Characterization of *Bacillus thuringiensis* (Bacillaceae) strains pathogenic to *Myzus persicae* (Hemiptera: Aphididae)

*Mary Carmen Torres-Quintero[1], Iván Arenas-Sosa[1], Víctor Manuel Hernández-Velázquez[1], Ramón Suárez-Rodríguez[1], and Guadalupe Peña-Chora[2,]**

Abstract

Forty strains of *Bacillus thuringiensis* Berliner (Baciliales: Bacillaceae) that were isolated from corpses of Hemiptera were assessed against *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), an aphid that is considered one of the most destructive pests affecting the agricultural economy. Seventeen strains were significantly different from the control according to the LSD test (α = 0.05). They caused mortality rates ranging from 64.4 to 88.9% at 10 ng/µL total protein concentration, and from 71.1 to 91.1% at 100 ng/µL total protein concentration. The virulence (LC50) of these 17 strains was calculated, and 5 strains showed the highest virulence (GP777 = 10.63 ng/µL, HD1 = 9.10 ng/µL, GP528 = 7.86 ng/µL, GP402 = 7.12 ng/µL, and GP300 = 6.88 ng/µL). These strains have the potential to be used as an alternative to control *M. persicae* under field and covered conditions.

Key Words: in vitro; virulence; total protein; microbial control

Resumen

Cuarenta cepas de *Bacillus thuringiensis* Berliner (Baciliales: Bacillaceae), las cuales fueron aisladas de cadáveres de hemípteros fueron evaluadas contra *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), una especie de áfido que es considerada como una de las plagas más destructivas que afecta la economía de la agricultura. Todas las cepas fueron patógenas, sin embargo, 17 cepas mostraron diferencia significativa de acuerdo a la prueba de LSD (α = 0.05), las cuales mostraron un rango de mortalidad entre un 64.40 a 88.87 % con 10 ng/µL y entre un 71.10 a 91.10% con 100 ng/µL de concentración de proteína total. La virulencia Concentración Letal 50 de éstas 17 cepas fue determinada y sólo cuatro cepas produjeron la mayor virulencia (GP777= 10.63 ng/µL, HD1 = 9.10 ng/µL, GP528= 7.86 ng/µL, GP402= 7.12 ng/µL, GP300= 6.88 ng/µL). Estas cepas tienen el potencial para ser empleadas como una alternativa para el control de esta plaga bajo condiciones de campo y de cubierta.

Palabras Clave*: in vitro*; virulencia; proteina total; control microbiano

Aphids (Hemiptera: Aphididae) belong to the phytophagous group, and several species constitute worldwide major pests because they are polyphagous and negatively impact several economically important crops. Therefore, these insects are considered the most destructive pests affecting the agricultural economy (Pascal et al. 2010). *Myzus persicae* (Sulcer) is one polyphagous species of major economic importance because this aphid can transmit more than 100 viruses, causing a number of diseases including yellow leaf curl, beet mosaic virus, cucurbit aphid-borne yellow virus, plum pox virus, and tobacco mosaic virus (Blackman & Eastop 1984; Manachini et al. 2007). Current management of this pest unfortunately relies exclusively on the application of chemical insecticides, which leads to the development of resistance (Chougule & Bonning 2012), subsequent increase in the number of requisite applications, and the use of active ingredients that cause harmful effects to non-target insects, humans, and the environment (Foster et al. 2000; Lacey et al. 2001). Therefore, alternative techniques for control should be implemented.

One of these techniques could be the use of the entomopathogenic bacterium *Bacillus thuringiensis* Berliner (Baciliales: Bacillaceae). This bacterium produces one or various crystalline inclusions that are composed of insecticidal crystal proteins (ICPs) or δ-endotoxins. These ICPs have highly specific activity against several orders of insects, increasing their attractiveness for their use as a biological control agent (Aronson 1993, 2000). The ICPs are toxic to the Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, and Mallophaga species, as well as other organisms, such as nematodes, mites, and protozoa (Van Frankenhuyzen 2009). The use of these proteins has shown great efficacy against lepidopteran, coleopteran and dipteran pests; however, ICPs have not been successful in controlling Hemiptera (Schnepf et al. 1998).

Several reports have demonstrated low levels of toxicity to aphids at high concentrations of toxins from *B. thuringiensis.* Porcar et al. (2009) found low to moderate toxicity of Cry3A, Cry4Aa, and Cry11Aa to the pea aphid *Acyrthosiphon pisum* Harris. Walters & English (1995)

[1]Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos. Avenida Universidad 1001 Colonia Chamilpa, Cuernavaca, Morelos. México. CP 62209; E-mail: mark_tq@hotmail.com (M. C. T.-Q.), arsoivan@gmail.com (I. A.-S.), vmanuel@uaem.mx (V. M. H.-V.), rsuarez@uaem.mx (R. S.-R.)
[2]Centro de Investigaciones Biológicas, Universidad Autónoma del Estado de Morelos**.** Avenida Universidad 1001 Colonia Chamilpa, Cuernavaca, Morelos, México. CP 62209; E-mail: penacg@uaem.mx (G. P.-C.)
*Corresponding author; E-mail: penacg@uaem.mx (G. P.-C.)

used feeding bioassays that indicated some toxicity of the *B. thuringiensis* toxins Cry2, Cry3A, and Cry4 against the potato aphid *Macrosiphum euphorbiae* Thomas. Huarong et al. (2011) obtained low toxicity with Cry1Ac and Cry3A against *A. pisum*. However, Sattar & Maiti (2011) obtained high toxicity with the toxin Vip2Ae (homologue protein of Vip2A). Analyses with transgenic plants expressing Cry toxins on aphids showed minor effects on aphid survival and fecundity, as well as significant beneficial effects on aphid populations (Burgio et al. 2007, 2011; Schuler et al. 2005).

In previous research, we showed that *B. thuringiensis* strains induced physical changes and mortality in *M. persicae* (Torres-Quintero et al. 2015). In this work, we characterized 40 *B. thuringiensis* strains isolated from corpses of Hemiptera, and we identified the strains that were pathogenic and highly virulent to *M. persicae*.

Materials and Methods

SOURCE OF *M. PERSICAE* FOR LABORATORY BIOASSAYS

Native populations of *M. persicae* were collected from lettuce crops in the state of Morelos, Mexico, and kept in a greenhouse. All stages of the insects were maintained on chili plants (*Capsicum annuum* var. *aviculare*; Solanaceae). The plants were put into cages (90 × 90 × 90 cm) covered with a mesh to exclude predators or parasitoids. The insects used in the bioassays were from the 4th generation.

ORIGIN OF *B. THURINGIENSIS* STRAINS ASSESSED

The *B. thuringiensis* strains were obtained from the collection at the Laboratory of Plant Parasitology of the Center of Biological Research of the Autonomous University of the State of Morelos, Mexico (CIB-UAEM). Forty strains from this collection were selected because they had been isolated from corpses of hemipteran insects (Table 1). The commercial strain HD1 (active against Lepidoptera) was used as a negative control, and the strain GP139 was used as a positive control because it is pathogenic to *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Salazar-Magallon et al. 2015).

PRODUCTION OF SPORE–CRYSTAL SUSPENSION

The *B. thuringiensis* strains were grown in solid nutrient HCT (Bacto Tryptone (Difco) 5; Casamino acids (Difco) 2; pH adjusted to 7.5). After sterilization, KH_2PO_4, 3.4 g/L; $MgSO_4.7H_2O$, 0.012 g/L; $MnSO_4.4H_2O$, 0.003 g/L; $ZnSO_4.7H_2O$, 0.0028 g/L; $Fe(SO_4)_3.7H_2O$, 0.02 g/L; $CaCl_2.2H_2O$, 0.147 g/L; and glucose, 3 g/L were added, and strains were incubated at 30 °C for 72 h. After complete sporulation, the spores and crystals were collected in sterile water with 1 mM of phenylmethylsulfonyl fluoride, and the total protein concentration was determined by the protein dye method of Bradford (1976) using bovine serum albumin as a standard.

PATHOGENICITY BIOASSAYS

The in vitro feeding bioassays were performed with the spore–crystal complex produced by the *B. thuringiensis* strains using 4th instar nymphs of *M. persicae*. The bioassays for aphid feeding were prepared as described by Torres-Quintero et al. (2013). The basic liquid diet consisted of 5% yeast extract and 30% sucrose in distilled water at pH 7.0 (Jancovich et al. 1997). For each strain, 2 concentrations of total protein were used: 10 ng/µL and 100 ng/µL. A plain liquid diet was used as untreated control. A completely randomized design was used, and each experimental unit consisted of a feeding chamber with 20 aphids. All bioassays were carried out in triplicates. The mortality of aphids was determined at 72 h.

VIRULENCE BIOASSAYS (LC50 AND LC90) AND PROTEIN PROFILES OF THE STRAINS

Virulence was determined with 7 concentrations of total protein (1, 2, 4, 6, 8, 10, and 12 ng/µL) and a control without protein. Each treatment was applied in the same way as in the pathogenicity bioassays. The mortality was determined at 72 h (Torres-Quintero et al. 2015). The protein profiles of all the strains evaluated in bioassays of virulence were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

STATISTICAL ANALYSES

The percentage of mortality was analyzed by ANOVA and multiple comparisons of means (least significant difference "LSD"); both analyses were carried out with the SAS system for Windows 9.0 (SAS Institute 2002). Probit analysis of mortality data was performed to estimate the lethal concentrations (LC50 and LC90) with the program PoloPlus (Robertson et al. 2003).

Results

PATHOGENICITY BIOASSAYS

Of the 40 *B. thuringiensis* strains evaluated for their pathogenicity to *M. persicae*, 17 strains were significantly different from the spore-crystal-free control according to the LSD test ($\alpha = 0.05$), with mortality rates between 64.4 and 88.9% at 10 ng/µL and between 71.1 and 91.1% at 100 ng/µL, with the exception of strains GP865 and GP399, which only were significant at 100 ng/µL (Table 2). Among the strains that were significantly different from the spore-crystal-free control, there were 6 strains that produced a mortality rate above 80% (GP209, GP338, GP762, GP780, GP528, and GP322), 6 strains that were above 70% mortality (GP402, GP778, GP300, GP382, GP782, and GP640), and 3 strains were above 60% mortality (GP238, GP777, and GP60). The strain GP139 that was used as a positive control caused mortality rates above 80%. The

Table 1. Host origins of the *Bacillus thuringiensis* strains evaluated in this study.

Family (Insecta)	*B. thuringiensis* strains
Aphididae	GP15, GP47, GP53, GP54, GP55, GP56, GP59, GP60, GP209, GP210, GP238, GP338, GP382, GP402, GP640, GP670, GP762, GP777, GP778, GP779, GP780, GP782, GP839
Cercopidae	GP49, GP528
Psyllidae	GP132, GP202, GP280, GP308, GP322, GP336, GP847, GP850, GP865
Phylloxeridae	GP233
Cicadellidae	GP631
Coccidae	GP274, GP300, GP399
Diaspididae	GP297

Table 2. Toxicity of *Bacillus thuringiensis* strains to *Myzus persicae.*

	% Mortality			% Mortality	
Strain	Concentration 10 ng/µL	Concentration 100 ng/µL	Strain	Concentration 10 ng/µL	Concentration 100 ng/µL
GP322	88.87 ± 4.43*	68.87 ± 5.88**	GP839	46.63 ± 10.17	30.67 ± 4.67
GP762	86.63 ± 6.67*	84.43 ± 4.43**	GP847	43.30 ± 16.70	36.70 ± 30.00
GP139	84.40 ± 5.88*	90.62 ± 6.33**	GP47	42.20 ± 11.10	57.73 ± 4.43
GP528	84.40 ± 8.90 *	66.63 ± 26.67**	GP59	42.20 ± 5.88	53.30 ± 7.68
GP780	82.20 ± 5.88*	88.87 ± 4.43**	GP15	40.00 ± 0.00	57.73 ± 4.43
GP209	82.20 ± 5.88*	82.80 ± 7.41**	GP53	40.00 ± 20.00	51.03 ± 11.82
GP338	81.09 ± 8.69*	90.77 ± 2.53**	GP56	40.00 ± 20.00	28.67 ± 11.33
GP402	77.73 ± 12.37*	75.50 ± 11.10**	GP336	39.95 ± 26.65	53.30 ± 20.00
GP778	77.73 ± 11.13*	75.13 ± 12.64**	GP280	37.77 ± 9.68	26.63 ± 6.67
GP300	76.65 ± 3.35*	77.73 ± 12.37**	GP233	37.73 ± 4.43	60.00 ± 6.60
GP382	73.00 ± 9.99*	79.97 ± 10.17**	GP308	35.53 ± 2.23	36.65 ± 13.40
GP782	71.07 ± 4.47*	84.40 ± 8.90**	GP55	33.46 ± 13.46	33.37 ± 7.74
HD1	71.07 ± 2.23*	82.17 ± 8.02**	GP631	33.10 ± 14.08	28.87 ± 4.43
GP640	71.07 ± 15.53*	73.30 ± 7.68**	GP202	30.00 ± 10.00	44.47 ± 5.89
GP777	68.83 ± 2.23*	68.84 ± 9.67**	GP54	28.63 ± 11.95	33.40 ± 10.10
GP238	64.43 ± 15.57*	71.10 ± 8.90**	GP274	26.63 ± 3.84	39.87 ± 6.93
GP60	64.40 ± 8.90*	71.10 ± 11.10**	GP865	26.60 ± 0.00	76.60 ± 3.35**
GP132	59.97 ± 10.17	64.47 ± 11.10	GP779	24.43 ± 4.43	44.20 ± 11.20
GP210	57.73 ± 12.37	57.73 ± 19.73	GP670	23.30 ± 10.00	36.65 ± 10.00
GP297	53.30 ± 16.76	55.53 ± 21.21	GP49	22.20 ± 5.88	37.77 ± 9.68
GP850	51.07 ± 8.00	48.63 ± 11.95	Control	14.73 ± 3.18	14.73 ± 3.18
GP399	50.87 ± 22.76	79.97 ± 3.84**			

Mean ± standard error. Asterisks (* for 10ng/µL and ** for 100ng/µL) denote a significant difference from the spore-crystal-free control according to the LSD test (α = 0.05).

strain HD1 used as a negative control was surprisingly toxic against *M. persicae*, with mortality rates above 70%. Of the 17 strains that caused high mortality, 12 were isolated from dead insects belonging to Aphididae, 2 from Psyllidae, 2 from Coccidae, and 1 from Cercopidae.

VIRULENCE OF 17 BT STRAINS TO *M*. *PERSICAE* AND PROTEIN PROFILES OF THE STRAINS

To determine the virulence (LC50) of the *B. thuringiensis* strains against *M. persicae*, bioassays were carried out with the spore–crystal suspensions using 7 concentrations. Three strains were highly virulent and had LC50 values from 4 to 5 ng/µL (GP640, GP399, and GP238), 9 strains had values from 6 to 7 ng/µL (GP322, GP139, GP762, GP338, GP300, GP402, GP382, GP528, and GP782), and 6 strains had values above 9 ng/µL (HD1, GP209, GP777, GP778, GP60, and GP780) (Table 3). However, comparing the LC90 values, we observed that some of the strains required 13- to 42-fold higher concentrations, e.g., GP60 (257.3 ng/µL), GP780 (370.7 ng/µL), and GP782 (478.7 ng/µL), compared with strains in which low concentrations were effective, e.g., GP528 (11.48 ng/µL), HD1 (11.79 ng/µL), GP402 (13.9 ng/µL), GP300 (14.20 ng/µL), and GP777 (19.2 ng/µL) (Table 3). The protein profiles of the strains showed major bands with the following molecular weights: 30, 65, 70, 75, 100, 110, 120, 130, 200, and 250 kDa (Fig. 1).

Discussion

The aim of this study was to characterize 40 strains of *B. thuringiensis* isolates from corpses of hemipteran insects for the purpose of finding strains pathogenic to *M*. *persicae*, one of the most important agricultural pests worldwide. There is little information about the toxicity of *B. thuringiensis* proteins to aphids. Previous studies reported the activity of some toxins against several species; however, none are commercially available. Pathogenicity is the ability of an organism to infect a host and cause a disease. Furthermore, it is a requirement for virulence. However, in some cases, the organism can infect the host but not cause its death (Stephen & Elkinton 2004). The bioassays performed in this study with the spore–crystal suspensions of *B. thuringiensis* showed that 17 strains were pathogenic to *M. persicae* at both tested concentrations of 10 ng/µL and 100 ng/µL. They caused mortality rates of ≥60% statistically different from the spore-crystal-free control, with the exception of strains GP865 and GP399, which caused significant mortality only at 100 ng/µL. For this reason, these strains were not tested in the bioassays for the determination of virulence (LC50). The strains that were used as a positive control (GP139) and a negative control (HD1) were also toxic, with mortality rates above 70%. It is known that the commercial strain HD1 is specific for lepidopteran insects (Dulmage 1970); however, when HD1 was field tested on whitefly nymphs (Sternorrhyncha), the results indicated a decrease in the population (Radman et al. 1984). This strain also showed activity against *Tagosodes orizicolus* (Auchenorrhyncha), with mortality rates above 80% (Mora et al. 2007).

These results suggest that this strain can express toxins with activity against some hemipterans. The insecticidal protein genes that it has are: *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2A*, and *cry2B*. Few previous studies evaluated the efficacy of *B. thuringiensis* against sucking insects. For example, the spore–crystal complex LFB-039 of *B. thuringiensis* subsp. *morrisoni* was evaluated in *Triatoma vitticeps* Stål (Hemiptera: Reduviidae); however, this strain did not show activity (Lima et al. 1994). Another work evaluated the toxins Cry1Ac and Cry2Ab against *Lygus hesperus* Knight (Hemiptera: Miridae) without finding mortality (Brandt et al. 2004). Wellman-Desbiens & Coté (2005) also did not find Bt-induced mortality in *L. hesperus*. Conversely, a δ-endotoxin from *B. thuringiensis* subsp. *neoleonensis* (Cyt) was evaluated against *L. hesperus* and caused 68% mortality at 45 µg/mL (Stockhoff & Conlan 1998). Another study showed that 1 mg/mL of strain HD137 and 4 isolates of *Bacillus* sp. (23-O-to, 40-X-m, 43-S-d, and 26-O-to) were toxic to *T. orizicolus* with mortality rates of 19, 74, 96, 44, and 95%, respectively (Mora et al. 2007).

Table 3. Virulence of *Bacillus thuringiensis* strains to *Myzus persicae*.

		95% confidence interval			95% confidence interval		
Strain	LC50 (ng/µL)	Lower limit	Upper limit	LC90 (ng/µL)	Lower limit	Upper limit	χ^2
GP640	4.63	1.20	8.31	41.58	19.15	694.2	16.68
GP399	5.16	1.02	10.15	65.62	25.90	2,816	11.34
GP238	5.39	1.67	9.81	56.23	24.60	854.8	15.22
GP322	6.13	1.48	12.58	97.06	34.58	5,484	8.63
GP139	6.14	1.84	13.54	69.41	24.44	8,976.	41.20
GP762	6.18	2.79	10.02	49.86	24.34	417.5	15.76
GP338	6.58	3.52	10.63	47.18	21.07	1,695	19.06
GP300	6.88	5.26	8.01	14.20	10.84	43.60	21.52
GP402	7.12	5.03	8.46	13.91	10.88	34.38	14.45
GP382	7.62	4.38	12.04	84.49	38.39	599.5	11.68
GP528	7.86	6.23	9.46	11.48	9.52	20.23	44.91
GP782	7.95	2.82	23.08	478.76	92.84	2.57E+05	18.87
HD1	9.10	7.82	11.44	11.79	9.97	21.02	55.20
GP209	10.58	8.82	14.50	25.23	17.02	99.15	8.26
GP777	10.63	9.13	14.41	19.20	14.25	66.79	11.38
GP778	10.94	7.32	18.56	49.26	24.78	1,199	4.44
GP60	12.77	6.03	36.50	257.32	67.36	31,345	12.46
GP780	15.56	6.43	50.42	370.70	84.82	7.27E+05	19.39

The majority of the 17 strains identified as pathogenic in this study were effective at low protein concentration (10 ng/µL) causing mortality rates above 70%. However, the toxicity to *M. persicae* was not the same for all strains, which is most likely related to the expression of different proteins or other pathogenicity factors. Of the 17 effective strains, 12 were isolated from dead insects belonging to Aphididae, 2 were from Psyllidae, 2 were from Coccidae, and 1 was from Cercopidae. These results agree with the proposed idea that the insecticidal proteins from *B. thuringiensis* are specific for a certain insect group, and that the strains isolated from the insect corpse belonging to a particular order may be pathogenic to insects of the same order (Angus & Norris 1968; Dulmage 1970; Pinto et al. 2003).

There are other works that support our results: De Barjac & Thompson (1969) isolated a strain from a dead larva of *Galleria mellonella* L. (Lepidoptera: Pyralidae) that was identified as *B. thuringiensis* subsp. *thompsoni*, and when they conducted bioassays for pathogenicity with the same species and 2 other species of Lepidoptera, this strain was toxic. Konecka et al. (2007) analyzed 12 strains of *B. thuringiensis* that were isolated during an epizootic in the larvae of *Cydia pomonella* L. (Lepidoptera: Tortricidae), and when they tested the isolates against larvae from the same species, they were toxic. Krieg et al. (1983) isolated a strain from *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) that showed activity against several species of Coleoptera.

When we determined the virulence (LC50) of the 17 strains plus HD1 that were pathogenic to *M. persicae*, we found strains with values that were effective at rates from 4 to 5 ng/µL (GP238, GP399, and GP640), from 6 to 7 ng/µL (GP322, GP139, GP762, GP338, GP300, GP402, GP382, GP528, and GP782), from 8 to 9 ng/µL (GP865 and HD1), and others with values higher than 10 ng/µL (GP209, GP777, GP778, GP60, and GP780). These results suggest that the best strains for controlling *M. persicae* are those strains with an LC50 ranging from 4 to 5 ng/µL; however, the LC90 values of these 3 strains were 10 times greater, ranging from 42 to 66 ng/µL.

Conversely, there were 4 strains with LC90 values that did not change much with respect to their LC50 values, such as strain HD1 (LC50 = 9.10 ng/µL; LC90 = 11.79 ng/µL), GP777 (LC50 = 10 ng/µL; LC90 = 19.2 ng/µL), GP300 (LC50 = 6.88 ng/µL; LC90 = 14.20 ng/µL), and GP528 (LC50 = 7.86 ng/µL; LC90 = 11.48 ng/µL). The LC50 values of Cry2, Cry3, Cry11, and Cry4 toxins on *M. euphorbiae* were 200, 300, 350, and 400 ng/µL, respectively (Walters & English 1995). Porcar et

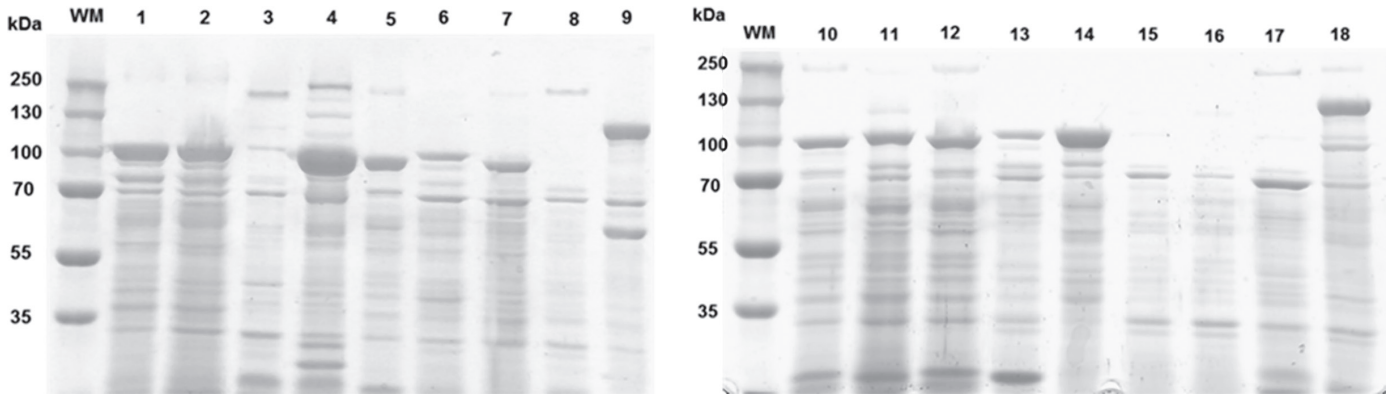

Fig. 1. Protein profiles of the strains virulent to *Myzus persicae*. Lane 1: GP640, Lane 2: GP399, Lane 3: GP238, Lane 4: GP322, Lane 5: GP139, Lane 6: GP762, Lane 7: GP339, Lane 8: GP300, Lane 9: HD1, Lane 10: GP402, Lane 11: GP382, Lane 12: GP528, Lane 13: GP782, Lane 14: GP209, Lane 15: GP777, Lane 16: GP778, Lane 17: GP60, Lane 18: GP780.

al. (2009) tested the toxins Cry3A, Cry4A, Cry11A, and Cyt1A against *A. pisum* and obtained LC50 values of 70 ng/µL for Cry3A and 100 ng/µL for the rest of the toxins; similarly, Huarong et al. (2011) showed that LC50 of Cry3A and Cry1A toxins against *A. pisum* where 500 ng/µL for both toxins. Palma et al. (2014) found a new *B. thuringiensis* protein that was toxic to *M. persicae* with an LC50 value of 32.7 ng/µL. Sattar & Maitti (2011) identified a homologous protein to Vip2A toxic to *Aphis gossypii* Glover (Hemiptera: Aphididae) with an LC50 value of 0.356 ng/µL.

Comparing our results with these, with the exception of the values reported by Sattar & Maitti (2011), the LC50 values of the strains evaluated in this study were up to 50 times lower. Da Costa et al. (2010) suggested that the best strains of *B. thuringiensis* to control *Aedes aegypti* L. (Diptera: Culicidae) were those with the lowest values of both LC50 and LC90. Accordingly, we suggest that the best strains to control *M. persicae* are those with the smallest values of both LC50 and LC90. The strains that meet these requirements are GP777, GP300, GP528, and HD1. These strains may have the potential to be used to suppress populations of the green peach aphid.

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