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Evaluation of industrial by-products for the production of *Bacillus thuringiensis* strain GP139 and the pathogenicity when applied to *Bemisia tabaci* nymphs

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Abstract

Three industrial by-products were evaluated to produce *Bacillus thuringiensis* strain GP139: molasses with soybean flour, nejayote with sucrose, cheese-whey with sucrose and HCT as a control media. *B. thuringiensis* cultures were grown in 250-mL shaking flasks containing 100 mL of medium, 5.9×10^8 spores/mL and 1.36 mg/mL of total protein were obtained using molasses with soybean flour medium, and 4.95×10^8 spores/mL and 0.78 mg/mL of total protein were produced with the cheese-whey plus sucrose medium. HCT medium yielded a 2.71×10^8 spores/mL and 0.84 mg/mL of total protein, and finally, the nejayote and sucrose medium yielded 2.04×10^8 spores/mL and 0.54 mg/mL of total protein. In bioassays conducted under greenhouse conditions, no statistically significant differences were observed in the mortality of *Bemisia tabaci* (Gennadius) (Hemiptera Aleyrodidae) nymphs treated with the *B. thuringiensis* spore-crystal complexes produced using the four media compared with chemical insecticide.

Key words: molasse, nejayote, cheese whey, whitefly.

Introduction

Industrial by-products discharged into natural waterways or municipal drainage lines typically without treatment are becoming the subject of environmental concern in industrial sectors, in government and in society in general. In most developed countries, a high percentage of industrial by-products are treated; unfortunately, in Mexico, only 20% is treated (Salmeron-Alcocer *et al*., 2003; Guerrero *et al*., 2010). In México, only few types of industrial by-products, such as whey, nejayote and molasses, are re-used.

The milk and dairy industry generates significant amounts of liquids, among which whey is the most abundant. Whey or cheese whey is the fluid that separates from the milk as it coagulates in the process of obtaining the cheese and consists of all the components of the milk that are not integrated into the coagulation of casein (Marwaha and Kennedy, 1988). The nixtamal industry (tortilla production) produces 16 to 22 million m^3 of wastewater per year. This waste is known as nejayote and is considered to be highly polluting effluent due to its organic and inorganic filler (Salmeron-Alcocer *et al*., 2003). Molasses is the by-product of the sugar industry and is widely used as a raw material for fermentation, such as in the production of ethanol and lactic acid, due to its abundance and low price compared with other available materials. Additionally, the waste and by-products of the sugar industry are relative pollutants as they contain many organic and inorganic nutrients that allow for recycling through the manufacture of other products, such as livestock feed and industrial ethanol, biomass, polymers, etc. As shown in the historical production of sugar cane, molasses and alcohol in Mexico, during the current harvest through the end of April 2013, the reported production of cane molasses (molasses) in the country was 1.72 million tons, a figure that is 120,000 tons greater than the cumulative harvest for the same time the previous year.

These three industrial by-products could be an alternative for the production *Bacillus thuringiensis* Berliner (Bacillales Bacillaceae) strain GP139, which was isolated from a *Bemisia tabaci* (Gennadius) (Hemiptera Aleyrodidae) corpse and exhibits toxic activity against *B. tabaci* (Peña and Bravo, 2002) and against the ectoparasite mite *Rhipicephalus microplus* (Canestrini) (Fernandez-Ruvalcaba *et al*., 2010). The strain was also characterized using PCR analysis and was shown to have the following genes: *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B*, *cry1D*, *cry2* and *s-layer gp1*. The aim of this work was to evaluate and developed a fermentation media made of low-cost industrial by-products to produce *B. thuringiensis* GP139 spore-crystal complex that is toxic against *B. tabaci*.

Materials and methods

Microorganism

B. thuringiensis strain GP 139 was used in this study and was from the collection of the Plant Parasitology Laboratory of the Center of Biological Research at the University of the State of Morelos, Mexico. This strain

Table 1. Composition (%) of the different by-products used in this work.

Nejayote (Velazco-Martínez *et al*., 1997)	
Moisture	97.85
Protein	7.42
Fiber crude	22.77
Ether extract	1.48
Nitrogen-free extract	55.67
Ash (minerals)	12.66
Calcium	13.13
Cane molasses (Vega-Baudrit *et al*., 2008)	
Water	20.00
Sucrose	30.00
Glucose	7.00
Other carbohydrates	4.10
Ash	12.00
Nitrogen compounds	4.00
Fat	0.40
Cheese whey (Guerrero-Rodriguez *et al*., 2010)	
Water	93-95
Lactose	4.5-5.3
Crude protein	0.6-1.1
Ash	0.5-0.7
Lactic acid	0.2

was isolated from a *B. tabaci* corpse (Peña and Bravo, 2002) and was maintained in the spore phase on filter paper in previously sterilized microcentrifuge tubes.

Inoculum preparation

The inoculum was prepared by adding three strips of filter paper containing spores to 5 mL of nutrient broth (NB) medium and incubating the culture overnight at 30 °C. The culture was placed in a 250-mL Erlenmeyer flask containing 100 mL of NB medium, and after 8 h of incubation at 30 °C in a rotary shaker operating at 150 rpm, the cell concentration was calculated using a Neubauer chamber. Each experimental medium was inoculated with 1×10^7 cells/mL.

Industrial by-product media and culture conditions

Three different industrial by-products (table 1) were used to formulate the following culture media: nejayote in its original concentration with 10 g/L of sucrose added as the carbon source (M_1) (Dominguez-Espinosa *et al*., 2002); 50 g/L of cane molasses as the carbon source supplemented with 50 g/L of soybean flour as the nitrogen source (M_2) (Abdel-Hameed *et al*., 1990); milk whey with 15 g/L of sucrose as the carbon source (M_3) (El-Bendary *et al*., 2008). Additionally, we used commercial HCT medium as the reference medium (M_4), which contained 5 gL^{-1} Bacto Tryptone (Difco) and 2 gL^{-1} Casamino acids (Difco); the pH was adjusted to 7.2, and after sterilization, 3.4 gL^{-1} KH_2PO_4, 0.012 gL^{-1} $MgSO_4.7H_2O$. 0.003 gL^{-1} $MnSO_4.4H_2O$, 0.0028 gL^{-1} $ZnSO_4.7H_2O$, 0.02 gL^{-1} Fe $(SO_4)_3.7H_2O$, 0.147 gL^{-1} $CaCl_2.2H_2O$, and 3 gL^{-1} glucose were added (Muñoz-Garay *et al*., 2009). In all four media, the pH was adjusted to 7.0 using 1 M NaOH and 30% HCl before sterilization. Then, 250-mL flasks with 100 mL of each culture medium were incubated for 72 h at 30 °C in a rotary shaker operated at 250 rpm. Three replicates of each experiment were performed.

pH kinetics

The pH was determined using a potentiometer (Fisher Scientific model 15) that was previously calibrated. Samples were taken at regular 2 hour intervals during the first 16 hours and subsequently at 22, 28, 34, 40, 52 and 72 hours; each determination was performed in triplicate.

Determination of the biomass, spore count, specific growth rate and doubling time

Aliquots of 5 mL of each medium (per sample flask) were taken at 2 h intervals during the first 18 h and at 6 h intervals from 18 h to 72 h. To determine the dry weight of the biomass, 5 mL of each culture was centrifuged at 7,000 rpm for 25 min. The supernatant was discarded, and the pellet containing the cells was recovered, washed three times and dried in a laminar flow chamber for 72 h. The spore counts were then determined using a Neubauer chamber (Bing-Lan and Yew-Min, 2000).

The specific growth rates were calculated using a linear regression of the natural logarithm of the biomass concentration versus time; the slope corresponded to the specific growth rate value. The doubling times were calculated using the following formula:

$$td = \frac{\ln(2)}{\mu}$$

where td is the duplication time (h) and μ is the specific growth rate (h-1).

Solubilization and estimation of total protein

A 500-μL sample of the spore-crystal complexes was collected, and the crystalized protein was solubilized in a 50 mM Na_2CO_3 solution at a pH of 10.5 that contained 0.2% β-mercaptoethanol, 80 mM NaCl, and 1 mM PMSF. The sample was shaken at 500 rpm for 2 h at 37 °C. The spore-crystal mixture was centrifuged at 14,000 rpm for 10 min, the supernatant was recovered, and the pellet containing the spores was discarded. The concentration of the total protein was determined using the Bradford method (Bradford, 1976). The absorbance at 595 nm was measured using a spectrophotometer.

Bioassay

Spore-crystal complexes from each of the different media were sprayed with an airbrush on *B. tabaci* nymphs in the third and fourth instar using the sprinkling method. *Solanum lycopersicum* (L.) plants were infested with *B. tabaci* adults 40 days before treatment. A concentration of 40 μg/mL of total protein from the culture of each of the four media was used. Additionally, we used a chemical insecticide, in which the active ingredient was omethoate (1 mL/L), as the positive control and water plus the dispersant (Dimethyl sulfosuccinate) (5 mL/L) as the negative control. Mortality was determined after 2, 4 and 8 days. Four repetitions per treatment were performed.

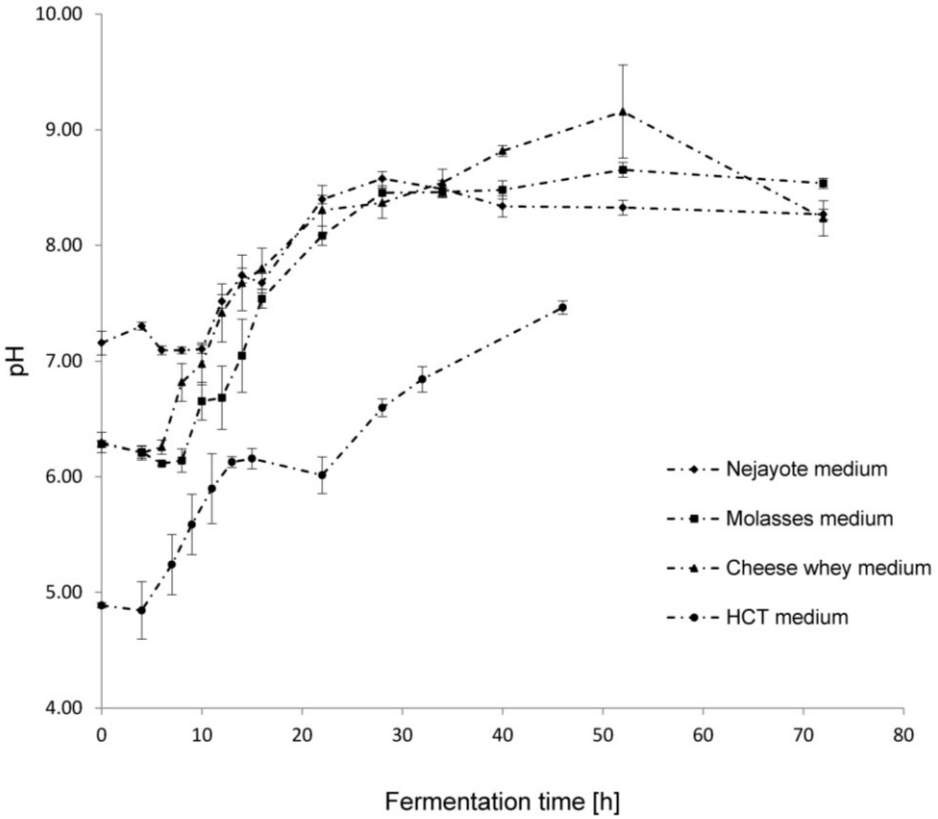

Figure 1. pH variations during fermentation of strain GP139 in four different media. (♦) M_1: Nejayote with sucrose medium, (■) M_2: Sugar cane molasses and soybean flour medium, (▲) M_3: Cheese-whey with sucrose medium, (●) M_4: HCT reference medium. Data are represented as the mean value ± SE.

Statistical analysis

The results related to the growth, the spore count, the total protein production, the specific growth rates and the doubling time are the average of three separate experiments. The mortality values are the average of four repetitions per treatment. All of the results were statistically analyzed using the Tukey test.

Additionally, the costs of the mean chemical insecticides used by farmers to control *B. tabaci* and the four media, HCT and three based on industrial by-products, are compared.

Results

pH kinetics

The pH kinetics are shown in figure 1. The pH values varied throughout fermentation in the four tested media. M_1, M_2 and M_3 maintained pH values of approximately 6-7 during the first 10 h; however, medium M_4 maintained a low pH value of approximately 5 during the first 6 h. After these periods, the pH values increased from 6-7 to 7-9 by 72 h of fermentation in all of the media tested.

Growth kinetics in three media composed of industrial by-products and in reference medium

The growth kinetics of strain GP139 in the four media are shown in figure 2. The fermentation process can be divided into three distinct phases, each with well-defined characteristics: phase I, which is when vegetative growth occurs; phase II, which is when the transition to sporulation occurs; and phase III, which is when spores mature and cells lyse (Berbert-Molina *et al*., 2008).

An adaptation phase was observed at approximately 10 h in the M_1 medium after which vegetative growth began; however, vegetative growth began immediately in the M_2, M_3 and M_4 media. Phase II was attained in the M_1 medium between 48 and 60 h of fermentation, between 26 and 34 h of fermentation in the M_2 medium, between 48 and 60 h of fermentation in the M_3 medium and between 24 and 32 h of fermentation in the M_4 medium. Phase III was observed after 34 h of fermentation in the M_1 medium, after 28 h of fermentation in the M_2 medium, after 52 h of fermentation in the M_3 medium and after 22 h of fermentation in the M_4 medium.

The biomass produced in the different media varied. As shown in table 2, the largest biomass (6.15 ± 0.32 m gDW/L) was obtained in the M_3 medium at 52 h of fermentation. This was followed by the M_2 medium at 24 h of fermentation and finally by the M_4 and M_1, in which the smallest biomass was attained and was observed at 22 and 32 h of fermentation, respectively.

However, the greatest spore count and protein concentration was obtained in the M_2 medium (5.90×10^8 ± 0.85 spores/mL and 1.36 ± 0.045 mg/mL of total protein). The M_3 medium also had a high spore count and protein concentration (4.95×10^8 ± 0.85 and 0.78 ± 0.030). The M_4 and M_1 media produced the lowest spore

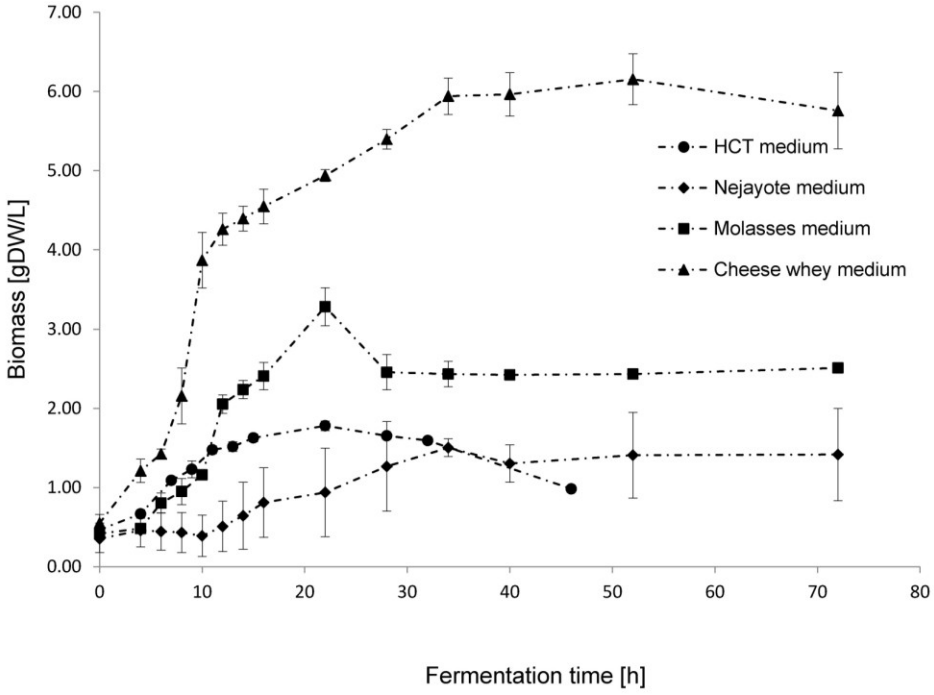

Figure 2. Kinetics of the growth of strain GP139 in four different media. (♦) M_1: Nejayote with sucrose medium, (■) M_2: Sugar cane molasses and soybean flour medium, (▲) M_3: Cheese-whey with sucrose medium, (●) M_4: HCT reference medium. Data are represented as the mean value ± SE.

Table 2. Kinetics results obtained for *B. thuringiensis* strain GP139 grown in four different media.

Medium	Biomass (gDW/L)	Spore count (spo/mL)	Protein (mg/mL)	μ (h^{-1})	Doubling time (h)
M_1	1.50 ± 0.11c	$2.04x10^8$ ± 0.16 c	0.54 ± 0.014 c	0.45 ± 0.006 b	16.3 ± 2.35 c
M_2	3.28 ± 0.24 b	$5.90x10^8$ ± 0.85 a	1.36 ± 0.045 a	0.107 ± 0.009 b	6.6 ± 0.60 b
M_3	6.15 ± 0.32 a	$4.95 x10^8$ ± 0.85 b	0.78 ± 0.030 b	0.162 ± 0.006 a	4.3 ± 0.66 a
M_4	1.78 ± 0.06 c	$2.71x10^8$ ± 0.79 c	0.84 ± 0.018 b	0.094 ± 0.013 b	7.6 ± 0.98 b
CV	11.43	12.45	5.71	16.56	17.72

The significance of the differences was assessed using Tukey's test ($P < 0.05$). Three repetitions were performed, and the values provided are the mean values ± SE. CV: coefficient of variation.

count and lowest protein concentration. Additionally, the spore-crystal complexes were obtained between 24 and 30 h in the M_2 and M_4 media, whereas this complex was obtained in the M_1 and M_3 media after approximately 60-72 h of fermentation.

Additionally, biomass production in terms of the specific growth rate and the doubling time was calculated. The M_2 (0.107 ± 0.009 h^{-1} and 6.6 ± 0.61 h) and the M_3 medium supported the greatest specific growth rate and the lowest doubling time, respectively, followed by the M_4 medium, whereas the lowest specific growth rate and the greatest doubling time were associated with the M_1 medium.

Bioassay

To determine the effect of the GP139 spore-crystal complexes, *S. lycopersicum* plants infested with *B. tabaci* nymphs were sprinkled with 0.04 mg/mL of total protein. Table 3 shows the mortality values as percentages for the *B. tabaci* treated with the spore-crystal complexes produced by strain GP139 grown in the four different media. Additionally, the mortality percentage of the positive control (chemical insecticide) and the negative control (water + dispersant) were determined for the treatment conducted over the 8 days of the bioassay.

The spore-crystal complexes produced using the four different media, which were applied at a concentration of 0.04 mg/mL, produced the same level of mortality as the chemical insecticide: an 88-92% mortality of the third and fourth instar *B. tabaci* nymphs. The criteria for mortality were dehydration, deformation and collapse of the larvae. All dead treated nymphs exhibited a collapsed structure compared with the negative control nymphs, which had an undamaged structure.

Discussion

B. thuringiensis produces insecticidal proteins referred to as δ-endotoxins during sporulation (Schnepf *et al*., 1998). Mass production of these proteins is required for marketability and depends on an optimal and inexpen-

Table 3. Bioassay data obtained from treating *B. tabaci* nymphs on *S. lycopersicum* plants with spore-crystal complexes produced in four different media and applied at 40 µg/mL of total protein.

Treatment	After 2 days		After 4 days		After 8 days	
	n	Mortality (%)	n	Mortality (%)	n	Mortality (%)
M_1	94	83.69 ± 9.75 a	132	86.68 ± 5.29 a	113	96.33 ± 2.40 a
M_2	119	82.32 ± 9.65 a	221	90.20 ± 3.63 a	228	94.53 ± 3.77 a
M_3	154	86.47 ± 4.57 a	145	98.89 ± 1.11 a	195	90.93 ± 5.44 a
M_4	169	83.40 ± 9.22 a	221	89.44 ± 3.27 a	148	89.10 ± 5.75 a
Chemical insecticide	151	89.51 ± 7.24 a	139	83.15 ± 11.49 a	209	90.92 ± 6.41 a
Water + dispersant	178	21.57 ± 5.64 b	146	31.40 ± 2.68 b	110	32.09 ± 8.62 b
VC		18.6		14.2		14.1

The significance of the differences was assessed using Tukey's test ($P < 0.05$). Four repetitions were performed, and the values provided are the mean values ± SE. VC: variation coefficient. Abbott test was applied to calculate the percentage of corrected mortality.

sive fermentation media, which could be based on different carbon and nitrogen sources (Avignone-Rossa and Mignone, 1995). The pH of the fermentation medium is an indirect measure of the consumption of carbohydrates by *B. thuringiensis*. Some studies (Yousten and Rogoff, 1969; Morris *et al*., 1996; Amin *et al*., 2008) found that variations in pH due to the carbohydrates being oxidized to some form of organic acid (e.g., acetic acid) during the first 10 hours and then being reoxidized to carbon dioxide. Additionally, reports have shown that the pH in cultures of *B. thuringiensis* increased after 10 hours due to the release of nitrogen-containing compounds, which were primarily proteins (Kraemer-Schafhalter and Moser, 1996).

In this study, different cell growth profiles were observed in all four of the media. The largest biomass of strain GP139 was obtained using the M_3 medium, and the biomass amount was significantly different from the biomass obtained in the reference medium followed by the biomass in the M_2 medium, which was 48% smaller than the biomass obtained in the M_3 medium, which was significantly different from that obtained in the reference medium. Using the M_4 medium, a biomass was obtained that was 72% smaller than the biomass obtained in the M_3 medium, and finally, the biomass obtained in the M_1 medium was 76% smaller than the biomass obtained in the M_3 medium.

The different biomass content obtained may be due to the different types of nutrients present in the four media (table 1). Cultures of *B. thuringiensis* that have an excessive carbon source have been reported to contain heterogeneous cell populations and have a prolonged exponential growth phase (Keshavarzi *et al*., 2005; Khodiar *et al*., 2008; Valicente and Mourão, 2008). Moreover, the medium must contain glutamic acid, glycine, arginine, methionine and aspartic acid, which stimulate the growth of *B. thuringiensis* and optimize its protein production, and these amino acids are present in whey and soybean meal (El-Bendary *et al*., 2008; Rajalakshmi and Shethna, 1977; Rajalakshmi and Shethna, 1980; Prabakaran and Hoti, 2008).

We are reporting the effect of different culture media on the production of spore-crystal complexes. It was shown that the maximum concentration of spores was obtained using the M_2 medium and that this value was significantly different from the values obtained using the other media, with the M_3 medium yielding a 16% lower concentration of spores, the M_4 medium yielding a 54% lower concentration of spores, and the M_1 medium yielding a 65% lower concentration of spores compared with the M_2 medium. Variations in spore concentrations are sometimes due to variations in the initial number of cells inoculated; however, in this study, a standardized initial cell concentration of 1×10^7 cells/mL was used, whereas other authors (Morris *et al*., 1996; Amin *et al*., 2008; Khodiar *et al*., 2008; Valicente and Mourão, 2008) used standardized initial cell concentrations of 1% of the final working volume. Therefore, the differences in the spore concentrations are likely due to the cumulative effects of the quantity and quality of carbohydrates and proteins in the culture media because the carbon source is directed toward the formation of the biomass (Abdel-Hameed *et al*., 1990; Morris *et al*., 1996; Keshavarzim *et al*., 2005; Khodiar *et al*., 2008; Valicente and Mourão, 2008; Rajalakshmi and Shethna, 1977; Prabakaran and Hoti, 2008).

Conversely, the maximum amount of total protein was produced using the M_2 medium followed by the M_4 medium, from which 39% less protein was obtained. Using the M_3 medium, 43% less protein was obtained than with the M_2 medium, and finally, the lowest amount of total protein was obtained using the M_1 medium, which was 62% less than with the M_2 medium. The variation in the amount of total protein obtained could be due to the concentration of crude protein and the amount of amino acids in the media, particularly arginine and glutamic acid, which are important for protein synthesis.

These amino acids comprise 45% of the amino acids in the insecticidal toxins that are produced by *B. thuringiensis*. The balance between the carbohydrates and proteins present in the culture media likely explains the differences in the production of *B. thuringiensis* insecticidal proteins in the media (Morris *et al*., 1996; Keshavarzi *et al*., 2005; Rajalakshmi and Shethna, 1977; Rajalakshmi and Shethna, 1980; Anderson and Jayaraman, 2003).

B. tabaci is a key pest of several crops and has developed resistance to the different groups of chemical insecticides that are used to control it (Basit *et al*., 2013; Castle and Prabhaker, 2013; Vassiliou *et al*., 2010);

Table 4. Costs (USD) in Mexico of chemical control and mass production of *B. thuringiensis* on four different media to control *B. tabaci*.

Chemical control	Cost/L	Cost/Ha
Fenpropathrín	57	28.5
Oxamyl	23	11.5
Imidacloprid	100	50
Bifenthrin	43	21.5
Imidacloprid + Betacyflutrin	62	31
Control with *B. thuringiensis*	Cost/L	Cost/Ha
HCT	7.5	188
Nejayote + sucrose	0.08	1.5
Molasses + soybean flour	0.09	2.5
Milk-whey + sucrose	0.1	2.6

therefore, an alternative could be the use of *B. thuringiensis*, but until now, there has not been any commercial bioinsecticide produced using this bacterium to control *B. tabaci* in this regard. One study tested nine different Cry proteins from *B. thuringiensis* on *B. tabaci*. This study showed that the nine proteins had no effect because *B. tabaci* does not have the necessary digestive proteases (Davidson *et al*., 1996). Another study (Al-Shayji and Shaheen, 2008) reported the mortality of 50 to 60% of first-instar nymphs of *B. tabaci* treated with a strain of *B. thuringiensis* isolated in Kuwait at a concentration of 500 µg/mL. Our results are better than those reported previously because mortalities of up to 90% were achieved in the third- and fourth-instar nymphs treated with spore-crystal complexes at a concentration of 40 µg/mL, which highlights the importance of continuing to investigate the use of this entomopathogenic agent to control this aggressive global crop pest. Additionally, the spore-crystal complexes produced in three of the tested media were as effective as the chemical insecticide. Moreover, the costs to produce the quantity of total protein needed to control *B. tabaci* were much less expensive with the three alternative culture media compared with the commercial medium and the commercial chemical insecticides (table 4) that are commonly used to control *B. tabaci* in México. Application costs are not included.

Conclusion

The use of chemical insecticides to control insect pests has adverse effects on non-target organisms, such as beneficial insects and birds; leaves residues in the environment; and can lead to resistance, among other issues. These effects are most visible in countries where there is no strict control on the use of these chemicals. An alternative to these pesticides is the use of microorganisms, such as *B. thuringiensis*. In this work, alternative culture media are proposed to produce spore-crystal complexes without affecting the pathogenicity of this bacterium.

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