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Baseline Susceptibility of Western Corn Rootworm (Coleoptera: Crysomelidae) to Cry3Bb1 *Bacillus thuringiensis* Toxin

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ABSTRACT Susceptibility to Cry3Bb1 toxin from *Bacillus thuringiensis* (Bt) was determined for western corn rootworm, *Diabrotica virgifera virgifera* LeConte, neonates from both laboratory and field populations collected from across the Corn Belt. Rootworm larvae were exposed to artificial diet treated with increasing Cry3Bb1 concentrations, and mortality and growth inhibition were evaluated after 4–7 d. The range of variation in Bt susceptibility indicated by growth inhibition was similar to that indicated by mortality. Although interpopulation variation in susceptibility was observed, the magnitude of the differences was comparable with the variability observed between generations of the same population. In general, the toxin was not highly toxic to larvae and estimated LC_{50} and EC_{50} values were several times higher than those reported for lepidopteran-specific Cry toxins by using similar bioassay techniques. These results suggest that the observed susceptibility differences reflect natural variation in Bt susceptibility among rootworm populations and provide a baseline for estimating potential shifts in susceptibility that might result from selection and exposure to Cry3Bb1-expressing corn hybrids.

KEY WORDS Bt susceptibility, geographic variability, bioassay, *Diabrotica virgifera virgifera*

INCREASING PUBLIC CONCERN ABOUT environmental hazards and widespread resistance in pest populations is threatening the continued effectiveness of conventional insecticides. This threat is especially true for the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, which is the most serious pest of field corn, *Zea mays* L., in North America. Crop rotation and chemical control have been the primary management strategies (Levine and Oloumi-Sadeghi 1991), although *D. v. virgifera* has become increasingly difficult to control due to its sequential ability to evolve resistance to different classes of small-molecule insecticides (Ball and Weekman 1962, Meinke et al. 1998, Zhou et al. 2002). In areas where crop rotation has been the primary management strategy, rootworms also have evolved a "behavioral" resistance involving oviposition in nonhost crops (O'Neal et al. 2001, Levine et al. 2002). Eggs deposited outside cornfields can cause significant damage to corn planted in these fields the following year. Soil insecticides such as organophosphates and pyrethroids are still effective, but they pose significant environmental and human health risks and are therefore unlikely to provide viable and long-term management options.

In 2003, the Monsanto Company announced it had received a registration from the U.S. Environmental Protection Agency for the sale of its YieldGard Rootworm-protected corn hybrids. This event (referred to as MON 863) constitutively expresses the Cry3Bb1 toxin derived from *Bacillus thuringiensis* (Bt) in both root and above ground tissue, although only rootworm larvae are affected by the toxin (EPA 2003). This technology offers several important advantages over conventional rootworm management techniques, including reduced applicator exposure to insecticides and a narrower spectrum of activity, and it does not require special application equipment or calibration (Vaughn et al. 2005). However, as with other transgenic Bt crops, the risk of resistance development is perceived as being high, especially for MON 863, which does not express a high dose of toxin such that significant survival and adult emergence from expressing plants have been reported (Vaughn et al. 2005).

The development of Bt resistance in target pests threatens the continued effectiveness of Bt technology. The possibility of resistance development highlights the need to develop and implement resistance management strategies to prevent or delay the evolution of resistance to Bt (Hokkanen and Deacon 1994). These strategies are dependent on the development of effective resistance monitoring programs capable of early detection of resistance that allow implementation of appropriate management decisions in a timely manner (Dennehey 1987). The initial steps in implementing such programs include development of appropriate bioassay techniques and establishment of baseline susceptibility data among populations

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across the geographic range of the target species. With this information, potential population susceptibility changes in response to selection with Bt can be identified (Fischhoff 1996).

Documenting the variation in susceptibility to Bt toxins among geographically distinct populations has become a common theme in establishing sustainable resistance management programs. The objective of the current study was to establish a baseline of susceptibility to Cry3Bb1 toxins from geographically distinct populations of western corn rootworms.

Materials and Methods

Insects. Western corn rootworm populations used for bioassays were either collected from the field or obtained from laboratory populations. Generally, laboratory populations were initiated from field-collected adults during 1995–1996 and reared for five to seven generations at the USDA–ARS Northern Grain Insects Research Laboratory in Brookings, SD, by using standard rearing techniques (Jackson 1986). Field populations were collected in 2002 as adults and were maintained on fresh corn silks, corn ears, and a water source according to Jackson (1986). Each collection consisted of at least 400 ovipositing females. The eggs from ovipositing females were collected in sifted soil by using standard techniques (Jackson 1986).

Eggs were shipped from the rearing facility and incubated at 25°C and 60% RH for 12 d. The eggs were then rinsed from the soil into a #60 sieve, removing as much fine soil and debris as possible. Eggs were poured into a 50-ml conical tube with excess water so that floating debris could be pored off. The remaining eggs were then suspended in 1.25 M $MgSO_4$, which causes the eggs to float, whereas soil and other debris sink to the bottom so that they can be suctioned with a Pasteur pipette. $MgSO_4$ flotation was repeated as necessary. The eggs were then disinfected to reduce microbial contamination by using techniques adapted from Pleau et al. (2002). Briefly, the eggs were cleaned first with Lysol brand disinfectant followed by 10% formalin for 2 min each and triple rinsed with sterile water. The eggs were pipetted onto Whatman no. 1 filter paper in petri dishes with vented lids and incubated at 28°C until hatching.

Bioassays. All bioassays were conducted by exposing neonates (<24 h after hatching) to treated artificial diet. Bioassays involved surface treatment of single wells of artificial diet dispensed into 96-well microtiter plates. The artificial diet used in all bioassays was prepared as described by Pleau et al. (2002). Bioassays were performed in 96-well microtiter plates (Falcon 353910, BD Biosciences, Franklin Lakes, NJ). Approximately 200 μl of diet was dispensed into each well by using an automated dispenser (Eppendorf 22-26-400-1, Brinkman Laboratories, Westbury, NY) and allowed to solidify. Six concentrations of Cry3Bb1 protein were used, and dilutions were made in 0.05% Triton X-100 nonionic detergent to obtain uniform spreading onto the diet surface. Each well was treated with 10 μl of the appropriate solution.

Purified crystal Cry3Bb1 protein was prepared from a *B. thuringiensis* strain (EG11231) containing the Cry3Bb1 gene. This strain was grown in C2 medium for 3 d at 28°C, at which time the cultures were fully sporulated and lysed. The spore and crystalline inclusions (i.e., crystals) were pelleted by centrifugation, and washed twice in 25 mM Tris-HCl, 0.005% Triton X-100, pH 7.5. The washed pellet was resuspended in 25 mM Tris-HCl, 2 mM $MgCl_2$, 50 mM phenylmethylsulfonyl fluoride, pH 7.5, and treated with benzonase nuclease at 25 U/ml for 1.5 h at ambient temperature before centrifugation to the capture treated pellet. The resulting pellet was extracted in 4 M NaBr and incubated at ambient temperature for 2 h with stirring. The 4 M NaBr extract was then clarified by multiple centrifugations and subsequent filtration to 0.22 μm. The filtered extract was dialyzed against 10 mM sodium acetate, pH 5.0, over 72-h period at which point a fine, white precipitate had fallen out of solution. This precipitated material was collected by centrifugation and washed once in 25 mM Tris-HCl, 0.005% Triton X-100, pH 7.5, before final suspension in same buffer.

Cry3Bb1 protein quantification was determined by spot densitometry where multiple loadings of the Cry3Bb1 protein were gel purified alongside bovine serum albumin (BSA) standards representing a five-point standard curve. The resulting Coomassie-stained gel was scanned and analyzed, and the Cry3Bb1 concentration was determined by direct comparison of band densities to the most linear portion of the BSA standard curve. Control treatments consisted of diet treated with 0.05% Triton X-100 only. Wells were allowed to air dry for 1 h, and one neonate was transferred into each well by using a camel's-hair paintbrush. Wells then were covered with Mylar plate sealers (Dynex Technologies, Chantilly, VA), and plates were held at 27°C, 24-h scotophase, and 80% RH. Mortality and individual larval weights were recorded 4–7 d later. All surviving larvae at each concentration were pooled, and the total weight was recorded.

Statistical Analysis. Only bioassays in which control mortality was <20% and that had at least three concentrations that produced mortality >0 and >100% were subjected to further statistical analysis. Bioassays were conducted in duplicate on three different dates, depending on availability of eggs, and they included at least five Bt concentrations that produced significant growth inhibition and mortality. Pooled weights of surviving larvae were recorded and transformed to percentage of growth inhibition relative to the controls, and these data were analyzed by nonlinear regression (SAS Institute 1988, Marçon et al. 1999). Mortality data were analyzed by probit analysis (Finney 1971) by using POLO-PC (LeOra Software 1987).

Results and Discussion

The susceptibility of neonate western corn rootworm larvae to the purified Cry3Bb1 protein for laboratory and field populations is presented in Tables 1 and 2, respectively. LC_{50} values ranged from 2.01 $\mu g/cm^2$ (Phelps Co., Nebraska) to 13.04 $\mu g/cm^2$ (Potter

Table 1. Susceptibility of western corn rootworm neonates exposed to the Cry3Bb1 toxin from *B. thuringiensis* as measured by growth inhibition and mortality from laboratory populations maintained for five or six generations

Population	*n*	EC_{50} (95% CI)[a,b]	Slope ± SE	LC_{50} (95% CL)[b]	χ^2	df
Monsanto lab (2/2001)	202	2.28 (1.55–3.01)	1.18 ± 0.20	5.57 (1.02–14.70)	3.45	3
Monsanto lab (3/2001)	192	8.73 (5.44–12.85)	1.00 ± 0.22	13.04 (5.01–27.77)	0.22	2
Phelps Co., NE (Keene)	275	1.85 (1.32–2.54)	1.32 ± 0.17	2.11 (1.38–3.00)	2.58	3
Phelps Co., NE (Nitchie)	418	2.10 (0.57–6.79)	1.13 ± 0.15	2.01 (0.75–3.75)	13.13	8
Phelps Co., NE (Wenz)	203	0.97 (0.51–1.52)	1.11 ± 0.21	2.18 (0.90–3.93)	0.99	3
Phelps Co., NE (Vandell)	137	2.12 (0.62–4.89)	1.25 ± 0.30	4.62 (1.90–8.23)	0.74	2
Clay Co., NE	198	2.71 (1.86–3.96)	1.17 ± 0.20	2.84 (1.29–5.10)	1.69	2
York Co., NE	275	3.14 (1.59–5.77)	0.94 ± 0.21	7.92 (3.62–14.75)	1.79	2
Saunders Co., NE	140	1.62 (0.62–3.45)	1.79 ± 0.59	2.38 (0.23–4.81)	0.77	3
Potter Co., SD	115	4.14 (2.77–5.19)	1.41 ± 0.39	10.79 (4.34–22.23)	1.80	2
Tippecanoe Co., IN	303	3.75 (2.41–5.81)	1.51 ± 0.21	4.14 (2.59–5.98)	2.86	3
Finney Co., KS	244	1.67 (1.41–1.98)	1.70 ± 0.21	2.36 (1.65–3.24)	2.47	3
Champaign Co., IL	292	1.95 (1.55–2.43)	1.08 ± 0.17	6.76 (3.91–11.04)	1.77	3
Center Co., PA	164	2.49 (2.12–2.93)	3.07 ± 0.84	2.06 (1.04–2.97)	0.15	2

[a] Concentration of Cry3Bb1 that produces 50% growth inhibition relative to untreated controls. Calculated by nonlinear regression fitted to a probit model. CI, confidence interval; CL, confidence limit.
[b] Micrograms of Cry3Bb1 per square centimeter of treated artificial diet.

Co., South Dakota) in laboratory populations representing a six-fold difference in susceptibility (Table 1). Similar results were obtained with larval progeny derived from field-collected adults (Table 2), although variability was slightly greater with LC_{50} values ranging from 0.74 $\mu g/cm^2$ (Winona Co, Minnesota) to 9.20 $\mu g/cm^2$ (Weld Co., Iowa). Similar variation was observed in the calculated EC_{50} values for both laboratory (6.6-fold) and field (12-fold) populations. The generally higher variability observed in the field populations may reflect a more heterogeneous environmental and genetic background of the field populations.

Sigmoid curves were observed for both mortality and growth inhibition (Fig. 1), although there was not clear separation in the response curves. The relative absence of sublethal effects is reflected in the similarity of LC_{50} and EC_{50} values (Tables 1 and 2) among the populations tested. These results suggest that Cry3Bb1 does not cause significant sublethal effects as has been noted previously for lepidopteran-active Cry toxins that cause significant growth inhibition at concentrations that do not cause mortality (Marçon et al. 1999, Siegfried et al. 2000). Therefore, the toxin does not seem to be causing inhibition of growth at sublethal concentrations, and Cry3Bb1 does not impact larval development at sublethal concentrations.

The results of these bioassays suggest that western corn rootworm larvae are in general not extremely sensitive to the Cry3Bb1 toxin because both LC_{50} and EC_{50} values are several orders of magnitude greater than values reported for lepidopteran-active Cry toxins by using similar bioassay methods. This lack of sensitivity is consistent with the designation of MON 863 as a low-to-medium dose product (EPA 2003). The designation of this technology as other than "high dose" is likely to be related to the inherent tolerance of *D. v. virgifera* to the Cry3Bb1 toxin. Although it is likely that there are multiple factors contributing to the survival of western corn rootworm larvae on MON 863 plants, it is also likely that the expression of toxin within these plants falls within the

Table 2. Susceptibility of western corn rootworm neonates derived from field populations exposed to the Cry3Bb1 toxin from *B. thuringiensis* as measured by growth inhibition and mortality

Population	*n*	EC_{50} (95% CI)[a,b]	Slope ± SE	LC_{50} (95% CL)[b]	χ^2	df
Yuma Co., CO	397	1.46 (0.60–3.39)	1.51 ± 0.20	3.14 (1.96–4.52)	1.79	3
Scott Co., IA	312	1.63 (1.00–2.58)	1.26 ± 0.20	1.49 (0.73–2.45)	1.14	3
Weld Co., IA	321	1.97 (0.92–3.88)	1.42 ± 0.18	9.20 (6.45–13.06)	1.33	3
Iroquois Co., IL	146	2.80 (2.22–3.50)	1.88 ± 0.45	4.22 (1.99–6.75)	1.96	2
Warren Co., IL	155	0.98 (0.76–1.24)	2.05 ± 0.48	0.74 (0.32–1.14)	1.91	2
Gove Co., KS	320	1.02 (0.28–2.39)	1.68 ± 0.31	4.90 (2.51–7.40)	0.99	3
Hamilton Co., NE	459	1.06 (0.91–1.21)	1.44 ± 0.15	2.03 (0.23–5.71)	12.12[c]	3
Clay Co., NE	293	1.47 (0.48–4.12)	1.49 ± 0.21	2.00 (0.69–3.86)	3.07	3
Seward Co., NE	277	1.22 (0.89–1.62)	1.42 ± 0.19	2.69 (0.82–5.79)	4.67	3
Winona, MN	494	0.52 (0.35–0.64)	1.91 ± 0.23	0.74 (0.23–1.31)	6.49	3
Ontario Co., NY	408	1.12 (0.88–1.39)	1.94 ± 0.19	1.88 (0.64–3.92)	18.13[c]	3
Lancaster Co., PA	289	2.26 (0.92–5.60)	1.16 ± 0.15	6.61 (4.49–9.84)	1.49	3

[a] Concentration of Cry3Bb1 that produces 50% growth inhibition relative to untreated controls. Calculated by nonlinear regression fitted to a probit model. CI, confidence interval; CL, confidence limit.
[b] Micrograms of Cry3Bb1 per square centimeter of treated artificial diet.
[c] χ^2 significant ($P < 0.05$).

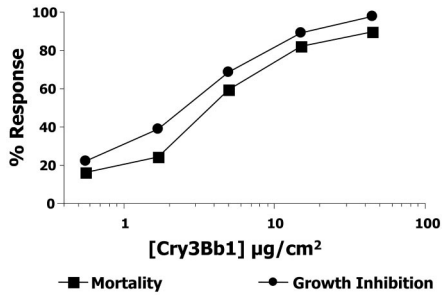

Fig. 1. Comparisons of response curves for mortality and growth inhibition of western corn rootworm neonate larvae exposed to increasing concentrations of the Cry3Bb1 *B. thuringiesnis* toxin.

normal distribution of tolerance among a susceptible homozygous population. Survival on MON 863 plants does not necessarily indicate a shift in the frequency of resistance alleles, and it is unlikely that initial frequency of major resistance-conferring alleles is any different than suggested for other species and other Bt endotoxins. Rather, it is more likely that expression of the toxin is comparable with a lethal dose that causes low-to-moderate mortality. As a consequence, a significant proportion of exposed populations may survive at the expressed levels and that MON 863 represents a truly low-to-moderate dose event.

Although variation in susceptibility to Cry3Bb1 was observed, the magnitude of the difference was relatively small and similar to other estimates of baseline variability among geographically distinct populations of other insect species. Stone and Sims (1993) found considerable interpopulation variation in *B. thuringiensis* susceptibility to Cry1Ac and formulated Dipel among U.S. populations of both corn earworm, *Helicoverpa zea* (Boddie), and tobacco budworm *Heliothis virescens* (16- and 4-fold, respectively). These data were reexamined by Sims et al. (1996), who suggested that interpopulation variation in *B. thuringiensis* susceptibility may reflect nongenetic variation or sampling error, because the populations tested represented a small sample, taken at one point in time, of considerably larger multivoltine populations. Similar levels of variability in susceptibility to Cry1Ab and Cry1Ac were observed among European corn borer populations (Marçon et al. 1999) that included populations of different voltine ecotypes and pheromone strains and among geographically distinct populations of *H. zea* exposed to Cry1Ab (Siegfried et al. 2000).

The variation in baseline susceptibility to *B. thuringiensis* toxins may reflect differences in vigor among parental populations (Rossiter et al. 1990), attributes that are the product of both genotype and the maternally determined nutritional status of the egg. This variation may be especially evident of field populations where a higher degree of variation in maternal status is likely.

Development of baseline susceptibility data represents the first step toward the development of a monitoring program designed to detect changes in susceptibility that may result from repeated and prolonged exposure to *B. thuringiensis* toxins. These data also may provide information that will allow development of diagnostic bioassays that would be more efficient in detection of resistant populations. However, it should be noted that because of the relative insensitivity of rootworm to the Cry3Bb1 toxin, designation of diagnostic concentrations equivalent to an LC_{99} may be difficult to achieve because of the large amounts of purified crystal toxins that would be required for large scale testing of field populations.

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