Susceptibility of Spanish Populations of the Corn Borers *Sesamia nonagrioides* (Lepidoptera: Noctuidae) and *Ostrinia nubilalis* (Lepidoptera: Crambidae) to a *Bacillus thuringiensis* Endotoxin

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ABSTRACT Baseline susceptibility to the Cry1Ab delta-endotoxin from *Bacillus thuringiensis* (Berliner) was determined for four populations of *Sesamia nonagrioides* (Lefebvre) and two populations of *Ostrinia nubilalis* (Hübner) from Spain. This study shows that *S. nonagrioides* is at least as susceptible as *O. nubilalis* to *B. thuringiensis* Cry1Ab protein. We found small differences in susceptibility among the Spanish populations of *S. nonagrioides* that can be attributed to natural variation, because there are no records of B. *thuringiensis* products being used on corn crops in Spain. There were no differences in susceptibility to Cry1Ab toxin between the two populations of *O. nubilalis*.

KEY WORDS European corn borer, *Ostrinia nubilalis*, *Sesamia nonagrioides*, *Bacillus thuringiensis*, *B. thuringiensis*-corn, insecticide resistance

SEVERE YIELD LOSSES caused by larval damage by the European corn borer, *Ostrinia nubilalis* (Hübner), have been reported in corn crops in the United States and Europe (Mason et al. 1996). *Sesamia nonagrioides* (Lefebvre), sometimes referred to as the Mediterranean corn borer, is one of the most damaging pests of corn in Spain (Castañera 1986) and other Mediterranean countries (Anglade 1972, Melamed-Madjar and Tam 1980).

Chemical control of these two species is particularly difficult because of their endophytic larval behavior, especially in *S. nonagrioides* whose larvae tunnel throughout the stem starting with the first instar. The use of genetically engineered corn plants expressing delta-endotoxins from *Bacillus thuringiensis* (Bt-corn) offers a rational strategy for the control of these two pests, reducing at the same time environmental costs associated with the use of conventional insecticides (Rice and Pilcher 1998). Since its registration in 1996, use of Bt-corn has spread quickly. In 1998, 6.7 millions hectares of Bt-corn were planted globally (James 1998). It is estimated that ≈22,000 ha of Bt-corn were grown in Spain for the first time in 1998, and a significant increase might be expected in the next few years.

Development of resistance in target pests to Bt-plants is the main risk for the success of this control tool (McGaughey and Whalon 1992) because laboratory selection assays show that many pests have the potential to develop resistance (see Tabashnik 1994 for a review). However, field resistance to *B. thuringiensis* products has been documented only in some populations of the diamondback moth, *Plutella xylostella* (L.), which had been treated with Bt-sprays (Tabashnik et al. 1990). In laboratory studies, the LC_{50}s of *O. nubilalis* populations increased 72.9-fold after only seven generations of selection pressure with a commercial formulation of *B. thuringiensis* (Huang et al. 1997), and this resistance was inherited as an incompletely dominant autosomal gene (Huang et al. 1999). It is expected that large-scale planting of Bt-plants could result in rapid selection for resistance in field populations of insects. Mascarenhas et al. (1998) reported a significant decrease in susceptibility to Bt insecticides in field strains of the soybean looper, *Pseudoplusia includens* (Walker), collected from Bt-cotton after two growing seasons.

To maintain the effectiveness of Bt-plants, it is necessary to detect changes in susceptibility through regular monitoring and to apply resistance management strategies to prevent or delay adaptation by pests (EPA 1998, Gould 1998). Variation in susceptibility to *B. thuringiensis* toxins among conspecific populations has been reported in many insect species (Tabashnik 1994). Sometimes it is not easy to distinguish natural variation among susceptible populations from low to moderate resistance (Koziel et al. 1993a).

In this context, it is of paramount importance to establish the natural susceptibility among geographical populations of corn borers to Bt toxins to help assess their potential evolution of resistance to Bt-corn. We report here on the baseline susceptibility of different geographical Spanish populations of *S. nonagrioides* and *O. nubilalis* to Cry1Ab toxin, the only Bt toxin expressed in Bt-corn grown in Spain.

Materials and Methods

Insect Collection. Corn borers were collected in four major Spanish corn growing regions: the southern

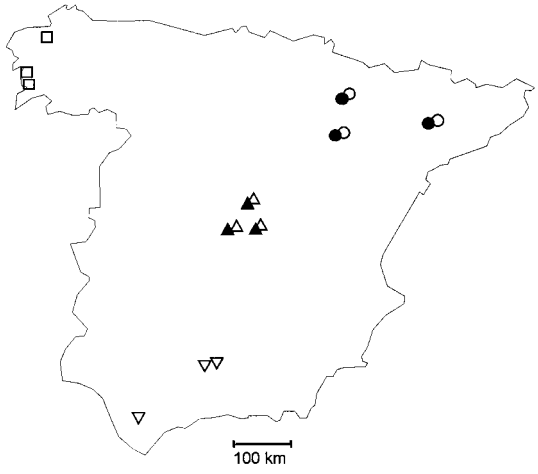

Fig. 1. Locations where *S. nonagriodes* (Madrid △; Andalucia ▽; Galicia □; Ebro ○) and *O. nubilalis* (Madrid ▲; Ebro ●) larvae were collected in Spain.

(Andalucia), central (Madrid), northwestern (Galicia), and northeastern (Ebro) regions of Spain. We collected 300-1000 larvae at 2–3 sites in three different locations in each region (Fig. 1). Bt-corn has never been cultivated at these sites. The larvae were collected in the fall of 1998 by dissecting corn stalks a few days before harvesting. All larvae were in diapause or entered diapause after collection, as determined by their developmental arrest once they reached the last instar. Four populations of *S. nonagrioides* were collected in Galicia (665 larvae), Madrid (793 larvae), Andalucia (661 larvae), and Ebro (857 larvae). *O. nubilalis* were collected in Ebro (644 larvae) and Madrid (345 larvae). Few *O. nubilalis* larvae were collected in Galicia, and none were collected in Andalucia. Field collected larvae were dipped in a solution of 0.01% phenylmercuric nitrate to prevent contamination by pathogens.

Insect Culture. Field-collected *O. nubilalis* larvae were placed in plastic cages (21 by 16 by 4 cm) containing corrugated cardboard (100 larvae per cage) and maintained in a growth chamber (Sanyo MLR-350 H, Sanyo, Japan) at 12 ± 0.5°C, 70 ± 5% RH, and a photoperiod of 12:12 (L:D) h. A meridic diet, modified from Poitout and Bues (1970) by the addition of 1.5 g of methyl p-hydroxybenzoate, 1.5 g fumidil-B, 1.2 g sorbic acid, and 0.6 g aureomycin, and with 9 g ascorbic acid (1.5× with respect to the original formulation of the diet) and 3 g benzoic acid (3×), was supplied to the larvae until they stopped feeding. After 2 mo at these conditions, diapause was broken by placing the larvae at 28 ± 0.5°C and continuous light without food; the cardboard was moistened daily until pupation. Egg masses were obtained by confining a minimum of 100 pairs (batches of five pairs) of adults in cylindrical plastic cages (12 cm diameter, 5 cm high). Cotton soaked with a solution of 10% honey in water was placed in the oviposition cages for feeding. The top of each cage was covered with a black waxed paper sheet, and all other sides were covered with filter paper. Egg masses were transferred to plastic containers (9.5 cm diameter, 3.5 cm high), provided with moistened filter paper, and incubated. Cages for oviposition and egg incubation were placed in an environmental chamber maintained at 24 ± 2°C, 70 ± 5% RH, and a photoperiod of 16:8 (L:D) h.

Field-collected *S. nonagrioides* larvae were maintained and diapause was broken as for *O. nubilalis*, except diapausing larvae were fed a meridic diet modified from Poitout and Bues (1970) by the addition of 1.6 g of Wesson's salt mixture, 1.5 g fumidil-B, 1 g methyl p-hydroxybenzoate, and 0.6 g aureomycin. Egg masses were obtained by confining a minimum of 100 pairs (batches of five pairs) of adults in ventilated plastic cylinders (12 cm in diameter, 30 cm high) containing five corn seedlings (20–30 cm high). Egg masses were removed from the corn leaf sheaths and placed in plastic containers (9.5 cm in diameter, 3.5 cm high), provided with moistened filter paper, and incubated. Environmental conditions for mating, oviposition, and egg incubation were as for *O. nubilalis*.

Bioassays. First instars (<24 h old) from the first laboratory generation were treated with different doses of a native *B. thuringiensis* Cry1Ab toxin on the surface of the diets described above. The *B. thuringiensis* protein was a sample of native Cry1Ab crystals purified (25% purity) from a bacterial culture provided by Novartis. Cry1Ab crystals were isolated from a *B. thuringiensis* subsp. *kurstaki* strain HD1–9, which produces only the Cry1Ab protein (Minnich and Aronson 1984, Carlton and Gonzalez 1985). This is the untruncated form of the Bt protein expressed in the Bt-corn grown in Spain. The crystals were resuspended by brief sonication in 0.1% Triton X-100. Doses, selected based on preliminary tests, were 12.3, 17.2, 22.1, 29.5, 36.8, 61.4, 98.2, 172, and 295 ng Cry1Ab/cm^2 of diet surface area for both species. Controls consisted of a Triton X-100 solution at the same concentration as the highest dose of toxin. Diets were dispensed in 128-cell (2 ml per cell) bioassay trays (Bio-Ba-128, Color-Dec Italy, Capezzano Pianore, Italy) and 50 μl of toxin solution was applied to the surface of the diet and kept at room temperature until dried. One neonate was placed in each cell and confined with a cover (Bio-Cv-16, Color-Dec Italy). A total of 48 larvae was tested at each dose, and larvae were taken from each of the 20 oviposition cages to preserve variability. *S. nonagrioides* was tested at one time, whereas the experiment was split into three blocks over time for *O. nubilalis*. Trays were incubated in a growth chamber at 26 ± 0.5°C, 70 ± 10% RH, and constant dark. Mortality (larvae not showing any reaction when prodded) was assessed after 7 d.

Data Analysis. For each population, the baseline susceptibility (LC_{50} and LC_{90}, at 95% confidence level) was established by probit analysis by using POLO-PC (LeOra 1987). Differences in susceptibility were compared by relative potency when slopes were not significant (i.e., regression lines were parallel) and by lethal dose ratios at the LD_{50} when one slope was

Table 1. Results of probit analysis indicating baseline susceptibility of field-collected larvae of *O. nubilalis* and *S. nonagrioides* from different Spanish corn growing areas to native Cry1Ab *B. thuringiensis* protein

Species	Geographic region	*n*	Slope ± SE[a]	LC_{50}[b] (95% CL)	LC_{90}[b] (95% CL)	Relative potency[c] (95% CL)	χ^2	df
S. nonagrioides	Madrid	468	1.84 ± 0.22a	23 (16–30)	113 (79–198)	1.00	7.88	7
	Andalucía	468	1.63 ± 0.20a	27 (16–39)	165 (105–362)	1.25 (0.89–1.76)	9.86	7
	Galicia	466	1.09 ± 0.23b	55 (19–115)	815 (292–20,567)	—	8.95	7
	Ebro	467	1.86 ± 0.21a	70 (56–87)	340 (243–554)	3.13 (2.24–4.47)	6.37	7
O. nubilalis	Ebro	430	1.55 ± 0.20a	109 (77–162)	733 (397–2,299)	4.58 (3.23–6.62)	33.82	22
	Madrid	428	2.10 ± 0.20a	104 (82–140)	426 (278–850)	4.94 (3.51–7.14)	40.48[d]	22

[a] Values followed by the same letter indicate that probit lines are parallel ($P < 0.05$).
[b] Doses expressed as nanograms of native Cry1Ab protein per square centimeter of diet surface area.
[c] Relative potency with respect to the most susceptible population (*S. nonagrioides* from Madrid). Relative potency was not calculated for the population of *S. nonagrioides* from Galicia because its slope was significantly different from slopes for other populations.
[d] Chi-square significant ($P < 0.05$).

significantly different from other slopes (Robertson and Preisler 1992).

Results

The *O. nubilalis* populations were more tolerant to the *B. thuringiensis* Cry1Ab native protein than the Madrid population (Table 1), the Andalucia population (relative potency with respect to Andalucia population of *S. nonagrioides*, 95% CL: Ebro –3.66, 2.59–5.27; Madrid –3.95, 2.81–5.68), the Ebro population (relative potency with respect to Ebro population of *S. nonagrioides*, 95% CL: Ebro –1.46, 1.04–2.05; Madrid –1.58, 1.13–2.20), and the Galicia population of *S. nonagrioides* (Lethal dose ratios at the LD_{50} with respect to Galicia population of *S. nonagrioides*, 95% CL: Ebro –1.96, 1.06–3.70; Madrid –1.89, 1.06–3.45).

The populations of *S. nonagrioides* from Madrid and Andalucia were more susceptible to the Cry1Ab toxin than the population from Ebro (relative potency, 95% CL: Ebro versus Madrid –3.13, 2.24–4.47; Ebro versus Andalucia –2.50, 1.80–3.55), but did not differ from each other in susceptibility (Table 1). It was not possible to calculate relative potency for the Galicia population because the slope was significantly different from other slopes. Lethal dose ratios at the LD_{50} of the Galicia population relative to the other *S. nonagrioides* populations were 2.41 (1.32–4.45) with respect to the Madrid population, 2.02 (1.09–3.78) with respect to the Andalucia population, and 0.79 (0.43–1.43) with respect to the Ebro population.

There was no difference in susceptibility to Cry1Ab toxin between the two populations of *O. nubilalis* (relative potency, 95% CL: 1.08, 0.77–1.53).

Discussion

Ostrinia nubilalis was less susceptible to *B. thuringiensis* than *S. nonagrioides.* The difference in susceptibility is not well understood but may be related to the processing, inactivation or binding affinity of the toxin in the insect midgut (Gill et al. 1992). It has also been suggested that greater tolerance to *B. thuringiensis* may be correlated to a broader host range (Stone and Sims 1993). Consequently, a more extensive complement of detoxifying enzymes to accommodate a diversity of hosts plants may have been developed in *O. nubilalis* because of its broader host range as compared with *S. nonagrioides*, which could also provide a greater tolerance to *B. thuringiensis.*

As reported in studies with other species (Tabashnik 1994), we found geographical differences in susceptibility to *B. thuringiensis* among populations of *S. nonagrioides* from Spain. However, these differences in susceptibility were small (3.1-fold as determined by their relative potencies with respect to the most susceptible population) when compared with the 42-fold differences reported for natural populations of Indianmeal moths, *Plodia interpunctella* (Hübner), and the 15-fold differences for almond moths, *Cadra cautella* (Walker) (Kinsinger and McGaughey 1979); the eightfold differences for the tobacco budworm, *Heliothis virescens* (F.), and the 16-fold differences for the corn earworm, *Helicoverpa zea* (Boddie) (Stone and Sims 1993); and the 99.7-fold differences for *Helicoverpa armigera* (Hübner) (Wu et al. 1999). The underlying causes of these differences in susceptibility within a species are mostly unknown, although in some instances it has been possible to associate lower levels of susceptibility with populations that were previously exposed to *B. thuringiensis* insecticides (McGaughey 1985, Tabashnik et al. 1990) or to Bt-plants (Mascarenhas et al. 1998). The differences in susceptibility among populations of *S. nonagrioides* observed in this study appear to be attributable to natural variation because there are no records of *B. thuringiensis* insecticide formulations being used on corn crops in Spain, which is its primary host. Moreover, all of the populations were collected in corn fields distant from those where Bt-corn was cultivated for the first time in 1998.

No difference in susceptibility to Cry1Ab toxin was observed between the two populations of *O. nubilalis.* Likewise, Huang et al. (1997) found small or no differences in susceptibility to a *B. thuringiensis* insecticide (Dipel ES) among five colonies of the European corn borer collected in different areas in Iowa and Kansas, and small differences (3.5-fold) were reported when using the purified protein (Marçon et al. 1999).

Transgenic corn plants expressing a truncated version of *B. thuringiensis* Cry1Ab delta-endotoxin are very effective against *O. nubilalis* (Koziel et al. 1993b,

Rice and Pilcher 1998). In laboratory assays, the LD_{50} of the Cry1Ab protein for this insect is 2–10 ng/cm^2 for U.S. populations (Carozzi and Koziel 1997, Marçon et al. 1999). However, the LD_{50} for two Spanish populations was ≈100 ng/cm^2. These differences in activity may be cause by natural variability between the American and the Spanish populations, by the source of the protein tested, or by the criteria used to assess mortality, because lower LD_{50}s would be expected if larvae with severe growth inhibition are considered as dead (Marçon et al. 1999). The susceptibility of field populations of *O. nubilalis* from the United States to *B. thuringiensis* insecticides has also been tested (Huang et al. 1997), but the results are not comparable because of the mixture of several toxins, spores, and formulation ingredients in the commercial products used by Huang et al. (1997). It has been recommended that susceptibility be tested with purified protein (as performed here), rather than with commercial formulations, because the purified forms may elucidate more readily shifts in susceptibility to transgenic plants (Stone and Sims 1993).

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