Title: Toxicity of several δ-endotoxins of *Bacillus thuringiensis* against *Helicoverpa armigera* (Lepidoptera: Noctuidae) from Spain.

Authors: C. Avilla[(1)], E. Vargas-Osuna[(2)], J. González-Cabrera[(3)], J. Ferré[(3)], J.E. González-Zamora[(1)]

Affiliations: [(1)] Departamento de Ciencias Agroforestales, Universidad de Sevilla. Carretera de Utrera, km 1. E-41013 Seville (Spain)

[(2)] Departamento de Ciencias y Recursos Agrícolas y Forestales. Universidad de Córdoba. Apartado 3048. E-14080 Córdoba (Spain)

[(3)] Departament de Genètica, Facultat CC. Biològiques, Universitat de València. Av. Dr. Moliner, 50. E-46100 Burjassot (Valencia, Spain)

Address for manuscript correspondence:

J.E. González-Zamora

Escuela Universitaria de Ingeniería Técnica Agrícola (Universidad de Sevilla)

Carretera de Utrera, km 1

E-41013 Sevilla (Spain)

E-mail: zamora@us.es

Phone number: +34-954 486 459

Fax number: +34-954 486 436

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ABSTRACT

Toxicity and larval growth inhibition of eleven insecticidal proteins of *Bacillus thuringiensis* were evaluated against neonate larvae of *Helicoverpa armigera*, a major pest of important crops in Spain and other countries, by a whole-diet contamination method. The most active toxins were Cry1Ac4 and Cry2Aa1, with LC_{50} values of 3.5 and 6.3 µg/ml, respectively. At the concentrations tested, Cry1Ac4, Cry2Aa1, Cry9Ca, Cry1Fa1, Cry1Ab3, Cry2Ab2, Cry1Da, and Cry1Ja1, produced a significant growth inhibition, whereas Cry1Aa3, Cry1Ca2, and Cry1Ea had no effect.

Keywords: *Bacillus thuringiensis*, *Helicoverpa armigera*, Microbial insect control, Cotton pests, Cry toxins, Growth inhibition, ICP**.**

INTRODUCTION

Helicoverpa armigera (Hübner) is an important pest of different crops (cotton, tomato, among others) in Spain and other countries. To prevent the damage that larvae produce in these crops, a variety of methods are used for their control, including the use of chemical pesticides and also microorganisms. The most important of the latter is *Bacillus thuringiensis* (Berliner), a bacterium which produces different proteins (δ-endotoxins) toxic to larvae of different species of Lepidoptera and other insects (Schnepf *et al*., 1998).

B. thuringiensis (*Bt*) has been used since the 50's of the 20th century by spraying its spores and crystals on the plants. In the late 80's and early 90's different cultivars, such as tobacco, tomato, potato, cotton, and corn, among others, were genetically engineered to express δ-endotoxins, which allowed them to be tolerant to insect attack (Schnepf *et al*., 1998). The use of insect resistant transgenic crops has been adopted in many countries because of the higher protection they provide and the reduction in pesticide applications, but there is concern about the development of resistance in target pest populations. Different strategies, such as the use of refuges, gene rotation, and gene stacking, have been proposed to avoid (or at least delay) the development of resistance.

To date, the only transgenic crop with a *Bt* gene authorized in Spain is *Bt*-corn, which incorporates a gene that codes for the Cry1Ab toxin. However, it is expected that other transgenic cultivars be authorized in the near future, in particular *Bt*-cotton (which expresses the Cry1Ac toxin) because of the important losses that *H.*

armigera can produce in this crop. Several authors have studied the effect of *Bt* toxins on *H. armigera* populations from China, India and Australia (Chakrabarti et al., 1998; Gajendra Babu et al., 2002; Liao et al., 2002; Kumar et al., 2004; Fengxia et al., 2004; Jalali et al., 2004). However, to our knowledge there is no published study describing the activity of *Bt* toxins in European populations. Since susceptibility to a given *Bt* toxin may vary among populations (González-Cabrera et al., 2001; Wu et al, 1999; Jalali et al., 2004), it is of great interest to determine the effect of *Bt* toxins on local populations of *H. armigera* prior to the possible introduction of transgenic cultivars and also for the more rational use of *Bt* spray formulations. The results obtained will also be useful to search for alternatives in the case resistance evolves in the target populations to some of the currently used *Bt* toxins.

MATERIALS AND METHODS

The *H. armigera* colony was established from larvae and egg masses collected in cotton fields in June-July 2001-02 in Andalucia, southern Spain. Bioassays were carried out by incorporating the *Bt* toxins into the artificial diet (Poitout and Bues, 1974). Cry1Aa3, Cry1Ab3, Cry1Ac4, Cry1Ca2, Cry1Da, Cry1Ea, Cry1Fa1, Cry1Ja1, Cry2Aa1, and Cry2Ab2 were purified from recombinant *Bt* strains EG1273, EG7077, EG11070, EG1081, EG7300, EG11901, EG11069, EG7279 (obtained from Ecogen Inc., Langhorne, Pa.), EG7543, and EG7699 (from Monsanto Co., Chesterfield, Mo.), respectively. *Bt* strains were grown at 29ºC for 48 h in CCY medium (Stewart et al., 1981) supplemented with the suitable antibiotic. Spores and crystals were collected by centrifugation at 22000 ×*g* for 10 min at 4ºC. The pellet was washed four times with 1 M NaCl/10 mM EDTA and suspended in 10 mM KCl. Protoxin inclusions were

solubilized in 50 mM sodium carbonate buffer, pH 10.5, containing 10 mM dithiothreitol. Protoxins were trypsin-activated (trypsin type XI: from bovine pancreas, Sigma Chemical, St. Louis, MO) at 37ºC for two hours (1 mg trypsin per 10 mg protoxin). Purified and trypsin activated Cry9Ca was obtained from Jeroen Van Rie (Bayer BioScience, Ghent, Belgium). Protein concentration was measured by the method of Bradford (Bradford, 1976).

The diet was poured into 100 ml beakers and the toxin was added to final concentrations of 1, 2, 4, 8, and 16 µg of toxin per ml of diet. A control was prepared with distilled water. The solidified diet was cut into small cylinders and placed into boxes of 30 mm diameter and 15 mm height, with no ventilation hole in the lid. One neonate larva was added to each box. Three groups of ten boxes were used with each concentration and the control. Mortality was determined after 7 days and surviving larvae were also weighed. Boxes were kept in a rearing room at 26±1ºC, 60±10% relative humidity, and a photoperiod of 16:8 (L:D).

The results of bioassays were evaluated by probit analysis and the 50% lethal concentration (LC_{50}) values were estimated using the POLO-PC program (LeOra Software, Berkeley, Calif.). The ratio between the weight of treated and untreated larvae at day seven was used to calculate larval growth inhibition. The effective concentrations that produce a reduction in larval growth of 50 % (EC_{50}) and 99 % (EC_{99}) were calculated by adjusting the curve as proposed by Sims et al. (1996).

RESULTS AND DISCUSSION

Among the eleven *Bt* toxins tested, Cry1Ac4 and Cry2Aa1 were the most toxic and produced a considerable mortality on *H. armigera* larvae (Table 1), with Cry1Ac4 being almost twice as much active as Cry2Aa1. These results are in agreement with those of other authors, who found Cry1Ac more or equally active than Cry2Aa with similar bioassay protocols (but using protoxins), although the LC_{50} ratio between toxins differs. Thus, the Cry1Ac/Cry2Aa LC_{50} ratio was 6.5-fold (Gajendra Babu et al., 2002) and 35-fold (Chakrabarti et al., 1998) in two independent populations from India, but it was non-significantly different in a population from Australia (Liao et al., 2002).

In the range of concentrations tested, the rest of toxins did not give enough mortality as to allow estimation of LC_{50} values. However, most of the toxins with no detectable lethal effect showed a clear effect on larval growth (Fig. 1), with several of them with an EC_{50} below or near 1 µg/ml or included in the range of concentrations tested (Table 1), like Cry9Ca, Cry1Fa1, Cry1Ab3, Cry2Ab2, Cry1Da, and Cry1Ja1. Toxins Cry1Aa3, Cry1Ca2, and Cry1Ea did not have any effect on either mortality or larval growth.

Only two studies with *H. armigera* have tested the toxicity of a considerable number of *Bt* toxins (Chakrabarti et al., 1998; Liao et al., 2002). For the Australian population Cry1Ac and Cry2Aa were the most active toxins, whereas for the Indian population these two toxins were the most active ones together with Cry1Aa, which gave no toxicity in our study. In agreement with our results, a severe effect on larval growth

has been observed for Cry1Ab, Cry1D and Cry1F in the Indian population (Chakrabarti et al., 1998), and Cry1Ab and Cry2Ab in the Australian population (Liao et al., 2002), and no effect (or negligible) for Cry1Ca and Cry1Ea in both populations.

The absolute values of LC_{50} estimated in our study for Cry1Ac4 (3.5 µg/ml) and Cry2Aa1 (6.3 µg/ml) are generally higher than those reported in previous papers using the similar bioassay conditions (diet incorporation): 0.02 µg/ml for Cry1Ac and 0.71 µg/ml for Cry2Aa (Chakrabarti et al., 1998), 0.14-0.18 µg/ml for Cry1Ac (Kranthi et al., 2000), 0.24 µg/ml for Cry1Ac and 1.57 µg/ml for Cry2Aa (Gajendra Babu et al., 2002), 0.17-0.54 µg/ml for Cry1Ac (Fengxia et al., 2004), 0.09-91 µg/ml (with a mean of 1.4 µg/ml for the 24 populations studied) for Cry1Ac (Wu et al., 1999), and 0.11-0.71 µg/ml for Cry1Ac (Jalali et al., 2004). These discrepancies might be due to differences in the nature and mode of application of the toxins, the method employed to estimate protein concentration, the temperature at which the bioassay was performed (this varies from 26 to 30ºC), and unquestionably, the origin of the insects. Regarding the nature of the toxin, our tests were carried out with purified and activated Cry proteins, whereas the rest of studies used protoxins or a mixture of crystals and spores. We have chosen activated toxins because we tried to simulate the form of toxin found in transgenic crops and to avoid the synergistic effect of spores. Transgenic crops seem to be the most widespread way of using *Bt* toxins in the future. The area dedicated to these cultivars is steadily growing since 1996 and it is expected to keep this tendency in the future (James, 2004).

In conclusion, the reported results represent the first study on the toxicity of *Bt* toxins to an European population of *H. armigera* . The Spanish population was very

susceptible to Cry1Ac4 and Cry2Aa1, as it has been found for populations of this species from other parts of the globe. Furthermore, growth inhibition was severe with Cry9Ca, Cry1Fa1, Cry1Ab3, Cry2Ab2, Cry1Da, and Cry1Ja1. Either in sprayable formulations or in *Bt*-crops, the combination/rotation of toxins with a different binding site is desirable from a resistance management point of view. The information on the toxicity of the different *Bt* toxins, along with that from binding studies of *Bt* toxins to binding sites in the midgut membrane (Estela et al., 2004), should allow to choose those toxins that, not binding to the same sites, are the most active against this pest.

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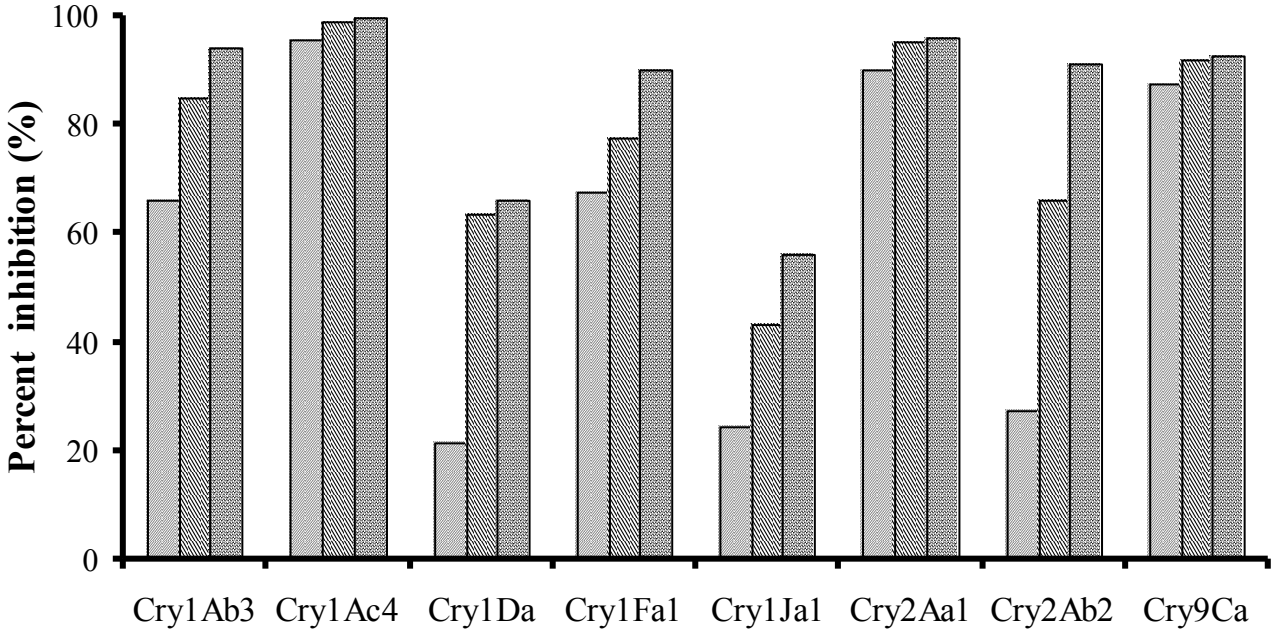
Table 1: Effect of activated *B. thuringiensis* toxins on mortality (LC_{50}) and larval growth rate (EC_{50} and EC_{90}) of neonate larvae of *H. armigera* exposed for 7 days.

Crystal protein	LC_{50} (µg/ml)	95% FL	EC_{50}±s.e. (µg/ml)	EC_{99}±s.e. (µg/ml)
Cry1Ac4	3.5	2.7-4.5	<<1.0[a]	3.7±0.5
Cry2Aa1	6.3	4.9-8.5	<<1.0	>16[b]
Cry9Ca	ND[c]		<<1.0	>16
Cry1Fa1	ND		<1.0[d]	>16
Cry1Ab3	ND		<1.0	>16
Cry2Ab2	ND		2.3±0.5	>16
Cry1Da	ND		3.8±1.6	>>16[e]
Cry1Ja1	ND		9.4±4.5	>>16
Cry1Aa3	ND		NE[f]	NE
Cry1Ca2	ND		NE	NE
Cry1Ea	ND		NE	NE

[a] EC_{50} was much lower than the lowest concentration tested (1 µg/ml).
[b] The EC_{99} was higher than the highest concentration tested (16 µg/ml).
[c] Not detectable because the mortality found at the highest concentration (16 µg/ml) was lower or near 50 % of the individuals and POLO PC (LeOra Software, Berkeley, Calif.) could not adjust to a regression line.
[d] EC_{50} was lower than the lowest concentration tested (1 µg/ml).
[e] The EC_{99} was much higher than the highest concentration tested (16 µg/ml).
[f] No effect on growth inhibition.

FIGURE LEGENDS

Fig. 1. Percent larval growth inhibition of *H. armigera* by different *B. thuringiensis* proteins at the concentrations: 1 µg/ml (▨), 4 µg/ml (▧), and 16 µg/ml (▩).


Percent inhibition (%)
100
80
60
40
20
0
Cry1Ab3
Cry1Ac4
Cry1Da
Cry1Fa1
Cry1Ja1
Cry2Aa1
Cry2Ab2
Cry9Ca