al.* 1997

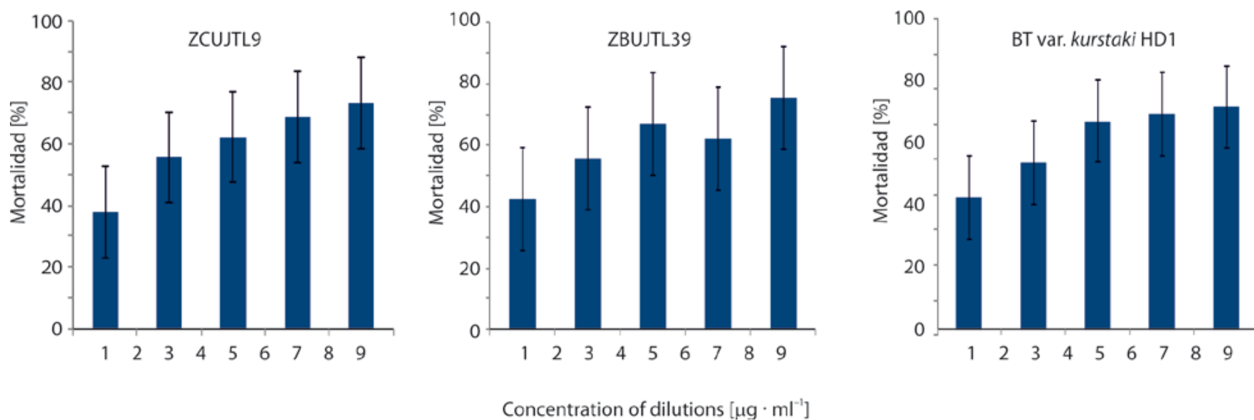

Fig. 2. Mortality rates obtained in the evaluation of dose-response of native Bt strain ZCUJTL9, ZBUJTL39 and reference strain Bt HD1 against second instar of *Bemisia tabaci* larvae. Serial dilutions: 1 μg · ml^{-1}, 3 μg · ml^{-1}, 5 μg · ml^{-1}, 7 μg · m^{-1}, 9 μg · ml^{-1}

Hemipteran-order crystals have been associated with bipyramidal, round, rectangular, spherical and uneven shapes (Asokan *et al.* 2014). However, the ZBUJTL21 strain had only triangular crystals, which have not been reported to be effective against this order of insects. Furthermore, this strain caused a mortality rate of a little more than 50% against *B. tabacci* (Table 1). Thus, it seems that the crystal shape does not determine biological activity.

According to the electrophoretic protein profile, all the native Bt strains of this study (except ZBUJTL34) showed possible biological activity against Hemipteran insects since different protein bands were identified. For instance, proteins belonging to Group III, with molecular weight bands between 35–50 kDa, were associated with proteins Cry1, Cry3, Cry6, Cry34, Cry35 and Cry1A, and Group IV, with molecular weight bands between 60–75 kDa, were associated with proteins Cry2 and Cry3 (Barloy *et al.* 1996; Zhang *et al.* 1997; Arango *et al.* 2002; Van Frankenhuyzen 2009).

The *cry*1 genes of Bt strains are frequently found in nature (Ramalakshmi and Udayasuriyan 2010). The insecticidal activity of these genes is highly limited against the order Hemipteran (Schnept *et al.* 1998).

In this study, *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1B and *cry*1C genes were identified in the 10 evaluated native strains, all with toxic activity against *B. tabaci*. Asokan *et al.* (2014) suggest that strains expressing different *Cry* proteins can be toxic against Hemipteran insects, e.g. *cry*1Ab genes have shown activity against this order. Gene *cry*1Ab was identified in the native strains ZBUJTL9, ZBUJTL11, ZBUJTL14, ZBUJTL23, ZBUJTL35 and ZBUJTL39 in the present study, indicating biological potential for the control of Hemipteran insects.

Toxins from Bt, such as Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Cry3Aa, Cry4Aa, Cry11Aa, Cry51Aa, Cyt1Aa and Cyt2Aa, have been evaluated against Hemipteran insects, specifically aphids (*Acyrthosiphom pisum* and *Macrosiphum euphorbiae*) (Walters and English 1995;

Sims 1997; Porcar *et al.* 2009; Li *et al.* 2011; Chougule *et al.* 2013), bedbugs (*L. ygusherperus* and *L. lineolaris*) (Baum *et al.* 2012), presenting low to moderate toxicity. However, it has been shown that toxin Cry1Ac can be activated by proteases of the bowel in the pea aphid (Li *et al.* 2011). According to Van Frankenhuyzen (2013), qualitative data indicates a similar toxicity level against Hemipteran insects, produced by the Cry1Ab and Cry1Ac toxins, with doses of 100–500 μg · ml^{-1}, and mortalities between 10 and 90%. Porcar *et al.* (2009) demonstrated a 25% mortality against the aphid, *Acyrthosiphon pisum*, produced by the Cry1Ab toxin at 500 μg · ml^{-1} of solubilized protein. In this study the genes *cry*1Ab and *cry*1Ac were identified in the strains ZCUJTL9, ZCUJTL11, ZBUJTL23 and even the HD1 (Dipel®), with possible biological activity against whitefly.

The two most toxic strains produced 69 and 67% of mortality (ZBUJTL9 and ZBUJTL39, respectively) at a concentration of 3 μg · ml^{-1}, thereby having a higher mortality percentage than reference strain *Bt* var. *kurstaki* (HD1). The LC_{50} for the strain ZBUJTL39 was 1.83 μg · ml^{-1} against second-instar whitefly nymphs, which was similar to that obtained with HD1 and ZCUJTL9 strains (2.16 to 1.85 μg · ml^{-1} respectively). Currently, there are no studies about whitefly mortality produced by Bt strains that register the LC_{50}. However, there are two reports of Bt biological activity against whitefly where bioassays have been carried out with a single dose. Al-Shayji and Shaheen (2008) obtained 68% of mortality against whitefly nymphs (at a concentration of 500 μg · ml^{-1}), and Salazar-Magallon *et al.* (2015) reported mortality rates of up to 90% against the same insect pest to 40 mg · ml^{-1}.

Bt was evaluated for the biological control of *B. tabaci* at a total protein concentration of 40 μg · ml^{-1} on *B. tabaci* nymphs in a strawberry crop under laboratory and greenhouse conditions. Bt had mortalities below 70% under laboratory conditions and under 50% under greenhouse conditions (Somoza-Vargas *et al.* 2018). In their study, 3rd and 4th instar nymphs were used while in our study we used 2nd instar nymphs. Apparently the susceptibility is much higher in this larval stage (Espinel *et al.* 2008) than in 3rd and 4th instar nymphs.

The results of microscopic, biochemical, molecular, and biological characterizations in many cases are related and can be complementary. As indicated above, the shape of the crystals shows biodiversity as does the number of toxins that a strain has, which is very important for the identification of more toxic strains (Ohbo and Aizawa 1986). In the present study it was observed that the strains with greater toxicity on the whitefly (mortality greater than 50%) had between two and four crystal shapes. The strain ZBUJTL39 showed three crystal forms and had one of the greatest toxicities. It seems that the number of crystals in a strain is related to the number of Cry proteins that it expresses and the toxicity on insects, in this case on whitefly. When the electrophoretic profiles of proteins were compared with the results of PCR amplification of cry genes, it was found that there was a correlation between them. Apparently, the protein bands with weights of 80–100 kDa are related with the presence of the *cry*1Aa genes in 100% of the cases (eight strains) and the *cry*1Ab genes in more than 60% of the strains that presented this protein weight. Additionally, four strains that produced mortalities above 60% also amplified the *cry*1Ba gene, and three of them also amplified the *cry*1Ca gene. As it can be seen, the information obtained from the microscopic, biochemical, molecular, and biological characterizations of Bt strains is complementary, thus making it possible to obtain an integral profile on the characteristics of each isolate, which is of great importance for its classification. Apparently, Bt strains that produce protein bands with weights of 80–100 kDa and the presence of genes *cry*1Aa and *cry*1Ab are related to toxicities greater than 50% on the whitefly.

ZBUJTL39 and ZCUJTL9 Bt strains showed evidence of insecticidal activity against second instar whitefly nymphs of tomato crops, representing an alternative for integrated management of this insect pest.

It is necessary to carry out further research to find strains that are more effective against *B. tabacci,* contributing to the biological control of this pest.

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