RESULTADOS

Como forma de presentación del presente trabajo doctoral, nos permitimos adjuntar las publicaciones que se tienen listas como resultado del desarrollo experimental del trabajo, de las cuales, la patente ya se tiene registrada, el capítulo ha sido aceptado para publicación y los artículos han sido revisados y se encuentran listos para ser enviados a revisión. El orden de presentación de los mismos en el texto es el siguiente:

Artículos:

I. Characterization of Mexican strains of *Bacillus thuringiensis* toxic for lepidopteran and coleopteran larvae

II. New granular formulations based on corn meal with different serovar. of *Bacillus thuringiensis*

III. Sprayable granule formulations for *Bacillus thuringiensis*

IV. *Bacillus thuringiensis* microencapsulated formulation for control of the coleopteran *Epilachna varivestis* Mulsant (Coleoptera: Coccinelidae)

Capítulo en Libro: *Bacillus thuringiensis* bioassays into artificial diets. 1996. *In*: Manual of techniques in insect pathology. L. A. Lacey . (ed.). Academic Press, England (en prensa).

Patente: Microcapsulación de pesticidas (en trámite).

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Characterization of Mexican strains of *Bacillus thuringiensis* toxic for lepidopteran and coleopteran larvae for EPA certification.

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ABSTRACT

Several *Bacillus thuringiensis* strains isolated from dead larvae and soil samples from cultivated fields located in northeast Mexico were characterized and compared with HD strains. These isolates were tested against lepidopteran (*Helothis virescens, Helicoverpa zea, Spodoptera exigua* and *Trichoplusia ni*) and coleopteran (*Epilachna varivestis*) pests and those showing high toxicity were subjected to the biological tests proposed by the EPA. We chose nine *B. thuringiensis* strains, five of the Howard Dulmage collection designated HD-73, HD-187, HD-193, HD-263 and HD-530 and four Mexican strains named C-4, C-9, GM-7 and GM-10. These strains were characterized by their flagellar serotype and identified as the serotypes *kurstaki* (HD-73, HD-187, HD-262, HD-530), *aizawai* (GM-7, GM-10), *galleriae* (HD-193), *morrisoni* (HD-530), and *kumamotoensis* (C-4, C-9). The results showed that there was no relation between they among the host range, serotype, molecular weight of the insecticidal crystal proteins (ICP), biochemical metabolism, antibiotic resistance and plasmid patterns. GM-7 and GM-10 were sensitive to R-41 and CP-51 phages. C-4 and C-9 strains showed toxicity against the coleopteran *E. varivestis*, and produced ulcers in mice, like a β-exotoxin producing strain (HD-41). Immunoblot of ICP's showed that strains killing lepidopteran larvae crossreacted with a polyclonal antiCryIAb and/or antiCryIAc antiserum in ca. 70 and 120-130 KDa protein bands. These results indicate that the CryIA is present.

KEY WORDS: *Bacillus thuringiensis*, characterization, coleopteran, lepidopteran.

Use of microbial control agents has specific advantages over synthetic chemical insecticides. Microbial agents are usually highly specific, affecting only the target pests, allowing beneficial insects and natural predators of the pests to survive. There is little or no environmental impact associated with use of microbial agents and they are safe for the workers handling them. *Bacillus thuringiensis* is the most widely used of these agents. Its major advantages are that it can be grown in bulk by conventional industrial liquid fermentation making it relatively inexpensive to produce, although it still costs more to produce than most synthetic chemical insecticides; it is stable in storage; it is pathogenic to many of the world's major crop pests; and, once applied, it does not persist in the environment. In addition, reports of resistance developing in a target pest in the field are rare (Meadows, 1992).

B. thuringiensis synthesizes a parasporal inclusion (crystal) named δ-entotoxine, which is toxic to several insect orders and it can be composed of one or several insecticidal crystal proteins (ICP). *B. thuringiensis* spore preparations have been registered as insecticides since 1961, and considerable toxicologic data has been collected over the years. Prior to the early 1970's, some *B. thuringiensis* serovar. *kurstaki* spore preparations contained a toxic substance know as β-exotoxin. This toxin is a nucleotide analog that inhib its RNA synthesis and is distinct from δ-endotoxin (Goldburg & Tjaden, 1990). Because of this, for registration of *B. thuringiensis* strains for use in biological control, they need to be analyzed by both laboratory and field tests.

For experiments at the field level, the Environmental Protection Agency (US-EPA, 1988) published guidelines for those strains with high insecticidal potency.

Regulations are necesary to avoid contamination with *B. thuringiensis* products. The organism used needs be an authentic strain of *B. thuringiensis*, grown in pure culture with adequate controls to avoid any change in the characteristics of the original strain or contamination with other organisms. Before use, each lot needs to be tested by subcutaneus injections with one million or more spores into five 17 to 23 gram mice which remain healty for seven days post injection (Betz *et al*, 1990).

In Mexico, *B. thuringiensis* bioinsecticide use is still not extensive, because the manufacturing technology is in development and the availabe commercial products are imported and more expensive. We isolated 400 *B. thuringiensis* strains from soil or dead larvae. Of these, we selected four strains, two of which where highly toxic against lepidopteran and two which were toxic against lepidopteran and the coleopteran *Epilachna varivestis* (Mexican bean bettle). Because of the possibility of using these strains for bioinsecticide production in Mexico, we characterized four Mexican strains following the EPA (1988) and Betz *et al* (1990) recommendations and compared the differences vs five HD strains, which showed high toxicity 13 years after production by Howard Dulmage et al and storage in FCB-UANL (Galán-Wong, 1994).

Material and Methods

***B. thuringienis* strain history.** All the HD strains (HD-73, HD-187, HD-193, HD-263, and HD-530) were obtained from International Collection of Entomopathogenic *Bacilli* (Fac. de Ciencias Biologicas, UANL, Mexico). The *B. thuringienis* isolated in Mexico, C-4, C-9, GM-7, and GM-10 are placed in the same collection.

Biochemical data. The strains were biochemically tested following the guidelines given in Bergy's Manual (Sneath *et al*, 1986), for *B. thuringiensis*. The growth and fermentation were tested in a different media containing salts with 10.0 g/1000 ml of sorbitol, glucose, arabinose, melobiose, sucrose, rafinose, lactose, glycine, manittol, urea, maltose, dulcitol and celobiose as carbon source. Other characteristics were gelatinase activity; arginin, lysine, and ornitin decarboxylase; growth on thioglycolate; α and β hemolysis, and oxidase, peroxidase, caseinase, amylase, and catalase activity using Difco™ media following the manufacturer's guidelines. All the tests were done using tubes or petri dishes, with three replications. Each strain was inoculated and the tubes and dishes were incubated at 37°C for 48-72 h.

Flagellar antigen serotype analysis. The serotype analysis was based on serological tests using the *B. thuringiensis* antigenic flagellar reaction (H antigen, De Barjac & Bonnefoi, 1962; De Barjac y Frachon, 1990).

Antibiotic resistance patterns. The strains were grown on nutritive broth (Difco™) for 18 h and grown petri dishes with nutritive agar (Difco™). At this time antibiotic multidiscs for Gram (+) (Rigaux™) were placed. After this, the cultures were incubated at

37°C for 24-48h. The inhibition zones of susceptible strains to each antibiotic were detected visually.

Protein profile of the insecticidal δ-endotoxin produced. The strains were growed on nutrient agar (Difco) petri dishes. The dishes were incubated at 30°C until 95% sporulation (3-4 days). Double distilled water (5 ml) was added and the cultures were harvested by centrifugation at 12,000 rpm for 10 min at 4 °C in a JA-21. The pellets were washed two or three times with double distilled water and loaded on a polyethylene glycol 4000 (PEG) *gradient. The gradient was prepared with* 11.0 g PEG (Sigma Chemical Corp. St Louis, *MO, USA); 34.0 ml of 1.0 M* pH *7.4 phosphate buffer; and* 5 *ml spore-crystals* mixture. The mitxure was centrifuged at 1000 rpm for 3 min. The crystal phase (*interface*) was separated and washed twice with double distilled water and stored at -20°C until needed. The crystals were mixed with an equal volume of double-strength SDS-PAGE sample buffer (Tris 0.25 M, SDS 4%, glycerol 20%, and 2-β-mercaptoethanol 10%) and heated at 100°C for 5 min (Nagamatsu *et al*, 1984). The extracts were assayed by SDS-PAGE carried out in 10% polyacrylamide on 1.5 mm thick gel. Gels were run in a VERY SMALL II VERTICAL SLAB UNIT (Hoefer). 10% acrylamide gels were stained with 0.1% Coomassie brilliant blue (Laemmli, 1970). Molecular weights were estimated by *comparison* to these standard proteins (molecular weights in parentheses -KDa-): α_2-Macroglobulin reduced (170), β-*galactosidase (116.4), fructose-6-phostate-kinase* (85.2), glutamate dehydrogenase (55.6), aldolase (39.2), triosephosphate isomerase (26.6).

Inmunoblot. Discontinous buffer SDS-PAGE was performed in 10% polyacrylamide gels. Separated proteins were transferred to nitrocelulose membranes

using a Tris-glycine buffer. After transfer, membranes were blocked in TBS buffer containing 2% Tween 20. The membranes were then incubated in TBS -antiCry. The antiCry used were polyclonal antiCryIAb, antiCryIAc, and antiCryIIIa. The nitrocellulose membranes were then washed and developed as previously described (Towin *et al*, 1979).

Plasmid profiles. Plasmid profiles were detected using Gonzalez' method (Gonzalez *et* al, 1981) modified as following. Nine strains were reactived on petri dishes on nutritive agar for 18 h at 37°C, and grown in nutritive broth for 14-22 h at the same temperature (before sporulation). The culture was centrifugated at 10,000 rpm for 3 min, and washed one time with water. We used the Gonzales technique after this. Gels were stained with 0.5 mg of ethidium bromide per ml and photographed through a red filter on Polaroid type 107 film.

Bioassays. The strain was tested against both first instar larvae of the coleopteran *E. varivestis* on bean leaves and the lepidopteran *Helicoverpa zea, Heliothis virescens, Spodoptera exigua*, and *Trichoplusia ni* using diet incorporation bioassays. For *E. varivestis*, the leaves were inoculated with 100 ml of each concentration on an marked area of 33 cm^2 and allowed to dry. The concentrations employed were 10, 25, 50, 100, and 250 mg/50 ml of spore-crystal complex or active ingredient of the microencapsulated formulation. A disk was cut from each leaf and put in a plastic dish. Each leaf was infested with three larvae using five repetitions by treatment and one negative control. The dishes were maintained in a room at 25°C ± 3°C, and 65% ± 5% relative humidity (RH) for five days and supplied with one leaf each third day. The LD_{50} was obtained using the Probit statistical method (SAS, 1989). The

bioassays with the lepidopteran *Helicoverpa zea*, *Heliothis zea*, *Spodoptera* exigua, and *Trichoplusia ni* were made using first instar larvae and artificial media (Castro-Franco *et al*, 1995). The concentrations employed were 5, 10, 13, 15, 25, 50, and 100 µg/ml, with three replications and 25 larvae/replication, one larva per container. Each treatment was maintained at 28°C ± 5°C and 65% ± 5% of RH for one week. LD_{50}'s were obtained in the same way as the coleopteran larvae.

Sensibility to phages. We used the R-41 and CP-51 phages. These phages and *Bacillus cereus* strain NRRL 569 were supplied by Molecular Biology laboratory (FCB, UANL, México). The phages were grown on *B. cereus*, and separated by filtation using 0.22m Milipore™ membranes to obtain the phage solution. GM-7, GM-10, and HD-73 strains were grown on YP medium (Krieg, 1981), for 24-48 hrs, at 37°C. After this, 200 µl of the culture was mixed on 3 ml YPA media (semisolid), and transfered to a nutritive agar petri dish. When the YPA was solid, one drop of phage solution was added. Each dish was incubated at 37°C for 24-48 h. The inhibition zones were visually detected.

Mouse subcutaneus cytotoxicity test. This test was based on Betz *et al* (1990). Mexican strains were tested using the HD-41 strain as a positive control. It is reported to be a β-exotoxin producer (Arévalo-Niño & Galán-Wong, 1994). Each strain was grown on nutritive broth (Difco™) until sporulation. Spore numbers were counted using a New Bawer camera. Tubes were centrifuged at 2,000 rpm for spore precipitation and the pellets were resuspended in phosphate buffer (PBS). Six mice of 17-23 g were subcutaneously injected with approximately one million two thousand PBS-spores of each strain and a negative control injected with PBS only. The mice were maintained for eight days and then cytotoxic

activity was determined. In the case of infection or death of the mice, to be sure that the ulcers were produced by the strain injected, we took a tissue sample (0.5 grams approximately) and ground it in a sterilized mortar in PBS buffer. After this, we took 10 μl of this solution and inoculated it on nutritive broth tubes. Tubes were incubated for 36 h at 35 °C (until sporulation). Then the tubes were subjected to thermic shock in water at 80 °C for 10 min and inoculated into petri dishes with nutritive agar. The inoculated dishes were place at 35 °C for 48-36 h to grow *B. thuringiensis*. In addition, the tissue samples were histhopathologically examined by the Giemsa technique.

Results

***B. thuringiensis* strains history.** C-4 and C-9 strains were isolated form dead larvae of *E. varivestis* in Durango, Mexico. GM-7 and GM-10 were isolated from cultivated soil located in Valle del Yaqui, Son., Mexico. All Mexican strains have bipyramidal crystal. The size of the GM-7 crystal is approximately 1.6μ, and the GM-10 crystal is approximately 0.87μ.

Biochemical and morphological data. The *B. thuringiensis* strains showed differences between each carbon source utilization. All the strains fermented glucose and arabinose, asimilate maltose, dulcitol and celobiose, except HD-530 strain, which fermented (acid production) the two last carbon sources. The rest of the strains were variable metabolism with the differents source carbone (Table 1). We can see the same respect the metabolic activity on differents substrates (Table 2). All the strains produced acid on ornitin decarboxylase, can hydrolized casein, and exhibed catalase activity. No one grow on citrate or produced oxydase. All given negative response to indol.

Flagellar antigen serotype analysis. Serological results showed that C-4 and C-9 strains are serovar *kumamotoensis*; GM-7 and GM-10 strains are *aizawai*; HD-73, HD-187 and HD-263 strains are *kurstaki*; the HD-193 strain is *galleriae*; and the HD-530 strain is *morrisoni*.

Antibiotic resistance patterns. In this test, in general, GM-10, HD-187HD-263, and HD-530 were more resistant to antibiotics (Table 3). Al the strains were sensitive to cefuroxime (30 mg), erithromycin (15 mg), gentamicin (10 mg), penfloxacine (5 mg),

and tetracycline (30 mg). Only C-4, GM-7 and HD-530 were sensitive to trimethoprim-sulfamethoxasol.

Description of the insecticidal toxins produced. Protein profiles showed that all the strains tested had a protein band of 120-130 Kda. Inmunoblot assays showed that the strains crossreacted with a polyclonal antiCryIAb antiserum in a band of ca. 120 KDa. The C-9 and HD-193 crossreacted with a polyclonal antiCryIAc antiserum, in a band of ca. 120 and ca. 70 KDa respectively (Fig 1 y 2).

Plasmid profiles. *B. thuringiensis* strains tested showed variable plasmid profiles (Fig 3). C-4, GM-10, HD-73, and HD-263 showed five different plasmids; GM-7 four, HD-187 three, and C-9 and HD-193 two plasmids.

Bioassays for insect toxicity. In general, the HD-263 strain (*kurstaki*) was shown to be more toxic against the lepidopteran larvae, better than the HD-1-S-1980 control (LD_{50} -µg/ml- of 3.5, 15.6, 18.8 and 5.6 with HD-263; vs 22.3, 16.7, 26.3 and 19.8 with HD-1-S against *H. virescens, H. zea, S. exigua* and *T. ni* respectively)(Table 4). GM-7 and GM-10 were as toxic as HD strains against *H. virescens* and *T. ni* larvae, but less toxic against *S. exigua* larvae *vs* HD-1-S (LD_{50} -µg/ml- of 30.8 and 35.5 with GM-7 and GM-10 *vs* 31.7, 31.0 and 22.3 with HD-187, HD-193 and HD-1-S against *H. virescens* respectively; 73.7 and 44.4 *vs* 39.8, 92.8 and 26.3 against *S. exigua* respectively; 22.2 and 18.5 *vs* 25.7, 17.4 and 19.8 against *T. ni*, respectively). C-4 shown the lowest toxicity value compared to GM-7 and GM-10 (LD_{50} of 241 *vs* 30.8 and 35.4 against *H.* virescens; 66.3 *vs* 73.3 and 44.4 against *S. exigua;* 29.3 *vs* 22.2 and 18.5 against *T. ni*, respectively). C-9 was least toxic against lepidopteran larvae (LD_{50}

of 219.35 µg/ml against *T. ni*), but showed more activity against *E. varivestis* larvae than the C-4 strain (LD_{50} of 397 µg/ml *vs* 587µg/ml of C-4) . C-4 and C-9 showed toxicity against *E. varivestis* larvae only (Table 4).

Mouse subcutaneous toxicity test. Cytotoxic tests demonstrated that C-4 can produce ulcers similar to that of the HD-41 strain (β-exotoxin producer). Of six mice tested, two C-4 injected showed ulcers of 0.4 and 0.7 cm and two HD-41 mice injected showed ulcers of 0.5, and 0.8 cm. The C-9 strain produced larger ulcers than the HD-41 strain. Of six mice, four showed ulceres of 0.7, 0.8, 1.0, and 1.3 cm, respectively. We can reisolate *B. thuringiensis* of the C-9 and HD-41 ulcers, but not of the C-4 ulcers. Histopathologic examinations revealed nectrotic and lymphocytic areas in the ulcer caused by all the strains.

Discussion

The posibility of using strains of B. thuringiensis with high insecticide activity has lead to numerous studies, among wich are field trials to control crop pests. The proof of results obtained in laboratories and greenhouses is carried out in this manner.

As with a chemiucal insecticides, registration of biological pesticides is the first step in wide spread commercialization, In order to obtain registration, it is necessary to characterize the strains of *B. thuringiensis*. Therefore, we consider it essential to evaluate the Mexican strains that show activity against coleopteran and lepidopteran larvae according to the protocol put forth by the EPA.

The results show the strains isolated in Mexico have insecticidal activity similar to those in the HD collection (LD_{50} of 30.8 and 35.5 µg/ml for GM-7 and GM-10 compared with 31.7, 31.0 and 3.5 µg/ml for HD-187, HD-193 and HD-263 respectively against *H. virescens*; 73.7 and 44.4 µg/ml compared with 39.8, 92.8 and 18.8 µg/ml respectively against *S. exigua*; 22.2 aand 18.5 µg/ml compared with 25.7, 17.4 and 5.6 µg/ml respectivelyagainst *T. ni*). In addition, the different degrees of activity of the different serovars and strains against different insects that were observed were in agreement with the earlier report of Percy and Fast (1983). In this respect, C-4 showed the lowest toxicity compared with GM-7 and GM-10. GM-7 and GM-10 also were susceptible to attack by phages PC-51 and R-41, which can be undesirable in the field (Debabov *et al*, 1984). Therefore, it is desirable that the strain be free of phages (Barack *et al*, 1988). However, EPA omits this requeriment (US-EPA, 1988).

The molecular weight of the PCI from strains with activity against lepidopteran corresponds to that reported for these serovar (ca. 120 KDa). Strains C-9 and HD-193 possess a protein of ca. 120 and 70 KDa respectively that reacts with AntiCryIAc. We also found that strains that demonstrate toxic activity against coleopteran are capable of producing damage similar to that reported for β-exotoxin producing strains in female Balb/C mice. Of six mice injected per group, C-9 caused raw ulcers 0.7 to 1.3 cm in four of them; C-4 produced similar ulcers (0.5 cm) in only two mice and the positive control, HD-41, caused ulcers between 0.4 and 0.8 cm in three of the mice.

B. thuringiensis was reisolated from the lesions only from mice inoculated with spores of HD-41 and C-9. From these results, only GM-7 and GM-10 can be used safety in the field (Betz *et al*, 1990).

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Table 1. *B. thuringiensis* assimilation and fermentation of several carbon sources

Carbon source	*Bacillus thuringiensis strains*[1]							
	C-4	C-9	GM-7	GM-10	HD-187	HD-193	HD-263	HD-530
Sorbitol	+	+	+	a	+	a	+	a
Melobiose	+	+	a	+	a	a	a	a
Saccharose	a	a	a	-	-	-	-	a
Rafinose	+	+	+	+	a	a	+	a
Lactose	a	+	-	+	a	a	a	a
Glycine	+	+	-	a	+	+	+	-

1. + growth, - no growth, a acid production

Table 2. Some biochemical responses of *Bacillus thuringiensis strains*

	Bacillus thuringiensis strains[1]							
Strain activity[2]	C-4	C-9	GM-7	GM-10	HD-187	HD-193	HD-263	HD-530
Arginine decarboxylase	a	a	b	a	a	b	a	a
Lysine decarboxylase	a	b	b	a	a	b	b	a
Blood hemolysis	β	+	+	β	+	α	-	+
Lechitinase	-	-	-	-	-	+	-	+
Amylase	-	-	-	+/-	+/-	+/-	+	+/-

1. a acid production, b alcali production, + growth, - no growth, α and β hemolysis

Table 3. *Bacillus thuringiensis* antibiotic resistance patterns

		Bacillus thuringiensis strains							
Antibiotic[1]	Dose	C-4	C-9	GM-7	GM-10	HD-187	HD-193	HD-263	HD-530
Ampicillin	10 μg	-	-	+	+	+	-	+/-	-
Cefalotoxin	30 mg	-	+	+/-	+	+/-	-	+	+
Ceftaximide	30 mg	-	+	+/-	+/-	+	-	+	+
Dicloxacillin	1 mg	+	+	+	+	-	+	+	-
Penicillin	10 UI	-	+	+	+	+	+/-	+/-	+
Trimesuxsol[2]	26 mg	-	+	-	+/-	+	+	+	-

[1]. + Inhibition, - Resistancy, +/- Slighty inhibition.
[2]. Trimethoprim-sulfametoxasol

Table 4. LD$_{50}$ and LD$_{90}$ of six strains of *B. thuringiensis* for lepidopteran larvae.

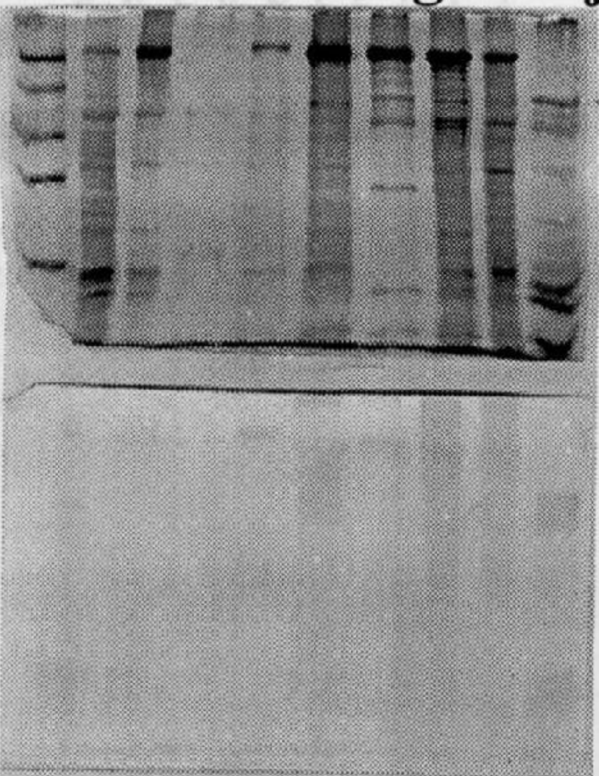
Strains		*Helicoverpa virescens*	Conf. Interval	*H. zea*	Conf. Interval	*Spodoptera exigua*	Conf. Interval	*Trichoplusia ni.*	Conf. Interval
HD-1-S	LD$_{50}$	22.3	18.2-27.48	16.7	13.5-28.8	26.3	21.5-32.1	19.8	12.8-29.3
	LD$_{90}$	95.3	57.1-257.3	71.2	34.6-290	127.9	81.0-311	82.5	58.3-187
C-4	LD$_{50}$	241	45.2-1287	24.3	19.9-37.2	66.3	39.2-112	29.3	18.2-43.7
	LD$_{90}$	5110	157-4.4x10^{6}	88.6	52.2-196	1195	264-2.2x10^{5}	772	140-2.1 x10^{5}
C-9	LD$_{50}$		n.d.		n.d.		n.d.	219	156-668
	LD$_{90}$		n.d.		n.d.		n.d.	5762	398-2.5x10^{5}
GM-7	LD$_{50}$	30.8	24.8-38.4		n.d.	73.7	26.3-145	22.2	10.6-44.2
	LD$_{90}$	162	97.1-438		n.d.	300	167-3366	86.1	57.9-203
GM-10	LD$_{50}$	35.4	27.5-45.6		n.d.	44.4	29.3-68.9	18.5	12.8-39.4
	LD$_{90}$	227	120-775		n.d.	248	178-472	69.4	34.6-99.3
HD-187	LD$_{50}$	31.7	23.5-42.8	19.3	12.7-30.2	39.8	20.4-53.4	25.7	10.0-42.9
	LD$_{90}$	175	82.3-762	439	106-6893	289	91.8-2685	854	29-3.3 x10^{5}
HD-193	LD$_{50}$	31.0	22.7-42.2	14.4	10.6-27.7	92.8	34.3-250.7	27.4	15.0-34.2
	LD$_{90}$	190	84.0-940	94.4	53.7-246	1794	151-2.1 x10^{6}	53.6	38.7-101
HD-263	LD$_{50}$	3.50	1.1-11.0	15.6	9.74-25.3	18.8	14.7-24.0	5.61	4.57-6.88
	LD$_{90}$	51.1	32.8-120	177	45.9-2477	135	62.2-611	24.2	17.7-44.5

Against *E. varivestis* only C-4 (LD$_{50}$ = 587 ~388-2.8x10^{6} IC, µg/ml), and C-9 (LD$_{50}$ = 397 ~264-2.3x10^{5} IC, µg/ml).

Probit statistic (SAS, 1989) with the confidence interval of 95%. n. d. not determined

Supported by grant of CONACYT 3559-N9311

A



B

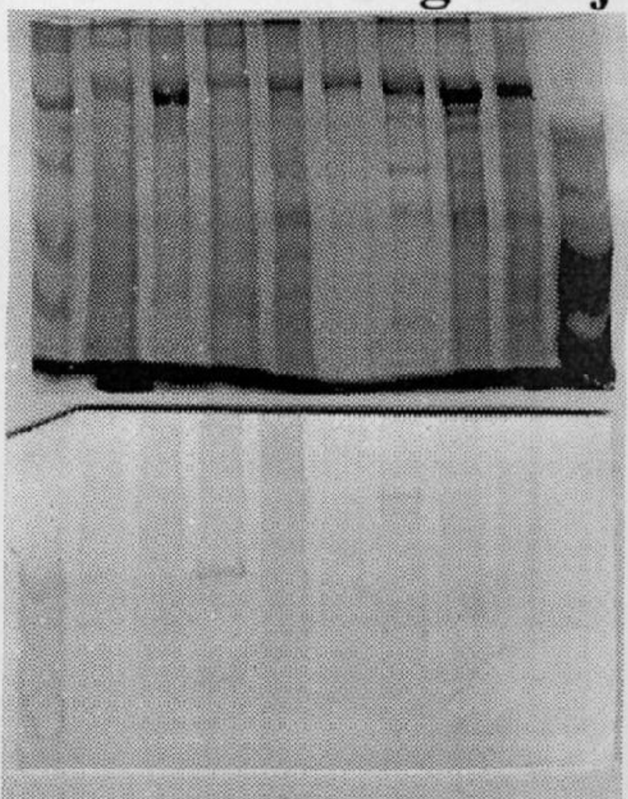
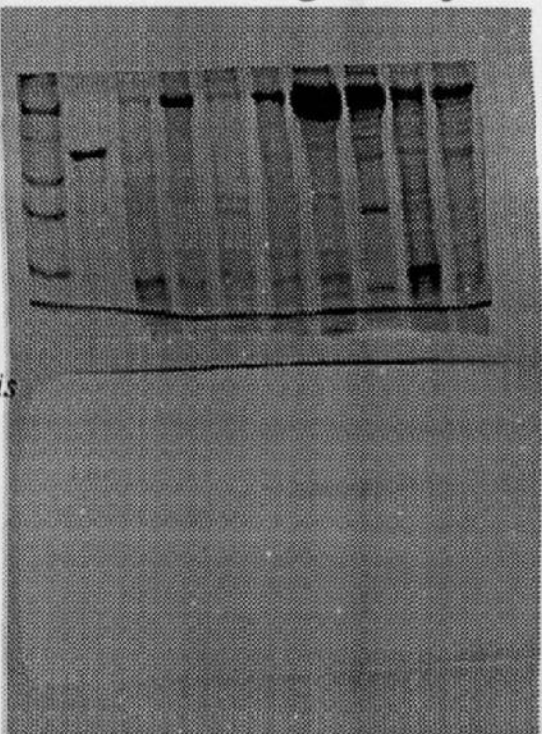


Fig. 1. Protein profiles and inmunoblot of Mexican and HD strains assayed with antiCryIAb (HD-1; A) and antiCryIAc (HD-73; B)

A



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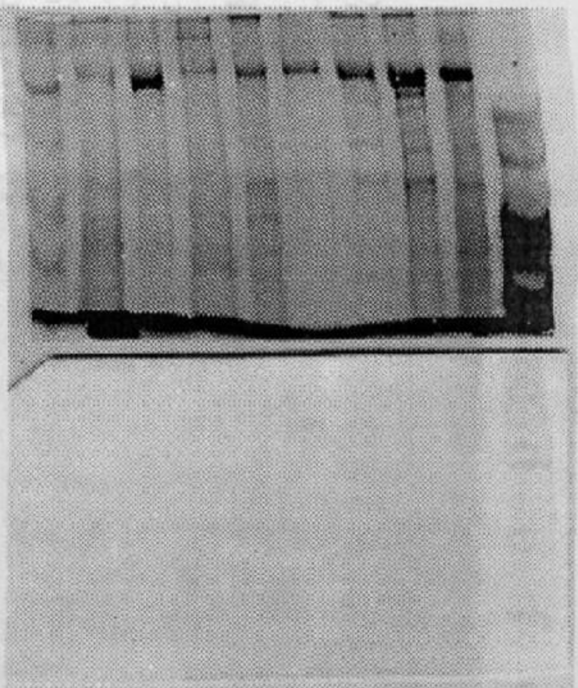


Fig. 2. Protein profiles and inmunoblot of Mexican and HD strains assayed with antiCryIIIA (*Bt tenebrionis*; A) and antiCryIIIC (GM-33; B)

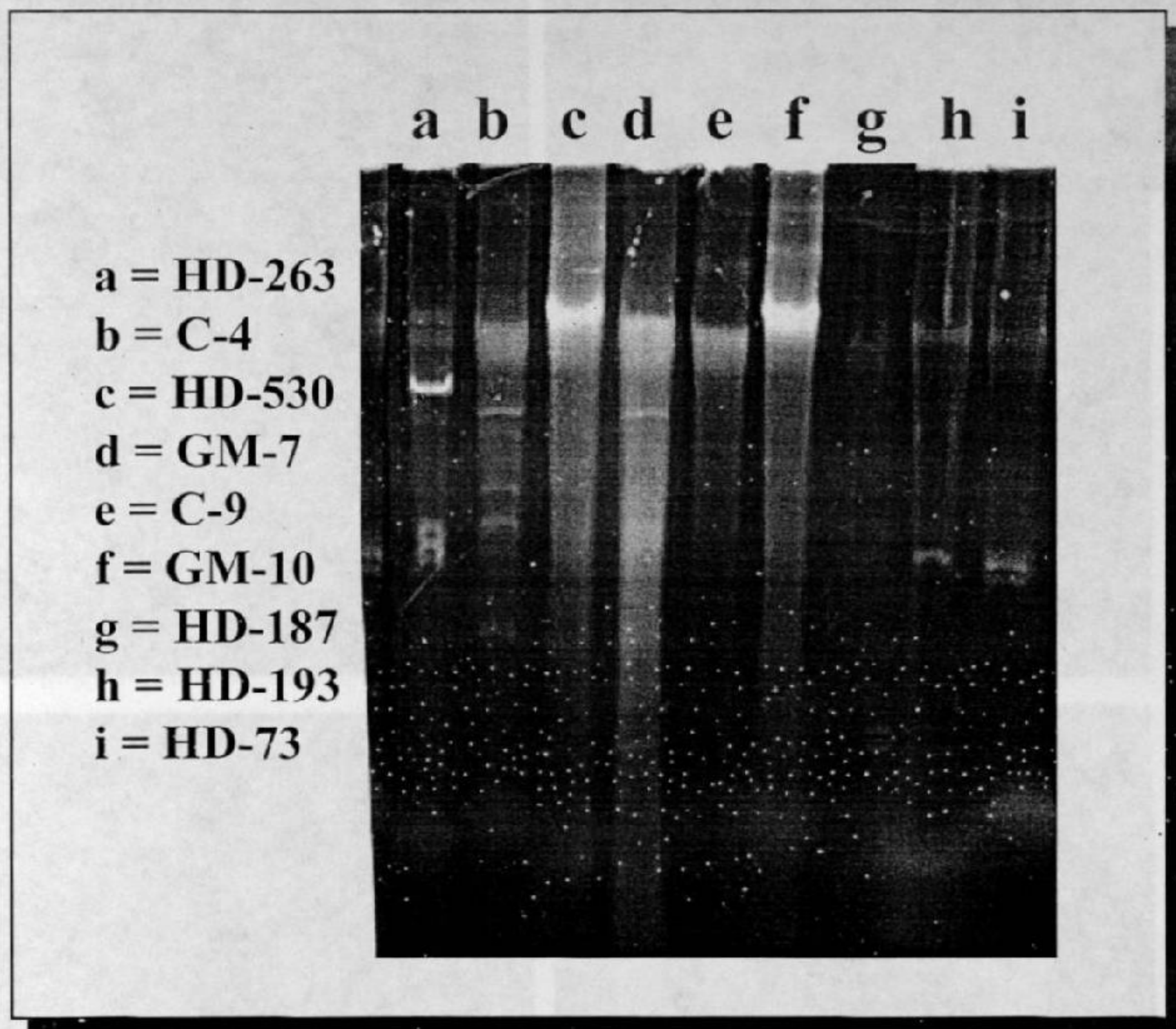
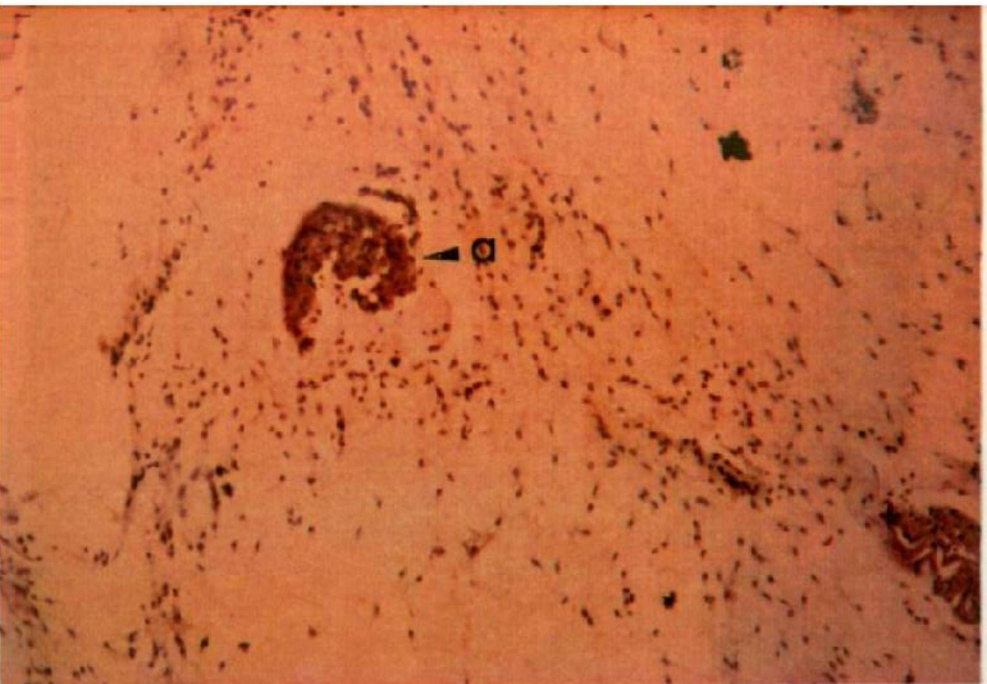
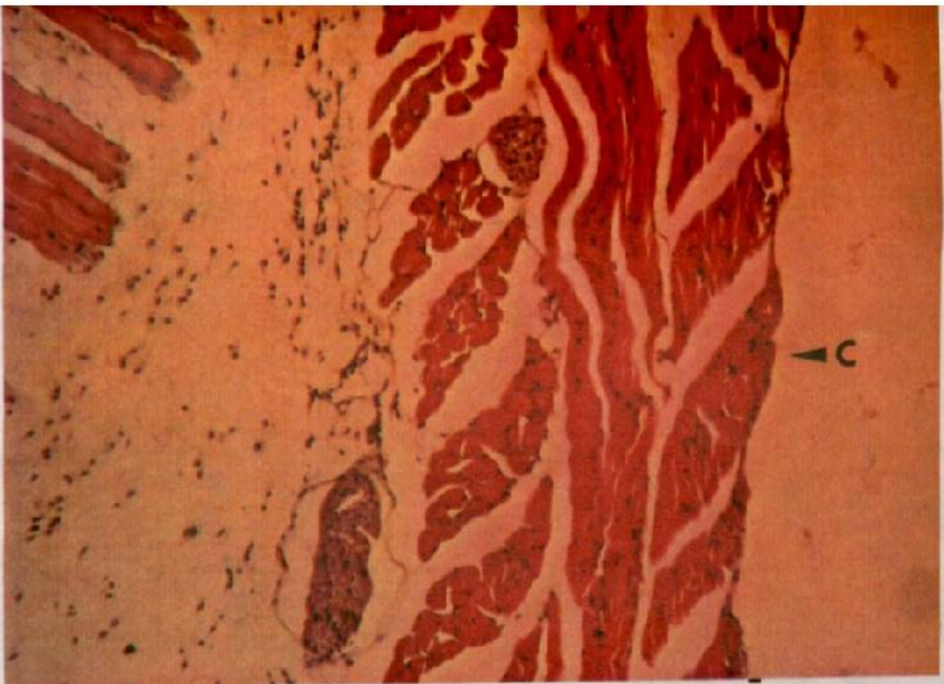
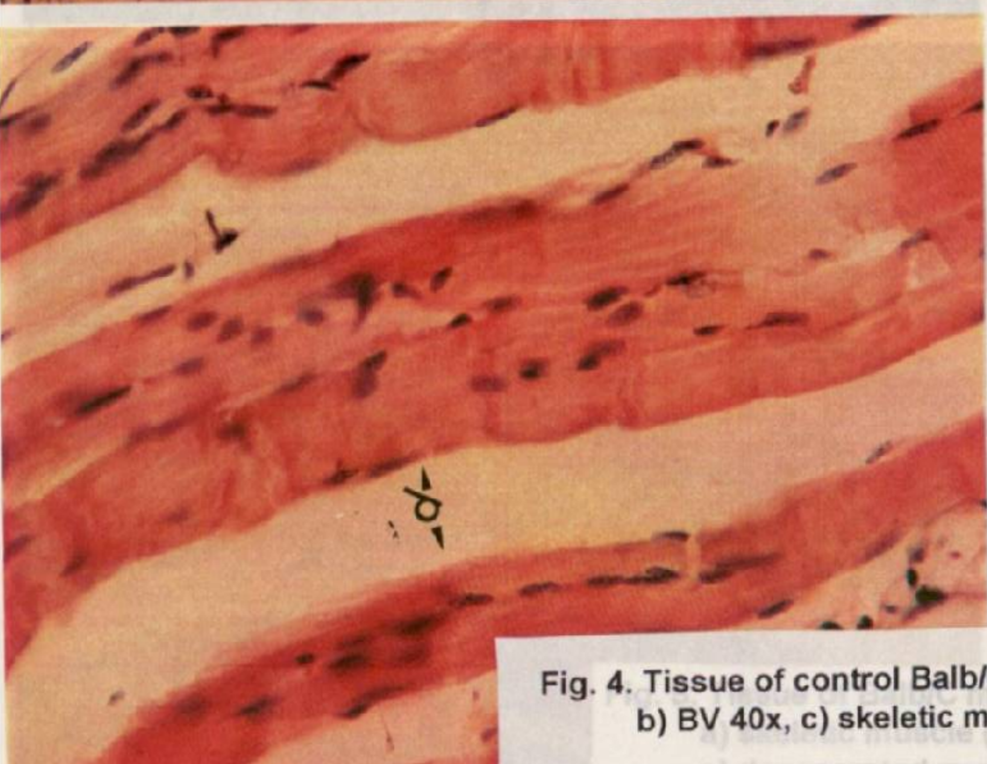


Fig. 3. Plasmid profiles of Mexican and HD strains







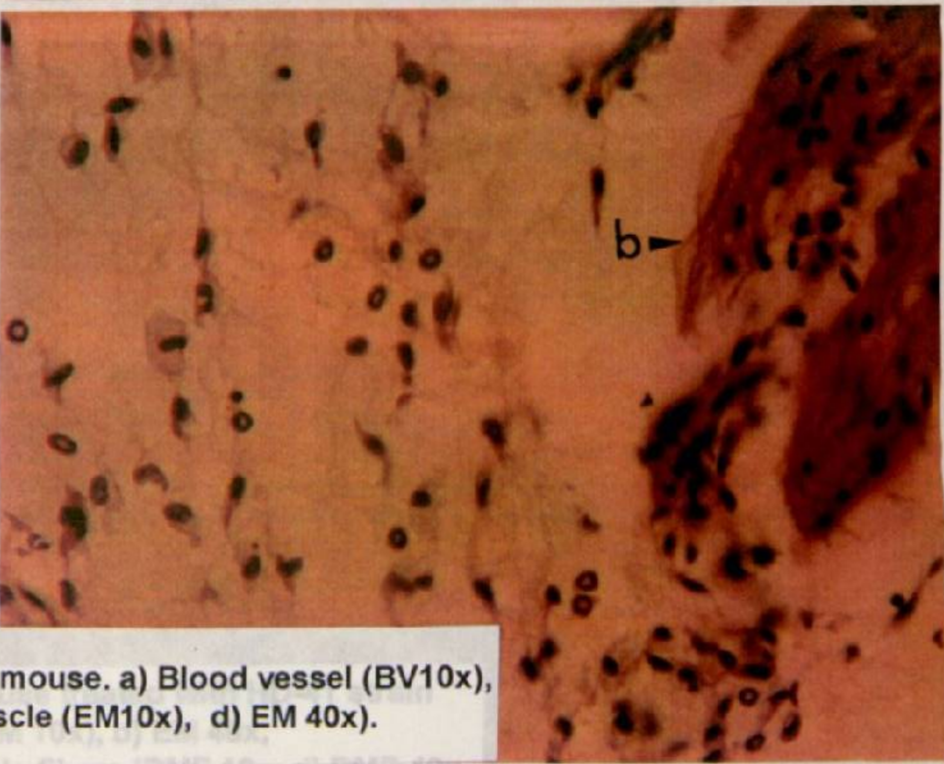
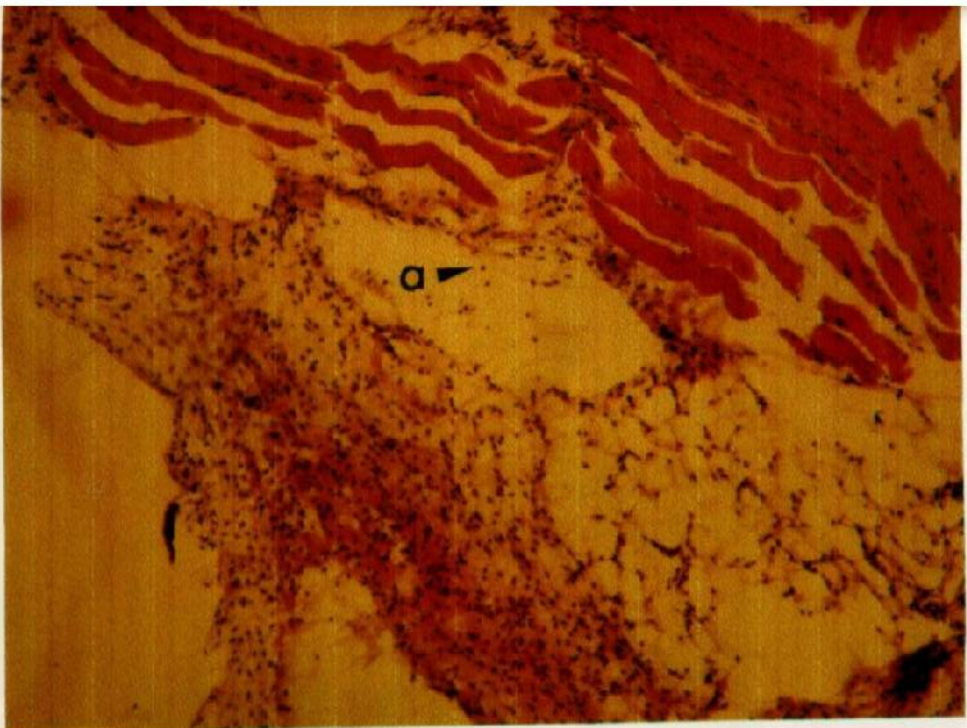
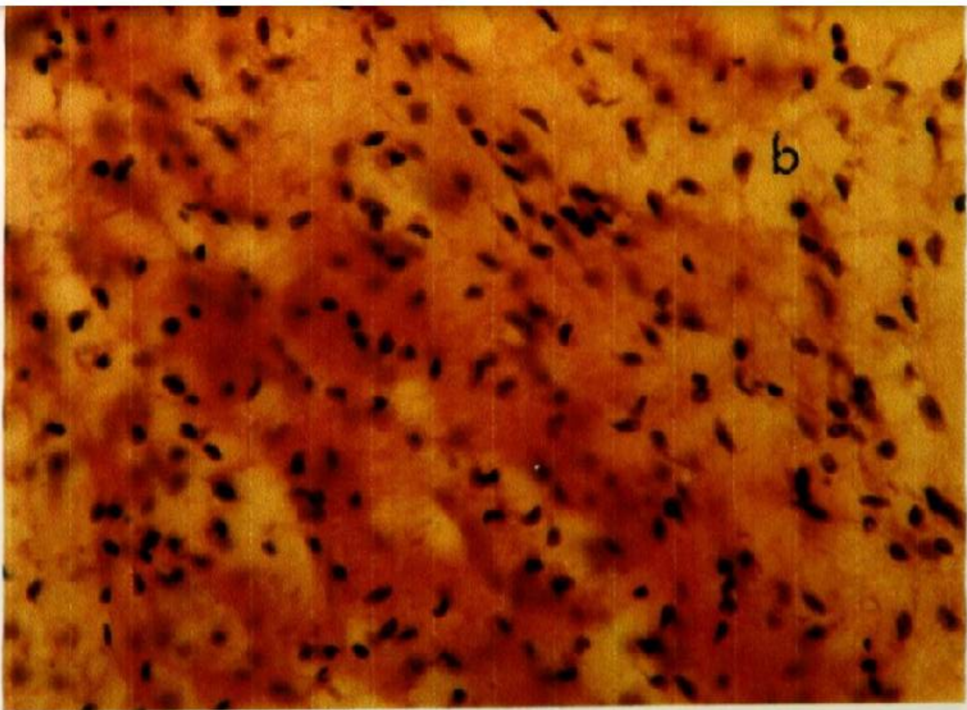
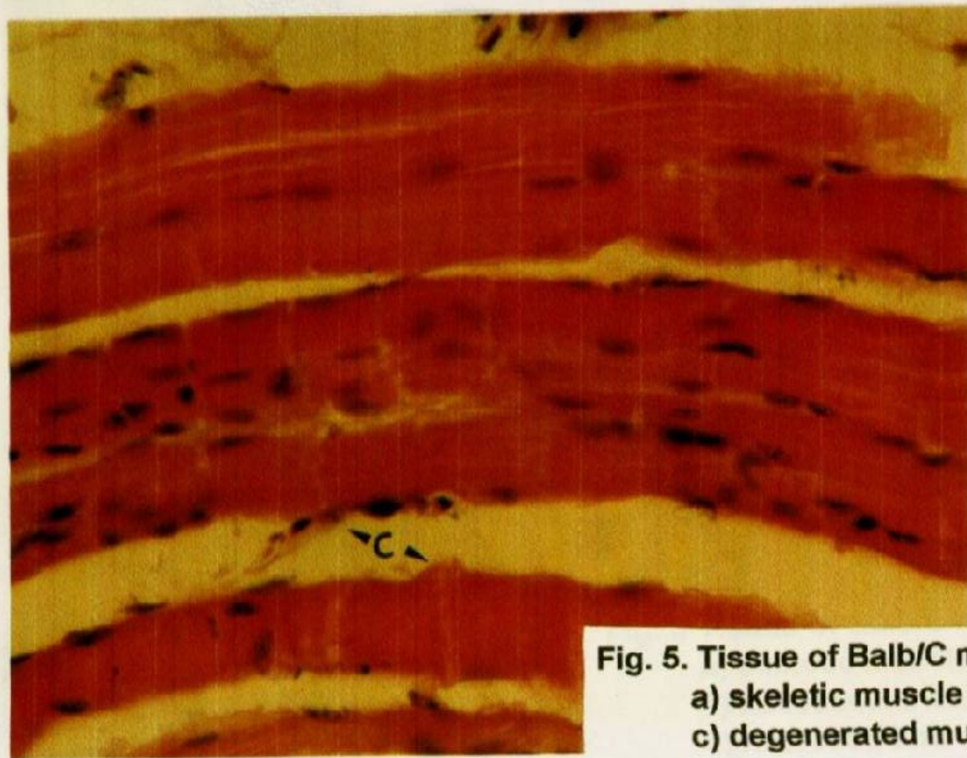


Fig. 4. Tissue of control Balb/C mouse. a) Blood vessel (BV10x), b) BV 40x, c) skeletic muscle (EM10x), d) EM 40x).







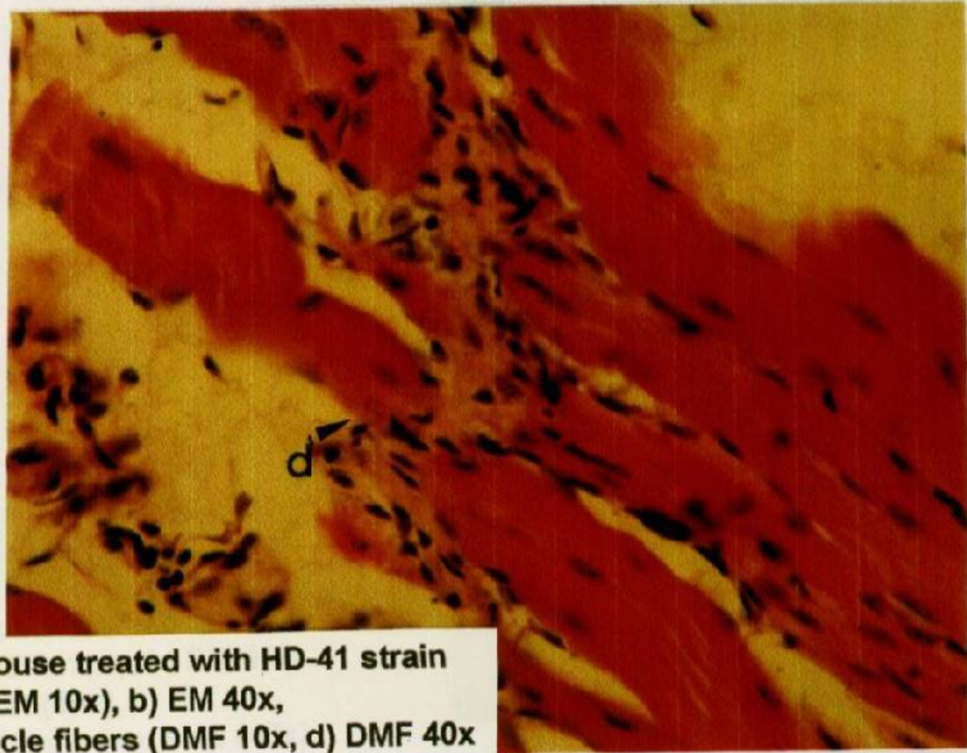
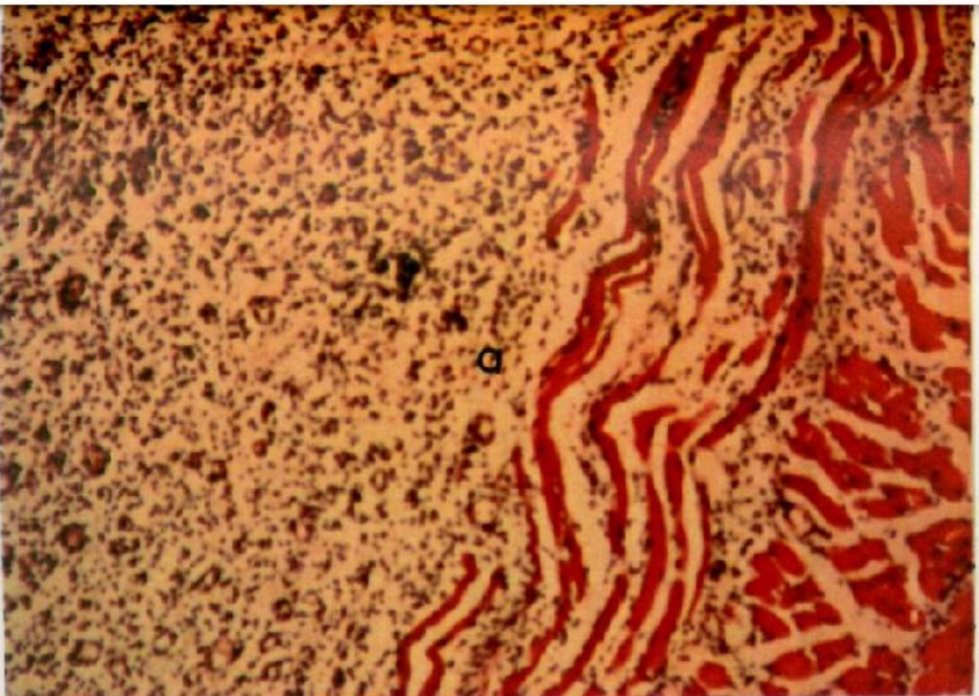
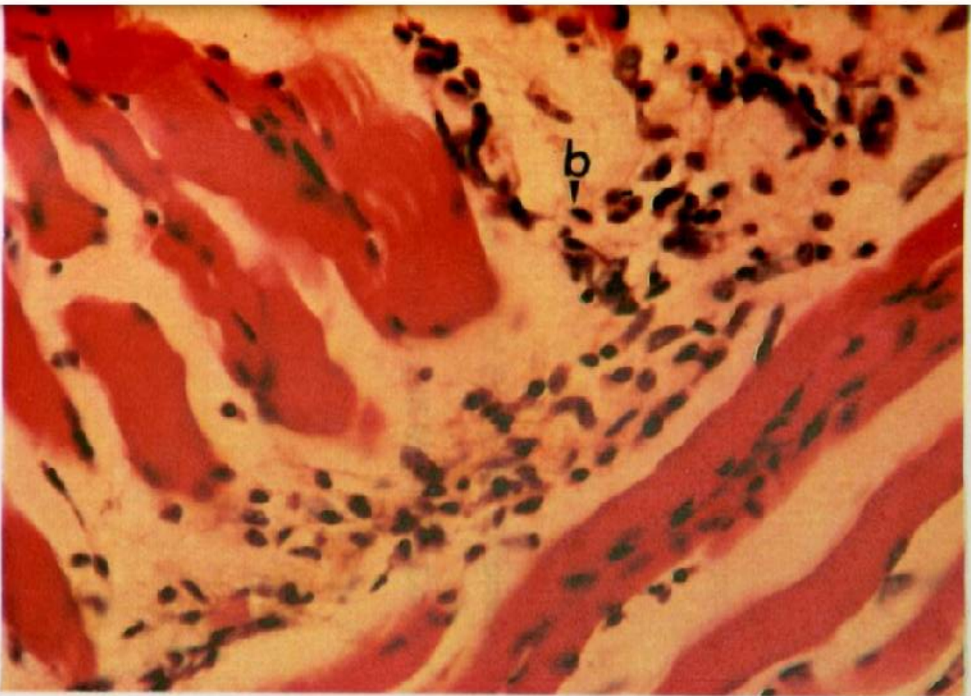
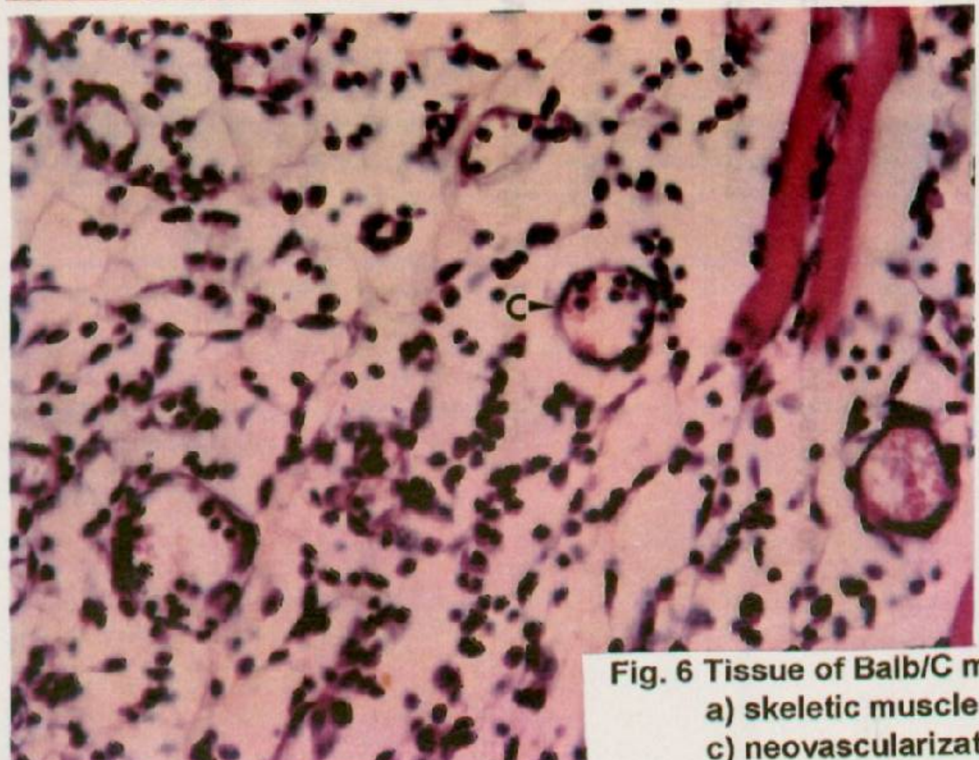


Fig. 5. Tissue of Balb/C mouse treated with HD-41 strain
a) skeletic muscle (EM 10x), b) EM 40x,
c) degenerated muscle fibers (DMF 10x, d) DMF 40x







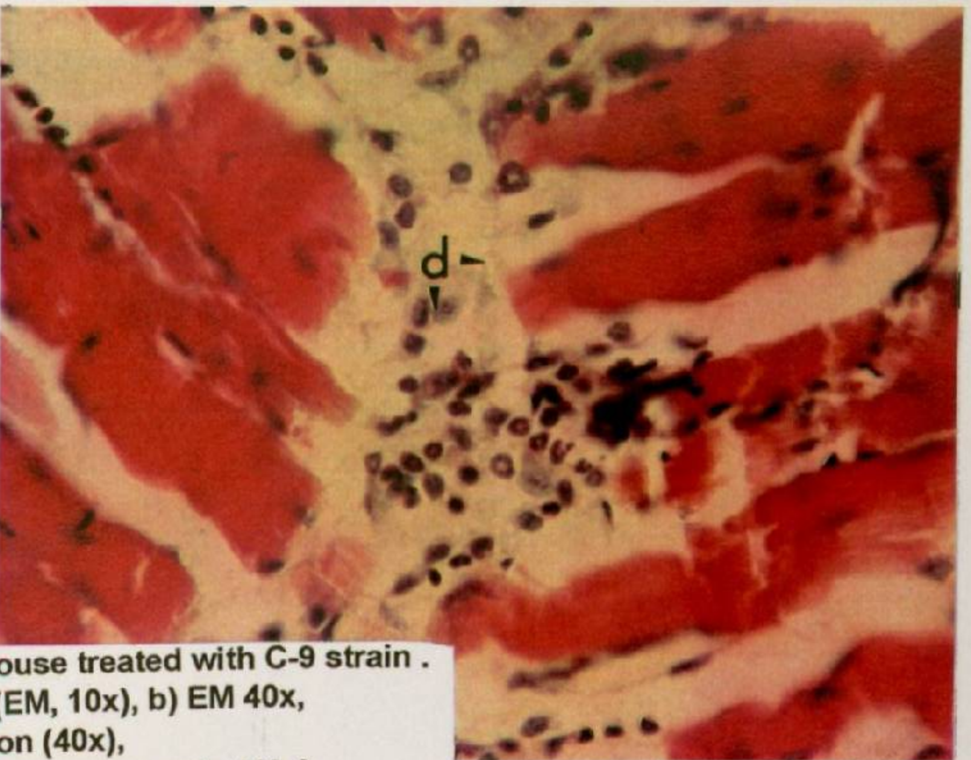


Fig. 6 Tissue of Balb/C mouse treated with C-9 strain .
a) skeletic muscle (EM, 10x), b) EM 40x,
c) neovascularization (40x),
d) necrosed and infiltrated muscle (40x).

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Biological and Microbial Control

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New granular formulations based on corn meal with different serovar. of *Bacillus thuringiensis*

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L. J. Galán-Wong and H. A. Luna-Olvera

ABSTRACT

Corn starch based formulations were used to compare the toxicity of six strains of *Bacillus thuringiensis* (*Bt*) belonging to four serovars, *aizawai*, *galleriae*, *kurstaki*, and *kumamotoensis*, against four lepidoteran species, *Helicoverpa zea*, *H. virescens*, *Spodoptera exigua*, and *Trichoplusia ni*. Three of the strains were from the Howard Dulmage collection (HD) maintained at the University of Nuevo Leon (UANL) and three were isolated in Mexico. Laboratory bioassay results were used to select strains for field experiments.

Field tests were conducted on corn plots in a semi-arid region in central Mexico during 1994 and 1995 using two concentrations of spore-crystals, 2 and 4%, comparing the six experimental strains with a chemical insecticide (carbaryl) and a commercial bioinsecticide (Dipel™). In 1994 the yields of corn obtained from two HD strains (*galleriae* and *kurstaki*) and one Mexican strain (*aizawai*) were significantly higher ($p<0.05$) than from the biological insecticides but were not significantly different from the carbaryl. The most dramatic differences were observed when a 4% spore-crystal complex was used. The other strains were not significantly different from the controls. These tests indicate that *Bt* survived in the gelatinized starch matrix complex.

In the 1995 field test, the three most toxic strains were evaluated individually or by mixing two at a time in the same granular formulation at a total dose of 3%, and a formulation prepared two years earlier was also tested. The highest yields of corn were

obtained with strain HD-263 (*kurstaki*), which had originally shown the highest efficacy in bioassays, and with strain GM-10 (*aizawai*), formulated two years earlier. This suggested that the product can be produced and retain shelf-life suitable for commercialization.

KEY WORDS: Granular formulation, *Bacillus thuringiensis*, bioinsecticide, lepidopteran.

Methods of application of *Bt* to crops have been modified significantly in recent years in order to increase toxicity for target insects and increase residual time. The active ingredient has been applied as a liquid spray. One formulation was composed of spore-crystals mixed in water with other additives or emollients used primarily with chemical insecticides. The mixture was sprayed directly on the plants. Moreover, there have been reports of the possible utilization of granular formulations (Angus & Lüthy, 1971; Amhed *et al*, 1973). Using corn starch had given better results than the liquid preparations, but the disadvantage of granular formulations is that they are lost in the air or the soil and only can be used in crops like corn and cotton (Couch, 1978). For this reason additional materials were added as stabilizing, potentiating, and adhering agents. Positive results were observed using baits of corn flour, or adherents such as carbohydrates including sucrose (Norris, 1978). Encapsulating agents such as alginate-clay had been shown to prolong residual time (Fravel *et al*, 1985), or bacterial alginates like a good option to prepare biocontrol formulations (De Lucca *et al*, 1990). Granular formulations of *Bt* spore-crystals based on corn starch have been shown to be effective bioinsecticides (Dunkle and Shasha, 1988). Additives to these materials can be used, such as solar protectants like congo red, to impregnate the granules to protect from ultraviolet light (Dunkle and Shasha, 1989) as well as feeding stimulants like Coax™ (Bartelet *et al*, 1990) or adhesives such as 2-propanol to prevent rain washoff (McGuire

& Shasha, 1992). Another UV protectant reported is melanin for increased mosquitocidal activity of *Bt* serovar. *israelensis* (Liu *et al*, 1993).

In Mexico there has not been much commercialization of products based on *Bt* partly because of the high cost of imported products and partly because the biotechnology of production is still developing. Highly effective strains of *Bt* have been isolated and the technology for the production of inexpensive granular fornulations using gelatinized corn starch based on an industrial product for making tortillas (nixtamalized flour) has been developed (Galán-Wong & Padilla-Rodríguez, 1991; Castro-Franco, 1994). The solar protectant tested successfully to make these granules was malachite green. Sucrose, vegetable oil and isopropanol were used as stabilizers. In this work, we will demonstrate the feasibility of production and use of the described granular formulations based on the activity of different serovars. of *Bt* for the control of lepidopteran larvae in the field.

Materials and methods

***B. thuringiensis* strains.** The six strains that were used for the production of spore-crystal complexes of *Bt* were from the lot HD; 187, and 263 (*kurstaki*) and 193 (*galleriae*), and three isolated in Mexico, GM-7 and GM-10, (*aizawai*), and C-4 (*kumomatoensis*). All of these were obtained from International Collection of Entomopathogenic Bacilli (Facultad de Ciencias Biológicas, UANL, Monterrey, N. L., México).

Production of the Active Ingredient (AI). The strains were propagated in tubes of nutritive agar, and at 24 hours, 250 ml Erlenmeyer flasks containing 50 ml of tryptose phosphate sterile broth (Difco) were inoculated. The broth was agitated at 250 rpm in a mechanical stirrer at 30°C (Incubator Shaker Series 25, New Brunswick Sci, Inc.). After 18 hours 500 ml Erlenmeyer flasks containing 150 ml of sterile fermentation media were inoculated. A volume of approximately 1% v/v of an 18 hour culture of each *Bt* strain (described above) was used as the inoculation in each case. The fermentation media containing soy flour, 20 g; corn soaking liquid, 10 g; molasses, 20 g; calcium carbonate, 1 g; distilled water, 1000 ml was homogenized and the pH was adjusted to 7.0±0.2. The media was autoclaved for 2 hours and agitated until it reached 100% oxygen saturation (O. S.). The 500 ml Ehrlenmeyer flasks were agitated and 18 hr later were used to inoculate 14 liter fermentation flasks (New Brunswick Sci, Inc., model MF-214) containing

9 liters of fermentation media. Fermentation conditions were the following: temperature, 25±3°C; pH 7.0±1.0, agitation 700 rpm, aeration 1 vvm (volume air/volume media/minute). Starting at this time pH readings were made and microscopic examination was done to monitor the development of spores and crystals. pH adjustment and foam control were done automatically and temperature and OS were recorded continuously. When the culture contained 95% free spores, agitation was stopped and the pH was adjusted to 7.0. Then the extraction of spore-crystal complexes was done by coprecipitation with lactose (Dulmage *et al*, 1970).

Determination of LD_{50} and LD_{90}. To evaluate the toxic activity of the spore-crystal complexes obtained, bioassays using artificial diets of Shorei modified media (Castro-Franco *et al*, 1995) were performed. The assays were done using neonatal larvae of *Helicoverpa zea, H. virescens, Spodoptera exigua,* and *Trichoplusia ni.* Two concentrations of 50 and 500 mg/ml of the A. I. were tested. Afterwards, seven concentrations were selected, depending on preliminary results, and evaluated in triplicate, 25 larvae/replicate, each one in individual cups. Each lot of 25 larvae was mantained individually at 28°C and 55% relative humidity for eight days. Controls were maintained similarly except that an artificial diet with granules without *Bt* was used. Other controls consisted of a standard *Bt* (HD-1-S-1980) and one without *Bt* or granules (Beegle *et al*, 1986). The number of dead larvae were counted to obtain the LD_{50} and LD_{90} by the Probit method (SAS, 1989).

Inhibitory effect. In bioassays done previously, it was noted that surviving treated larvae were smaller than the controls. In order to evaluate this effect that other

authors have called the inhibitory dose, the larvae were weighed and the weights were compared with the controls. All living larvae were weighed and the average weight was calculated for each replicate. Although this effect was noted in all the strains, we tested only the strains GM-7 and GM-10 and compared them to the standard HD-1-S-1980. The assay was performed on larvae of *Helicoverpa*, *Spodoptera* and *Trichoplusia* grown on artificial diet containing 100, 50, 25, 15, 13, 10 and 5 mg/ml.

Preparation of formulations. The preparation of the granules was done by mixing 100 g of powdered sugar, 100 g of nixtamalized (pregelatinized, Maseca™) corn starch, 5 ml of vegetable oil, and 4.1, 6.15 or 8.2 g of spore-crystal complex from each strain, to obtaine granules of 2, 3 or 4% active ingredient respectively. The method of McGuire and Shasha (1992) was modified and the cool polymerization was done by combining 70 ml of water, 30 ml of isopropanol, 0.05 g of malachite green and 0.05 ml of formaldehyde, the latter as preservative. This was mixed with a spatula, left for 30 min and passed through 20 mesh sieve, forming granules. This was left to dry at room temperature until it reached 10% humidity. To evaluate the toxic activity of the strains and their shelf life, these preparations were evaluated after six months storage. Percent mortality was measured with *Trichoplusia ni* using artificial diets with concentration of 50 and 500 mg/ml as described above. Percent mortality was obtained using 75 larvae in three lots of 25 larvae per lot. The toxic activity of GM-10 was measured two years after preparation.

Field trials. Field trials were performed using corn (*Zea mays*) plots at the experiment station at URUZA, University of Chapingo, located in Bermejillo, Dgo.,

México. This is a semi-arid region in Central Mexico. In the first year (1994) the experimental design was a 2 x 8 x 4 factoral, corresponding to two levels of treatment (2 and 4%), six strains (C-4, GM-7, GM-10, HD-187, HD-193, and HD-263), chemical insecticide (carbaryl), the bioinsecticide Dipel 2x™ and the control; and four repetitions. Granules containing 2% A. I. were applied in the first block along with Dipel and the control. The plots were 4 x 10 meters for each treatment. The second block was treated with granules of the same strains at 4% A. I., carbaryl at recommended levels, and the control similar to the previous block. Artificial infestations with insects were not done in order to observe the effects of treatments in natural conditions. Only emergency irrigations were made, three altogether. When the presence of lepidopteran larvae was noted (usually after a rain) the number of larvae was counted on five plants per plot and the insecticides were applied. Larvae were counted again one week post treatment. In total three applications were done. The corn yield for each treatment was determined taking the total weigth of dry grains.

In the second period (1995), the experimental design was 10 x 4 factorial where 10 represented three strains (GM-10, HD-193, and HD-263), and combinations (GM-10 + HD-193, GM-10 + HD-263, and HD-193 + HD-263), all at level of 3% A. I., using 1.5 % of each one in a case of formulations with two strains, Dipel and carbaryl following manufacturer's recomendations, granules without A. I. and untreated control. Parameters evaluated were: percent of insect damage, relative damage, percent relative severity, and yield of grain (Castro-Franco, 1994).

Results

Production of active ingredient. We used fermentation process for this work. To mantain the pH of the fermentation media between 6.0 and 8.0, different batches of *Bt* required different amounts of base or acid. The same thing occurred with the antifoam agent and the time required to reach 95% free spore-crystals. Yield values of spore-crystals and paste were different for different batches. These results are shown in Table 1. HD-187 and HD-193 yielded the lowest values of product, but the lowest value of A. I. came from HD-193.

Determination of LD_{50} and LD_{90}. The Probit method was used to obtain the LD_{50} and LD_{90} of seven concentrations of AI for each strain and the standard HD-1-S-1980. (Table 2). These results show that for most insect species tested, the strain HD-263 was the most active and C-4, the least active.

Inhibitory effect of *Bt*. The growth inhibition demonstrated by the larvae cultivated on different doses of spore-crystals is shown in Table 3. The results show that the effect diminishes as concentration diminishes. However, the weights were always significantly less than the controls for all concentrations of A. I

Toxic activity of granular formulations. Percent age mortality of larvae (*T. ni*) after feeding on formulations produced six months and two years previously demonstrated that the activity was conserved for six months and two years, respectively (Table 4).

Field trails. Larval counts for the first trial (1994) are shown in Table 5. The number of larvae present decreased one week after the application of the different treatments except for the control. No larvae were found after the first application of chemical insecticide, but larvae were found in subsequent applications. The results of the second application of the granular formulation were the same.

With respect to harvest yield, for the 2% treatmnent, analysis of variance showed significant differences at a minimum of 1564.5 Kg/ha (Table 6). Using this value it was shown that the most effective strain is GM-10 and was comparable to GM-7, HD-193 and HD-187. Strains C-4, and HD-263 demonstrated values similar to Dipel™. In all cases, insecticide treatments were superior to the control. For the 4% treatment, analysis of variance showed significant differences at the minimum of 1372.1 Kg/ha (Table 6). GM-10, HD-263, and carbaryl shown the best control of pests; and GM-7, HD-193, and HD-187 were comparable. Strain C-4 had the lowest value and was barely superior to the control. In all cases, insecticide treatments were superior to the control. In general, the differences in yield were greater at 2% and the amount was greater at 4%.

For the second period (1995), analysis of variance showed significant differences of a minimum of 1065.4 Kg/ha (Table 7). Using this references value HD-263 and C. I. (Sevin™) produced the best results. GM-10 and GM-10 + HD-263 were comparable to HD-263 and C. I. and superior to HD-193 + HD-263 which were superior to the control and the corn starch base.

Discussion

The way to produce the active ingredient (fermentation media with molasses, corn liquor and soy flour) is an inexpensive and easy process, and a good option for production *Bt* strains for field trials. The active toxicity is well conserved by this production method. It was shown that the active ingredients of the different serovars of *Bt* retained their effectiveness when they are produced in by-products such as molasses, and corn liquor when combined soy flour. This production method was comparated with the used by people of Weslaco for the production of *Bt* strains (Dulmage & De Barjac, 1973) Also, strains isolated in Mexico showed toxic levels equal to the strains in the Howard Dulmage Collection and the standard HD-1 when they are used like active ingredient in laboratory bioassays or in granular formulations using gelatinized corn flour produced in Mexico. Maybe the production method helped to potentiate the toxic activity of the strain HD-263, like happend with other medias using *Bt* (Dulmage, 1971), and for this reason was more toxic in artificial diet than the HD-1-S. Besides, the standard employed was decreased its toxic activity with time. The nixtamalized corn is obtained from a process of cooking kernels of corn with calcium carbonate and alkali at high temperature. The corn starch present is pregelatinized by this process (Collison, 1968). The nixtamalized corn is an industrial product used to make tortillas. It was possible to produce granular formulations by conventional methods based on nixtamalized corn, a material cheap and easy to obtain in Mexico, at a low cost. With respect to cost, the

matrix used in this experiment had a value in Mexico of US $ 0.12 per Kg as opposed to US $0.44 per Kg for corn starch or US $ 1.32 per Kg for the pregelatinized starch Miragel ™ (McGuire *et al*, 1994).

The *kurstaki* and *aizawai* serovars were shown to have higher toxic activity versus lepidopteran larvae in laboratory bioassays, but each strain showed different activity for each lepidopteran insect. There seemed to be a pattern that *H. virescens* and *S. exigua* were more resistant to the various strains of *Bt* than *H. zea* or *T. ni*, but this did not hold true for all strains of *Bt*. Since the laboratory insects where raised in a open insect colony established at least eight years previously and not exposed to any insecticides, there had been little selection within the population. However, the introduction of new specimens from the field may have brought in genes for resistance. This supports other work that shows that different strains of *Bt* vary in their effectiveness against different species of insects (van Frankenhuyzen *et al*, 1992). In addition, the marked inhibitory effect of *Bt* on the growth of the larvae reduced their capacity to damage crops even though they were not all killed. Prevention of pupation at all concentrations of *Bt* breaks the life cycle which also reduces the population of the insects.

Bioassays and field trials showed that the granules alone were not toxic for the larvae. In the former, larval survival was similar to the controls. Corn yield was the same in the granular treatment without *Bt* as in the control. Mortality of larvae in the field trials could not be determined as to species because of difficulty in speciation. However, *S.exigua* is the most common pest in the test region. Field counts of larval mortality did

not seem to follow the same pattern as the laboratory bioassays. Also, the larval mortality did not seem to correlate with the yield of corn. For example, strain GM-10 showed good results in bioassays with diet incorporation, comparable to HD-193. However, in larval control in the field, GM-10 showed lower toxic activity than HD-193 as well as the others. Besides, C-4 showed the lowest toxic activity of all the strains in laboratory, but showed the same activity on larval counts in the field. This may have been for several reasons. One is that the number of larvae were counted on five plants only and the pretreatment counts were not done on the same plants as the post treatment plants. Another possibility is that there were other factors influencing production such as position in the field. There was a correlation, however, between the laboratory results and the yields in the field trials. Since the yields would have been influenced by the total damage done to the plants, the inhibitory effect may have reduced crop damage without reducing the number of larvae as much.

It was interesting to note that while the initial effect of the chemical insecticide was better than most bioinsecticides, in subsequent treatments its efficacy was greatly reduced. This would be difficult to explain on the basis of developing resistance because the adults would have dispersed to different plots and selection would not have been constant. However, there did seem to be an effect.

In the 1994 field trial, we saw differences between 2 and 4% level blocks. The lowest yield was the 2% level, in both treatment and control. This may has been because of the position in the field, because in the experimental design the 4% block was in the

interior of the field while the other block was next to the road. Despite this, the differences between the treatment versus the control can be shown in both levels.

For the results obtained in the field trials, we supposed that the malachite green can be used successfully as a solar protectant for treatment of corn plants with *Bt* as reported by Castro-Franco (1994).

***Table 1. Production of six strains of B. thuringiensis* in 14 liter fermentation flasks.**

Strains	Time (h)	Antifoam (ml)	Base (ml)[1]	Acid (ml)[2]	Paste	Spore-crystal A. I. (g)[3]
C-4	48	150	30	150	945.3	105.0
GM-7	48	325	50	200	960.2	108.3
GM-10	45	200	10	200	894.9	118.7
HD-187	45	350	35	175	613.5	107.0
HD-193	51	100	40	150	787.6	87.6
HD-263	48	150	30	150	945.3	105.0

[1] Base = 30% NaOH [2] Acid = 30% HCl [3] Paste = Sediemnts of fermentation products, spore-crystal complex after Dulmage's extraction (Dulmage *et al*, 1970. A . I. Active Ingredient

Table 2. LD_{50} and LD_{90} of six strains of *B. thuringiensis* for lepidopteran larvae.

Strains		*Helicoverpa virescens*	Conf. Interval	*H. zea*	Conf. Interval	*Spodoptera exigua*	Conf. Interval	*Trichoplusia ni*	Conf. Interval
HD-1-S	LD_{50}	22.3	18.2-27.48	16.7	13.5-28.8	26.3	21.5-32.1	19.8	12.8-29.3
	LD_{90}	95.3	57.1-257.3	71.2	34.6-290	127.9	81.0-311	82.5	58.3-187
C-4	LD_{50}	241	45.2-1287	24.3	19.9-37.2	66.3	39.2-112	29.3	18.2-43.7
	LD_{90}	5110	$157\text{-}4.4 \times 10^6$	88.6	52.2-196	1195	$264\text{-}2.2 \times 10^5$	772	$140\text{-}2.1 \times 10^5$
GM-7	LD_{50}	30.8	24.8-38.4		n.d.	73.7	26.3-145	22.2	10.6-44.2
	LD_{90}	162	97.1-438		n.d.	300	167-3366	86.1	57.9-203
GM-10	LD_{50}	35.4	27.5-45.6		n.d.	44.4	29.3-68.9	18.5	12.8-39.4
	LD_{90}	227	120-775		n.d.	248	178-472	69.4	34.6-99.3
HD-187	LD_{50}	31.7	23.5-42.8	19.3	12.7-30.2	39.8	20.4-53.4	25.7	10.0-42.9
	LD_{90}	175	82.3-762	439	106-6893	289	91.8-2685	854	$29\text{-}3.3 \times 10^5$
HD-193	LD_{50}	31.0	22.7-42.2	14.4	10.6-27.7	92.8	34.3-250.7	27.4	15.0-34.2
	LD_{90}	190	84.0-940	94.4	53.7-246	1794	$151\text{-}2.1 \times 10^6$	53.6	38.7-101
HD-263	LD_{50}	3.50	1.1-11.0	15.6	9.74-25.3	18.8	14.7-24.0	5.61	4.57-6.88
	LD_{90}	51.1	32.8-120	177	45.9-2477	135	62.2-611	24.2	17.7-44.5

Probit statistic (SAS, 1989) with the confidence interval of 95%. n. d. not determined

Table 2. LD_{50} and LD_{90} of six strains of *B. thuringiensis* for lepidopteran larvae.

Strains		*Helicoverpa virescens*	Conf. Interval	*H. zea*	Conf. Interval	*Spodoptera exigua*	Conf. Interval	*Trichoplusia ni.*	Conf. Interval
HD-1-S	LD_{50}	22.3	18.2-27.48	16.7	13.5-28.8	26.3	21.5-32.1	19.8	12.8-29.3
	LD_{90}	95.3	57.1-257.3	71.2	34.6-290	127.9	81.0-311	82.5	58.3-187
C-4	LD_{50}	241	45.2-1287	24.3	19.9-37.2	66.3	39.2-112	29.3	18.2-43.7
	LD_{90}	5110	157-$4.4x10^6$	88.6	52.2-196	1195	264-$2.2x10^5$	772	140-$2.1 x10^5$
GM-7	LD_{50}	30.8	24.8-38.4		n.d.	73.7	26.3-145	22.2	10.6-44.2
	LD_{90}	162	97.1-438		n.d.	300	167-3366	86.1	57.9-203
GM-10	LD_{50}	35.4	27.5-45.6		n.d.	44.4	29.3-68.9	18.5	12.8-39.4
	LD_{90}	227	120-775		n.d.	248	178-472	69.4	34.6-99.3
HD-187	LD_{50}	31.7	23.5-42.8	19.3	12.7-30.2	39.8	20.4-53.4	25.7	10.0-42.9
	LD_{90}	175	82.3-762	439	106-6893	289	91.8-2685	854	29-$3.3 x10^5$
HD-193	LD_{50}	31.0	22.7-42.2	14.4	10.6-27.7	92.8	34.3-250.7	27.4	15.0-34.2
	LD_{90}	190	84.0-940	94.4	53.7-246	1794	151-$2.1 x10^6$	53.6	38.7-101
HD-263	LD_{50}	3.50	1.1-11.0	15.6	9.74-25.3	18.8	14.7-24.0	5.61	4.57-6.88
	LD_{90}	51.1	32.8-120	177	45.9-2477	135	62.2-611	24.2	17.7-44.5

Probit statistic (SAS, 1989) with the confidence interval of 95%. n. d. not determined

Table 3. Inhibitory effect of three strains of *B. thuringiensis* on lepidopteran larvae.[1]

Dose		*Helicoverpa virescens*		*Spodoptera exigua*		*Trichoplusia ni*	
(mg/ml)	Strain	No. of dead larvae	Weight/larva (mg)	No. of dead larvae	Weight/larva (mg)	No. of dead larvae	Weight/larva (mg)
100	GM-7	64	1.16	n. d.	--	a.d.	--
	GM-10	59	1.28	a. d	--	a.d	--
	HD-1-S	n. d.	--	66	5.54	n. d.	--
50	GM-7	42	1.76	n. d.	--	69	1.63
	GM-10	41	1.68	74	6.62	67	6.37
	HD-1-S	n. d.	--	54	9.39	n. d.	--
25	GM-7	39	3.90	n. d.	--	66	2.67
	GM-10	38	3.26	65	5.36	61	7.7
	HD-1-S	n. d.	--	30	12.24	n. d.	--
15	GM-7	21	5.3	n. d.	--	50	6.62
	GM-10	20	5.21	62	8.77	42	8.4
	HD-1-S	n. d.	--	26	24.8	n. d.	--
13	GM-7	19	6.50	n. d.	--	35	31.5
	GM-10	17	6.31	54	9.48	36	11.8
	HD-1-S	n. d.	--	20	25.8	n. d.	--
10	GM-7	10	8.50	n. d.	--	28	31.5
	GM-10	15	8.52	56	10.99	37	25.8
	HD-1-S	n. d.	--	17	34.8	n. d.	--
5	GM-7	8	9.50	n. d.	--	10	37.4
	GM-10	7	8.99	40	20.6	25	38.2
	HD-1-S	n. d.	--	8	43.5	n. d.	--
-	Control	0	85.82	0	183.82	0	105.5

[1] average of three replications. 75 larvae per trial. n. d. not determined. a.d. all the larvae were dead

Table 4. Percent mortality of *T. ni* with *B. thuringiensis* in matrices of gelatinized corn[1]

	Bacillus thuringiensis strains							Control	Form[2]
Dose	C-4	GM-7	GM-10	GM-10[3]	HD-187	HD-193	HD-263	--	not *Bt*
50 mg/ml	54	100	94	100	23	100	100	0	0
500 mg/ml	100	100	100	100	100	100	100	0	0

[1] Total of 75 larvae with three replicates of 25 larvae each. Formulations produced six months previously
[2] Formulation without *Bt* [3] Formulation produced two years previously

Table 5. Larvae count before and after each formulation application.[1]

	APPLICATIONS						
Treatment	First		Second		Third		Percent
	before	after	before	after	before	after	total decrease
C-4	13	4	21	8	27	5	72.13
GM-7	16	7	35	5	21	5	76.38
GM-10	25	12	20	1	39	22	58.33
HD-187	19	6	19	4	29	14	64.18
HD-193	23	1	15	6	31	13	70.01
HD-263	31	5	14	5	28	9	73.97
Carbaryl	23	0	42	22	18	5	70.65
Control	13	12	19	18	31	62	+31.52[2]

[1] Average of three replications. [2] Percent total amount

Table 6. Corn yield (Kg/ha) resulting from application of bioinsecticides and a chemical insecticide[1]

A. I. [2]	Treatment								
	C-4	GM-7	GM-10	HD-187	HD-193	HD-263	Carbaryl	Dipel™	Control
2%	5413.7[cd]	5576.3[bcd]	7948.8[a]	6636.5[abc]	7190.7[ab]	6115.5[bcd]	n. d.	5628.6[bcd]	4906.4[d]
4%	7133.7[bc]	8605.6[ab]	8825.5[a]	7677.2[ab]	8375.5[ab]	8972.3[a]	8616.7[a]	n. d.	6064.7[c]

[1]Average of four replicates. [2] Percent of active ingredient. n. d. not determined. Values in the same line with the same letter are not significantly different from each other. Tukey' difference at $p< 0.05$

Table 7. Field trial of corn meal formulations with different serovars of *B. thuringiensis*

Treat.	HD-263	Carbaryl	10+263	GM-10	193+263	HD-193	10+193	Dipel™	Control	Granuls
Yield[1]	6344.6	6083.4	5961.2	5583.3	5513.7	5274.3	5101.5	5014.3	4720.3	4258.3
	a	ab	abc	abcd	abcd	bcde	bcde	bcde	de	e

[1] Kg/ha, average of three repetitions. Values with the same letter are not significantly different from each other. Range multiple analysis, $p< 0.05$

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SPRAYABLE GRANULE FORMULATIONS FOR *BACILLUS THURINGIENSIS*

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