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SELECTION AND CHARACTERIZATION OF MEXICAN STRAINS OF *BACILLUS THURZNGZENSZS* ACTIVE AGAINST FOUR MAJOR LEPIDOPTERAN MAIZE PESTS

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In order to isolate novel delta-endotoxins from ***Bacillus thuringiensis*** Berliner, a total of 426 native isolates (in varying numbers for each pest) were screened against four major maize pests: corn earworm, ***Helicoverpa zea;*** fall armyworm, ***Spodoptera frugiperda;*** southwestern corn borer, ***Diatraea graridiosella,*** and sugarcane borer, ***Diatraea saccharalis.*** Spore-crystal complexes from the isolates were integrated into semi-artificial diets of each pest and mortality was assessed 7 days after treatment. A total of 25 isolates were selected on the basis of highest larval mortality against at least one insect species. There was no correspondence of the most toxic isolates when tested against the four different insect species. Most of the 25 selected isolates caused higher toxicities against all four pests than the standard strain HD-I, regardless of not achieving 100% mortality in any bioassay. ***H. zea*** demonstrated the highest level of mortality (96%) and was susceptible to the largest number of isolates (98). None of the other insect species were found susceptible at levels greater than 60%. All the selected active strains were isolated from stored grain dusts (except for LBIT-167), and had bipyramidal crystals with Cry I-like proteins. Most isolates also formed an associated square (cubic) inclusion, with Cry 11-like proteins according to SDS-PAGE analysis of their parasporal bodies. The most active isolates will be subjected to further studies, in order to identify putative novel genes to be expressed in transgenic maize.

KEY-WORDS: ***Bacillus thuringiensis, Helicoverpa zea, Spodoptera frugiperda. Diatraea grandiosella, Diatraea saccharalis,*** mortality, maize.

Increased use of ***Bacillus thuringiensis*** Berliner in integrated pest management programs as bioinsecticide (Feitelson ***et al.,*** 1992) and development of transgenic plants expressing its toxins (Peferoen, 1992) have stimulated the search for more active strains of this bacterium against certain lepidopterous pests (Moar & Trumble, 1990). Contrary to strains of pathotypes B (mosquito active) and C (coleopteran active) of ***B. thuringiensis,*** pathotype ***A*** (lepidopteran active) range from highly active to almost innocuous. Variation in insecticidal activity also depends on the target species, as well as on the Cry-protein content of the parasporal bodies, acting individually or in combination (Gill ***et al,*** 1992). More than 80% of natural isolates of ***B. thuringiensis*** show the typical bipyramidal parasporal body of pathotype A; however, most of these isolates show low to moderate toxicity levels against highly susceptible species (Meadows ***et al.,*** 1992). This is the reason to select only the

highly toxic strains; however, because strains show some specificity against certain lepidopteran species due to their Cry-protein content and/or combination, strain selection should be directed towards particular target pest (Dulmage, 1975).

The most important lepidopteran pests of Mesoamerican maize are: corn earworm, ***Helicoverpa zea*** (Boddie); fall armyworm, ***Spodoptera frugiperda*** (J.E. Smith); southwestern corn borer, ***Diatraea grandiosella*** (Dyar); and sugarcane borer, ***D. saccharalis*** (Fabricius). These pests can account for more than 30% yield losses (Kumar & Mihm,1995).The increasing costs of chemical control, and the problems related to its use, make the search for alternative control measures extremely important. B. ***thuringiensis*** has been used as bioinsecticide against these pests, with varying results (Long ***et al.,*** 1961; Hensley ***et al.,*** 1961; Charpenter ***et al.,*** 1973; Gardner ***et al.,*** 1986; Hernández, 1988;; Ali & Young, 1993; Stone & Sims, 1993). B. ***thuringiensis*** commercial products are usually recommended to control ***S. frugiperda*** and ***H. zea,*** but not ***D. grandiosella*** and ***D. saccharalis.*** However, all four species are recognized for their moderate to low susceptibility to these products (Krieg & Langenbruch, 1981). Furthermore, the feeding habits of these insects, make them difficult to intoxicate with the sprayed products. For this reason, much effort is being directed towards the development of transgenic maize, expressing one or more B. ***thuringiensis*** toxins (Koziel ***et al.,*** 1993).

As part of a project to develop transgenic maize for management of lepidopteran pest populations, the International Maize and Wheat Improvement Center (CIMMYT) has conducted a selection of Mexican B. ***thuringiensis*** isolates collected by the Center for Research and Advanced Studies (CINVESTAV). Selected isolates may provide coding genes for specific and active toxins. This report presents a selection of toxic native isolates of B. ***thuringiensis*** from a total of 426 strains, against the four most important lepidopteran pests of maize in Mesoamerica, as well as a preliminary characterization of the selected isolates.

MATERIAL AND METHODS

BACTERIAL STRAINS

In a nationwide isolation program of Mexican B. ***thuringiensis*** strains, pasteurized samples of soil, grain dust, and insects were plated on nutrient agar and single colonies containing inclusion bodies were isolated and grown for further study (Sneath, 1986). Stocks were registered, freeze-dried and kept at – 20°C at the CINVESTAV's stock collection.

CULTURE CONDITIONS AND SPORE-CRYSTAL FORMULATION

Cultures of 426 isolates were grown in PMB medium (10 g peptonized milk, 10 g dextrose; 2 g yeast extract; 0.3 g $MgSO_4.7H_2O$; 20 mg $FeSO_4 7H_2O$; 20 mg $ZnSO_4.7H_2O$; 20 mg $MnSO_4$, in 1 l destilled water; pH 7.2-7.5), incubated at 30°C under agitation (340 rpm) until complete autolysis was achieved. The spore-crystal complexes were harvested by centrifugation at 10,000 rpm for 10 min at 4°C. Spores, parasporal bodies, and cell debris were washed three times in distilled water by centrifugation (10 min, 10,000 rpm). Pellets of spore-crystal complexes were freeze-dried and stored at – 20°C until bioassayed.

INSECT REARING AND BIOASSAY

Larvae of H. zea, S. frugiperda, D. grandiosella, D. saccharalis, were obtained from colonies maintained on artificial diets at 27°C with a photoperiod of 16:8 h (light:dark).

Artificial diets, prepared as described by Mihm (1982, 1983a, 1983b), were used for all tests. Each strain's freeze-dried spore-crystal complex was diluted in water, and mixed with a vortex mixer for 1 min., sonicated, and added to the diet at the rate of 50 µg per g diet. The same procedure was followed with the standard strain HD- 1. One hundred ml of the diet was mixed well with spore-crystal and poured into each well of a 32-well microtiter plate (Oliver). The diet was allowed to solidify and a single larva (one-day old larvae of S. frugiperda, D. grandiosella, and D. saccharalis and 2-day old larvae of H. zea) was added to each well. Microtiter plates were covered with polyester film lidding material (Oliver), and incubated in a growth chamber at 27°C and a photoperiod of 16:8 h (light:dark). Thirty-two insects were evaluated per strain and each treatment was replicated at least three times. Larval mortality was assessed afier 7 days. Mortality of the control larvae reared on a toxin-free diet and under the same conditions was recorded and used to correct the test mortality with Abbott's formula (Abbott, 1925).

PARASPORAL BODY MORPHOLOGY

In order to establish the crystal morphological type of the selected isolates, wet mounts of both intact and autolyzed sporangia of each isolate were observed under phase contrast microscopy. The three typical morphologies (bipyramidal, spherical-amorphous and flat-square) were discriminated. Inclusions associated with bipyramidal crystals were also identified. In order to try to correlate activity with crystal proportions, a classification of crystals and inclusions according to their size was developed as a means of discrimination between isolates.

SDS-PAGE ELECTROPHORESIS

Protein composition of the selected isolate's spore-crystal complexes was determined using SDS-PAGE essentially as described by Laemli and Favre (1973), with a 3% stacking gel and 10% running gel in a Bio-Rad mini-protean II cell slab vertical gel apparatus at 50 V for 15 min and 100 V for 1.5 hour. Gels were stained with Coomassie blue. The molecular masses of the putative parasporal body proteins were estimated by comparaison with those of the following proteins subjected to SDS-PAGE: carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), phosphorylase B (97.4 kDa), B-galactosidase (116.25 kDa), and myosin (205 kDa).

RESULTS

STRAIN ISOLATION

A total of 426 isolates (isolated from 522 source samples) was included in this study. Stored grain dusts were the most abundant and diverse source samples (82%), while soil and insect samples provided a smaller proportion of the total (11 and 7%, respectively). A majority of the isolates (84%) showed the presence of bipyramidal crystals, similar to those typical of isolates active against Lepidoptera (pathotype A). A few isolates exhibited crystals similar to those active against mosquitoes (8%) and Coleoptera (3%). The remaining 5% showed atypical crystal morphologies.

TABLE 1

Summary of different features shown by each of the more toxic Mexican Bacillus thuringiensis ***isolates, tested against first instar larvae of*** *H.* zea, *S.* frugiperda, D. grandiosella, ***and*** D. saccharalis

		Attribute of the isolate		
LBIT-Isolate	Source	BIP.	INCL.	PROT.
13	Maize	SML	SML	I/II
27	Sorghum	SML	SML	I/II
29	Maize	SML	SML	I/II
114	Beans	LRG	ABS	I
124	Maize	LRG	SML	I/II
128	Maize	NRM	SML.	I/II
135	Maize	SML	SML	I/II
139	Maize	LRG	ABS	I
144	Wheat	NRM	SML	I/II
149	Sorghum	LRG	ABS	I
151	Wheat	NRM	ABS	I
167	(Soil)	NRM	SML	I/II
174	Maize	NRM	SML	I/II
175	Maize	SML	ABS	I
178	Sorghum	LRG	SML	I/II
181	Maize	NRM	SML	I/II
193	Maize	NRM	ABS	I
199	Rice	NRM	SML	I/II
226	Sorghum	LRG	ABS	I
227	Sorghum	NRM	SML	I/II
236	Beans	NRM	SML	I/II
259	Sorghum	LRG	SML	I/II
290	Sorghum	NRM	ABS	I
301	Beans	NRM	LRG	I/II
437	Maize	SML	ABS	I

Source : Stored grain dust the strain was isolated from; BIP.: Size of the isolate's bipyramidal crystal (LRG: Large, NRM: Normal, SML: Small); INCL.: Presence and size of the cubic inclusion, associated to the bipyramidal crystal (ABS: Absent); PROT Presence of putative Cry I/Cry II proteins.

Almost all the 25 most toxic isolates tested against the four lepidopteran maize pests were isolated from dusts of stored maize, sorghum, beans, and wheat. Only isolate LBIT-167 was isolated from soil (table 1).

BIOASSAYS

Figure 1 shows the mortality distribution of a total of 352 isolates tested against D. ***grandiosella.*** Almost 99% of the isolates caused mortalities lower than 50%, with only 4 isolates inducing mortalities between 50 and 80%. No isolate caused higher mortalities. Similarly, all the 337 isolates tested against D. ***saccharulis*** exhibited mortalities lower than 50% (fig. 2). When a total of 156 isolates was tested against ***S. frugiperda,*** only 6 (4%) induced mortalities between 50% and 70%. No isolate caused mortalities greater than 70% (fig. 3). A different distribution was observed when 96 isolates were bioassayed against *H. zea,* as only 27% of the isolates tested exhibited a mortality lower than 50%. The remaining 73% of the isolates caused mortalities between 50 and 99%; however, no isolate showed 100% mortality under the conditions tested (fig. 4).

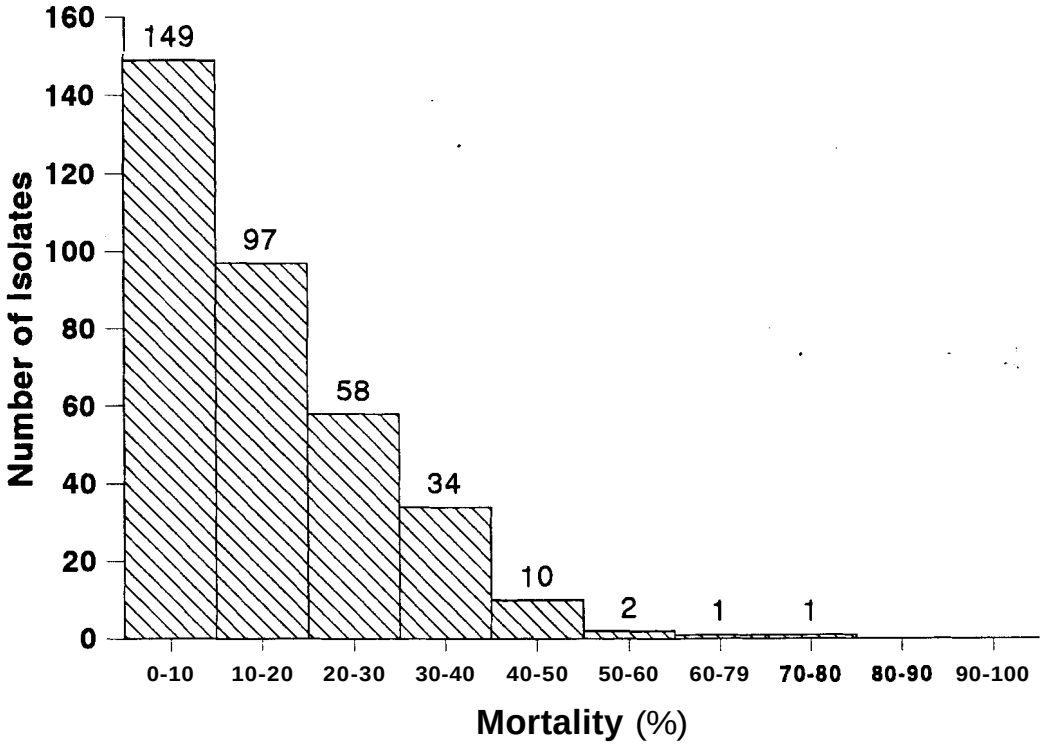

Fig. 1. Mortality distribution of spore-crystal complexes of *Bacillus thuringiensis* native isolates against D. *grandiosella* first instar larvae.

Table 2 shows the mortality levels obtained by the most active isolates, when tested against the four lepidopteran pests. These 25 isolates were selected on the bases of highest larval mortality against at least one insect species. Isolates LBIT-437, 139 and 259 caused the highest toxicity against D. ***grandiosella,*** while isolates LBIT-13, 236 and 29 induced the highest mortalities against D. ***saccharalis.*** On the other hand, isolates LBIT-193 and 27 caused the highest mortalities against S. ***frugiperda*** and isolates LBIT-227, 199, 144 and 149 induced the highest toxicities against H. ***zea.*** There was no correspondence of the most toxic isolates when tested against different insect species. An attempt to relate the toxicity levels of each strain between different insect species was made by regression analysis (data not shown); however, no significant regression was obtained, except when mortality levels recorded for D. ***grandiosella*** were related to those of the remaining species. Interestingly, the regression was negative.

When the performance of the native isolates was compared with that of the standard HD-I. 16,, 20, and 17 native isolates caused higher mortality than the standard, when tested against H. *zea,* S. ***frugiperda. D. grandiosella,*** and ***D. saccharalis,*** respectively (table 2).

CRYSTAL MORPHOLOGY

A close inspection of the parasporal bodies formed by the 25 selected isolates under phase contrast microscopy showed that all the isolates exhibit a bipyramidal crystal and 16

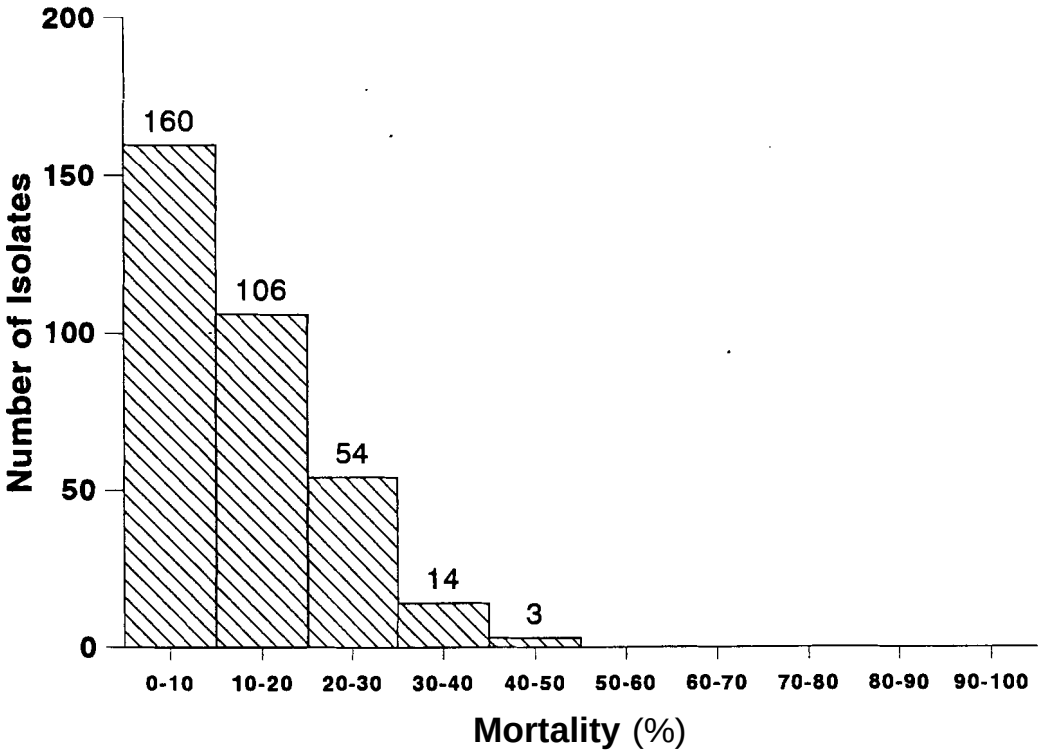

Fig. 2. Mortality distribution of spore-crystal complexes of *Bacillus thuringiensis* native isolates against D. *saccharalis* first instar larvae.

revealed a square (or cubic) inclusion along the main crystal. Bipyramidal crystals varied significantly in size and were arbitrarily classified as: small, normal and large, if they were smaller, equal or larger than 1 pm, respectively (table 1). When present, the cubic inclusion was always ca. 0.5 μm large, except for isolate LBIT-301, which showed a large cuboidal inclusion, even larger than the bipyramidal crystal.

Twelve isolates showed normal size bipyramidal crystals while 6 and 7 isolates exhibited smaller and larger crystals, respectively. The most frequent combination was a normal size bipyramidal crystal with a small inclusion; however, most of the isolates which produce significantly large bipyramidal crystals were not associated with cubic inclusions. When crystal morphology of the five most active isolates against each of the maize pests was compared. no specific morphology was associated to the activity, except that 14 out of the 19 isolates show the presence of cubic inclusions.

PEPTIDE CONTENT

When trying to correlate the diversity of crystal proteins with the toxicity levels found in the screening, SDS-PAGE analysis of the parasporal bodies produced by the 25 selecter isolates revealed two basic patterns. One included one to three proteins in the range of 125

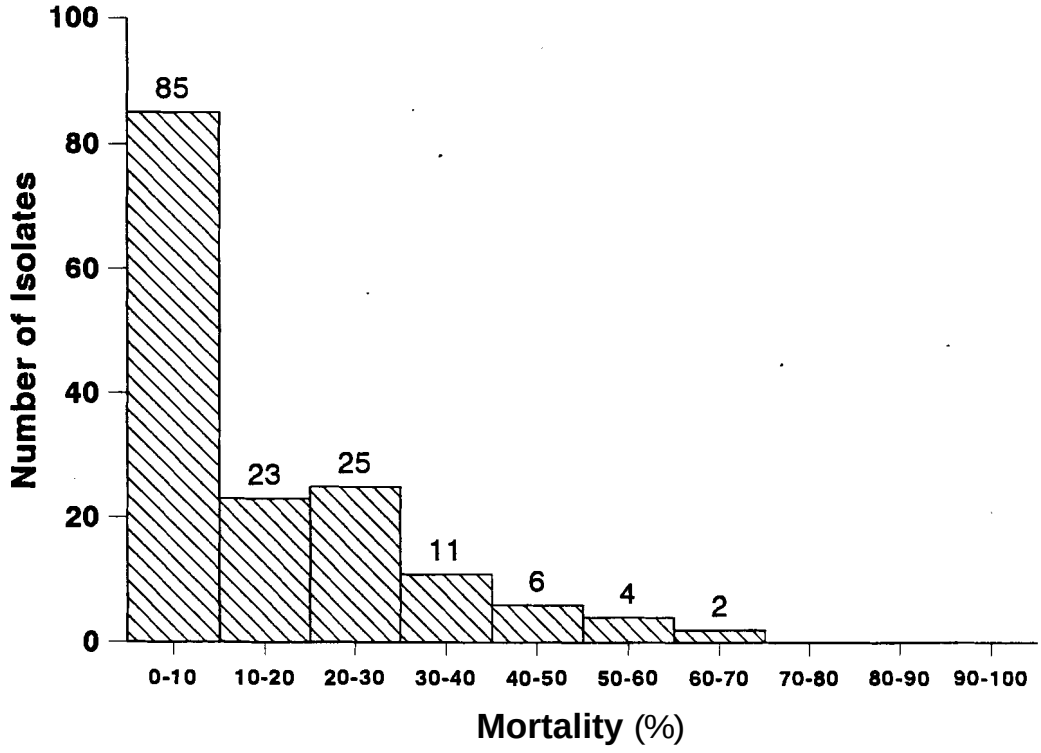

Fig. 3. Mortality distribution of spore-crystal complexes of *Bacillus thuringiensis* native isolates against S. *frugiperda* first instar larvae.

to 140 kDa, and a second included these proteins along with one or two proteins in the range of 60 to 65 kDa (fig. 5, table 1). According only to the molecular size and host range, the larger proteins may be related to the Cry I protoxins, while the smaller proteins may be related to the Cry II protoxins (Hofte & Whiteley, 1989). Most of the active strains show a combination of both types of protoxins; however, from the five most active strains against D. ***grandiosella,*** three show only Cry I-like protoxins.

DISCUSSION

Isolation of native B. ***thuringiensis*** strains from the total processed samples was higher than 80%, and higher than 86% from stored grain dusts. These results indicate the relative abundance of wild strains. All native isolates were tested in varying numbers against the four major lepidopteran maize pests and, regardless their higher proportion, an overwhelming majority of the selected isolates was obtained from grain dusts. Interestingly, in spite of maize dust being a major source of selected isolates (44%), the most active strains were isolated mostly from other sources. However, sorghum dust was also a major source of selected strains (28%). and it should be considered as an alternative host for at least some

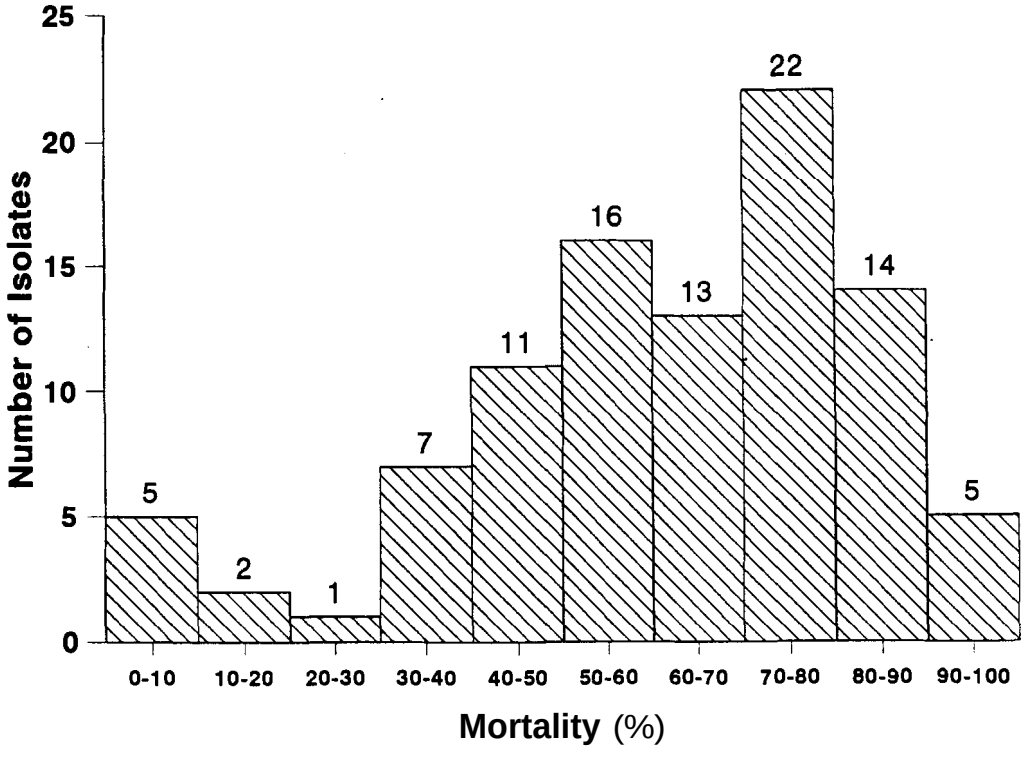

Fig. 4. Mortality distribution of spore-crystal complexes of *Bacillus rhuringiensis* native isolates against H. *zea* first instar larvae.

of the studied pests. In spite of this, there are previous reports where B. ***thuringiensis*** strains, isolated from grain dusts, show high activity against insects totally unrelated to grain (López-Meza & Ibarra, 1995).

Most of the selected isolates induced mortality levels higher than those induced by the standard HD-I. This clearly indicates their potential as a source of genes expressing active toxins. It is important to note that the dose tested was considerably high and the levels of toxicity were lower than those obtained against other highly susceptible insect species (i.e. the tobacco hornworm, ***Manduca sexta*** L.) (Schesser ***et al.,*** 1977); however, toxicity should be measured not only in terms of mortality, but also in terms of development reduction. This development effect has been recorded previously (De León & Ibarra, 1995), and it is possible that pest damage can be significantly reduced even when mortality is low (Arpaia & Ricchiuto, 1993; Schesser ***et al.,*** 1977). This effect was obvious in our bioassays against the four pests. as all the selected isolates (including HD-I) induced a significant size reduction in the larvae fed treated diet when compared with controls (data not shown). In other words, the use of moderately toxic proteins in transgenic maize is feasible, and it might be even helpful in the management of resistance development (McGaughey & Whalon, 1992).

According to the bioassays, *H. zea* is the most susceptible species of all four. This is clearly indicated not only by the highest mortalities obtained in the bioassays but also in the

TABLE 2

Mortality achieved by selected Mexican Bacillus thuringiensis *isolates, tested against first instar larvae of H.* zea, *S.* frugiperda, D. grandiosella, *and* D. saccharalis

	% Larval Mortality per Insect Species			
LBIT-Isolate	***H. zea***	***S. frugiperda***	***D. grandiosella***	***D. saccharalis***
13	53.5	20.1	18.8	40.9
27	56.4	62.2	20	17.2
29	74.5	4.7	22.6	34.3
114	43.3	3.2	48.3	1.6
124	69.9	4.8	31.7	31.7
128	71.8	6.6	53.3	18.3
135	70.7	38.9	19.7	16.9
139	42.9	28.9	60.7	1.7
144	92.3	35.6	41.9	15.6
149	92.3	40.9	22.6	9.4
151	80.6	47.4	29	15.6
167	88.8	16.7	38.7	6.3
174	81.3	41.3	12.9	26.7
175	84.5	43.4	19.4	9.4
178	92.5	8.4	9.7	0
181	84.5	52.3	25.8	12.5
193	54.1	65.2	3.2	15.6
199	93.4	25.7	16.1	3.2
226	—	50.4	23.3	20
227	96.6	23.9	6.3	33.3
236	—	8.4	12.5	37.6
259	46.6	3.9	56.3	3.2
290	89.9	0.7	18.8	3.3
301	—	53.8	29	3.3
437	—	—	71	—
HD-1	54.6	10.2	13.7	6.1

mortality distribution of all the tested strains, as most caused mortalities of H. *zea* higher than *50%.* The remaining three species are much less susceptible and they have been always considered as "recalcitrant" species (Krieg & Langenbruch, 1981). ***S. frugiperda*** is a moderately susceptible species; however, B. ***thuringiensis*** has been rarely recommended to control either D. ***grandiosella*** or ***D. saccharalis*** (Metcalf & Metcalf, 1993; Meister, 1986). These previous observations have been confirmed by our study, as none of the tested isolates induced mortalities higher than *50%* against D. ***saccharalis*** and only ***4*** caused mortalities higher than *50%* against D. ***grandiosella.*** Once this first screening of isolates is over, proper estimation of the LC_{50}'s using purified toxins is essential to establish toxicity levels from those isolates which caused the highest mortalities on each insect pest. However, these results are sufficient to initiate the identification of toxin-encoding genes from the most active isolates.

It should be noticed that, due to the fact that no correspondence was observed of the most active strains among the different pests, high specificity between the toxins and membrane receptors of each species can be expected. This becomes a problem, if the final goal aims toward the control of these pests by the expression of toxins in the plant tissues. Under the attack of several species, a proper control level might be achieved only if all the

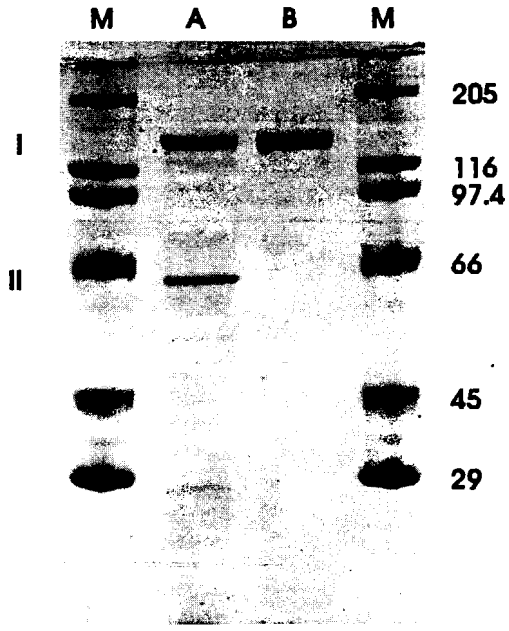

Fig. 5. SDS-polyacrylarnide gel showing the two basic patterns obtained from the 25 selected native isolates. Lane A: pattern oftwo types of Cry proteins; Lane B: pattern of one type of Cry proteins. I: putative Cry I proteins; II: putative Cry II proteins; M: molecular weight markers (size is in kDa).

different active toxins are expressed, which is impractical from the technical point of view. Furthermore, due to the moderate levels of toxicity shown against the ***Diatraea*** spp., high expression levels might be required to achieve optimum control. Previous problems with appropriate expression levels of B. ***thuringiensis*** toxins in plants were successfully overcome by the synthesis of genes, changing the original prokaryotic codon usage by an eukaryotic one (Koziel ***et al.,*** 1993). This solution might be applicable to the toxins reported here.

All the selected isolates form a bipyramidal crystal, typical of the pathotype A (active against Lepidoptera) (Krieg ***et al.,*** 1987). Also there was no correlation between toxicity levels and crystal size and/or the presence of the cubic inclusion (Donovan ***et al.***. 1988). It is known that the activity is rather related to a combination of the insect's ability to dissolve and partially digest the crystal proteins, as well as the affinity of the delta-endotoxins to membrane receptors and their ability to create membrane pores (Gill ***et al.,*** 1992). However, partial characterization of the crystal (including the peptide content) is helpful and provides useful information about each isolate. Based on molecular weights, peptide contents indicated the presence of putative Cry I proteins in the bipyramidal crystals, and of putative Cry II proteins in the cubic inclusions (when they were present). Proper identification of these proteins would require Western blot analysis, using specific (in some cases, monoclonal) antibodies. It is worth noticing that the preliminary characterization of the *25* selected isolates showed typical attributes of strains within the pathotype A in all isolates except one. The exception was isolate LBIT-437, whose bipyramidal crystal was rather narrow and its major protein content included bands of lower molecular weight

peptides than that reported for Cry I proteins. This isolate is currently being subjected to a thorough characterization, as it was the most active isolate against D. ***grandiosella*** and might produce a novel toxin, according to the unusual molecular weight.

Additionally, the most active isolates against each insect pest will be subjected to a thorough characterization, which will include serotyping, ultrastructural morphology of the parasporal body, plasmid pattern, differentiation of all Cry proteins, cloning and sequencing of the genes expressing the most active Cry proteins, etc. This information will provide the basis to proceed towards the successful development of transgenic maize, resistant to the attack of these major maize pests.

RÉSUMÉ

Selection et caractérisation d'isolats mexicains de ***Bacillus thuringiensis*** actifs contre quatre Lépidoptères majeurs du maïs

Les 426 souches de ***Bacillus thuringiensis*** Berliner isolées ont été testées sur quatre ravageurs importants du ma'is, ***Helicoverpa zea, Spodoptera frugiperda, Diatraea grandiosella, Diatraea saccharalis*** dans le but d'identifier de nouvelles delta endotoxines. Le complexe spore-cristal obtenu a partir de chacun des isolats a été incorporé dans un milieu nutritif artificiel pour insecte et la mortalité a été relevée apres sept jours de traitements. Vingt-cinq isolats ont été sélectionnés sur la base d'une toxicité élevée sur les larves d'au moins une espèce. Aucun des isolats actifs ne s'est révélé actif à la fois contre les quatre espèces d'insectes étudiées. La plupart des 25 isolats sélectionnés ont montré une toxicité supérieure à celle de la souche de reférence HD- 1, que la mortalité ait atteint ou non 100 %. ***H. zea*** s'est révélée être l'espèce la plus sensible (96 % de mortalité) au plus grand nombre d'isolats (98). Aucune des trois autre especes n'a montré plus de 60 % mortalité. Toutes les souches actives ont été isolées à partir de poussières de graines stockées (excepté l'isolat LBIT- 167) et produisaient des cristaux bipyramidaux. L'analyse du contenu protéique des cristaux en gel de polyacrylamide-SDS a montre que les cristaux bipyramidaux contenaient des protéines de type CryI et que les cristaux cubiques associés produits par la plupart des isolats contenaient des protéines de type CryII. Les souches les plus actives seront analysées de façon plus approfondie afin de détecter la presence potentielle de nouveaux gènes pouvant être exprimés chez les maïs transgéniques.

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