Selection for Cry3Bb1 Resistance in a Genetically Diverse Population of Nondiapausing Western Corn Rootworm (Coleoptera: Chrysomelidae)

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ABSTRACT Five short-diapause laboratory lines of western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), were selected for resistance to MON863, a variety of corn genetically modified with the *Bacillus thuringiensis* Berliner (Bt) transgene that expresses the Cry3Bb1 δ-endotoxin. Three of the selected lines were developed by incremental increase in the duration of exposure to MON863 over 11 generations (moderate selected lines). Two selected lines were developed from a control group by constant exposure to MON863 for at least 14 d posthatch over seven generations (intense selected lines). At the end of the experiment, survivorship, as measured by adult emergence, was ≈4 times higher in each of the selected lines reared on MON863 compared with control lines. Estimates of realized heritabilities (h^2) were 0.16 and 0.15 for the moderate and intense selected lines, respectively, and are consistent with h^2 estimates reported previously from a variety of pest insects. These lines provide data necessary for evaluating the potential for Bt resistance within diabroticite beetles and will be useful for developing improved insect resistance management strategies.

KEY WORDS *Diabrotica virgifera virgifera*, Bt resistance, MON863, insect resistance management

Development of resistance by western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), and related pests (*Diabrotica virgifera zea* Krysan & Smith, *Diabrotica barberi* Smith & Lawrence) to corn, *Zea mays* L., genetically modified with the *Bacillus thuringiensis* Berliner (Bt) transgene poses serious risks to the future viability of this crop. Usage of corn varieties incorporating Cry3Bb1, a δ-endotoxin that specifically targets corn rootworms (Ward et al. 2005), has increased dramatically since it was approved for use in the United States in 2003 (USEPA 2003). For example, total acreage of corn incorporating the Cry3Bb1 transgene increased from ≈161,874.26 ha (≈0.4 million acres) in 2003 to ≈13 million ha (≈32 million acres) in the United States in 2008 (Monsanto Corporation 2008). Although field resistance of western corn rootworm to Bt corn has not yet been documented, such an event is likely given the impressive history of adaptation to various control measures by this species (Metcalf 1983, Meinke et al. 1998, Levine et al. 2002). Despite significant risk of Bt resistance development within field populations, remarkably little is known about the biological mechanisms of Bt resistance in western corn rootworm (Gassmann et al. 2009, but see Kaiser-Alexnat 2009, Lefko et al. 2008, Meihls et al. 2008).

Although application of laboratory results to field settings can be problematic (Tabashnik 1992), characterization of Bt resistance is nevertheless important for field management strategies implemented to delay its development (Denholm and Rowland 1992, Alstad and Andow 1996, Elzen and Hardee 2003). For example, estimates of resistance parameters such as rate of resistance development and magnitude of resistance response are critical for insect resistance management (IRM) plans for Bt corn. These data are especially important for management of western corn rootworm in the United States, where adequacy of refuge size parameters required by the IRM strategy for Bt corn has been questioned (Powell 2003).

Here, we describe development of resistance to MON863 corn expressing Cry3Bb1 and also estimate the heritability of resistance within laboratory lines of western corn rootworm. Our study is intended to provide data for the characterization of Bt resistance within western corn rootworm and complement the few other studies (Lefko et al. 2008, Meihls et al. 2008) that have investigated Bt resistance in this pest species. Finally, we discuss the implications of our results for

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the IRM plan currently in place for Bt corn in the United States.

Materials and Methods

Genetically Diverse Base Population. A base population for Bt selection experiments was derived by initially crossing several virgin females of a nondiapausing line to males from one of four diapausing (D) western corn rootworm lines at the USDA–ARS North Central Agricultural Research Laboratory in Brookings, SD. The nondiapausing population from which females were sampled was naïve to Bt and has been maintained at the USDA–ARS laboratory for >30 yr (Branson 1976). Females were chosen to introduce the nondiapausing trait as diapause has a large maternal contribution (Krysan and Branson 1977). Diapause males of unknown age were wild-caught in 2004 from four different geographic regions across the Corn Belt (Hamilton Co., OH; Moody and Lake Cos., SD; Phillips Co., CO; and Will Co., IL) to capture genetic diversity across the Corn Belt. Males were aspirated from the wild colonies, and their sex was verified under the microscope (White 1977, Hammack and French 2007).

Each mating pair was placed in a separate cage. Experimental mating cages consisted of a plastic Genpak (Glens Falls, NY) AD32 750-ml hinged deli container. An opening ≈55 by 65 mm was cut into the bottom and covered with a glued nylon mesh (eight strands per centimeter) screen. The lids were cut from the base and the container inverted and pressed into the lid for use. The vent screen was now on top and food, water, and oviposition dishes were placed in the bottom of the cage. The food consisted of an artificial diet similar to that developed by Branson and Jackson (1988), except our diet substituted fructose for sucrose and added the antibiotics lincomycin and spectinomycin for bacterial and mycoplasmal control. Water was supplied in agar bars.

Oviposition dishes (60- by 15-mm petri dish) were placed in the cages for the first time 14 d after pairing. An oviposition dish consisted of ≈6 ml of soil that had been sifted through an 80-mesh screen and was thoroughly moistened with distilled water. Two slits were cut into the soil surface. A small hole, ≈5 mm in diameter, was drilled into the petri dish lid to allow the female access for oviposition. The dish lid was covered with a small piece of accordion-shaped sheet metal to partially darken dishes and thereby facilitate oviposition. Food and water were changed at least once a week and oviposition dishes were changed weekly. Males were removed once females began oviposition. Oviposition dishes were labeled and maintained in a growth chamber at 25°C and 45% RH. Eggs were washed from the dishes 7–10 d after removal from the cage and the number of eggs was recorded for each dish. Eggs were handled according to standard western corn rootworm rearing procedures (Jackson 1986) through the pupal stage.

The pupae were excavated from the soil, sexed (Krysan 1986) and then housed individually in a 44-ml bioassay cup containing ≈10 ml of dried and sifted 80-mesh soil moistened with 7 ml of distilled water to prevent desiccation. The pupae then were placed in an environmental chamber at 25°C and 60% RH in complete darkness and checked each day for eclosion. All newly emerged beetles were removed from the bioassay cups within 1 d after their cuticles had hardened, and only beetles that were mobile within the confines of the bioassay cup were selected for mating. Deformed beetles were not used. Newly emerged adults were individually isolated in the 750-ml Genpak plastic cages provisioned with plenty of fresh food and water and transferred to a growth chamber at 25°C, 45% RH, and a photoperiod of 14:10 (L:D) h.

At ≈1 wk of age, 28 virgin males and females, seven from each of the four locations, were transferred to a 30- by 30- by 60-cm, eight strands per cm mesh screen cage to promote random mating among the 56 males and females. Plenty of food and water were added to the cage. Males and females were housed together for a week and then provided with an oviposition dish. A new egg dish was added weekly for 5 wk. These offspring formed the genetically diverse base population and were used to select for short diapause.

Selection for Short Diapause. Progeny resulting from the random mating population were divided into eight independent lines containing ≈1,000 larvae each. These lines were then selected for early diapause for two generations. The 200 earliest hatching larvae each generation were used as parental stock in the subsequent generation, thereby selecting only those individuals with the shortest diapause periods. Five lines that did not demonstrate good survival to adult emergence were eliminated during the course of the experiment. After two generations of selection, all individuals from the three remaining early diapause lines were pooled and allowed to mate randomly to form a short diapause base population for subsequent experimentation.

Selecting for Cry3Bb1 Resistance. Five experimental lines were created initially from the short diapause base population for Cry3Bb1 selection experiments, three selected and two control lines. In total, 250 neonate larvae were placed into each of four 500-ml wide-mouthed Nalgene containers per line (1,000 individuals per line). Within each container, five kernels of sprouted corn (MON863 for selected lines and isoline for controls) that had germinated 72 h before the start of the experiment were overlain on moistened #3 germination paper. To achieve selection pressure without losing experimental lines, the duration of exposure to MON863 was gradually increased over the course of the experiment (Fig. 1), with a goal of achieving 20% survival to the adult stage in each successive generation. Exposure duration started at 24 h for generation 0, increased in 12-h increments through generation 4, and then increased in 24-h increments through generation 9, and then one final 12-h increment at generation 10, which exposed the larvae for 336 h, encompassing the first, second, and most of the third instar stages at 25°C (Jackson and Elliott 1988; Fig. 1). We used neonate larvae because the first instar

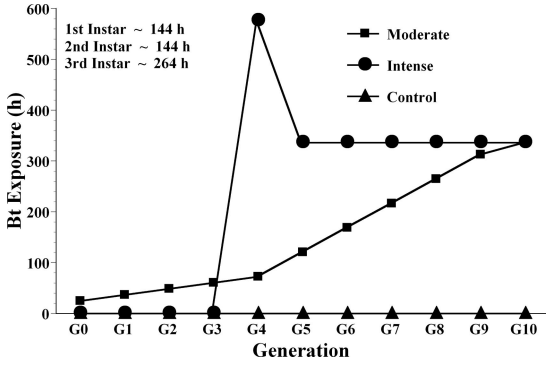

Fig. 1. Duration (hours) of western corn rootworm neonate larval exposure to MON863 roots expressing the Cry3Bb1 protein for moderate, intense, and control lines. The approximate time (hours) it takes to complete first, second, and third instars at 25°C also is shown.

stage is most susceptible to MON863 (B.W.F. and C.N., unpublished data). As exposure time increased with each generation and the corn rootworms adapted to the Cry3Bb1 toxin, additional 3-d-old sprouted MON863 corn was added as needed to exposure containers up to 168 h (7 d) or through generation 6. After exposure time, kernels and larvae from each exposure container were transferred to a 0.9-liter plastic deli rearing container (19.5- by 16.8- by 7-cm) that held an ample supply of 3-d-old isoline corn sprouts in soil. Due to the neonate larvae burrowing into the sprouts during feeding it was impossible to transfer these larvae without the sprout. Larvae not transferred with the sprout were carefully transferred by camel's hairbrush to the new deli rearing container. For generations 7–10, exposure was accomplished by placing the neonate larvae directly in the deli containers with several kernels of 3-d-old MON863 corn sprouts in soil. At 14 d for generations 0–6, the larvae and root mat from two deli containers were transferred to a larger 6-liter (32.5- by 26.5- by 9.5-cm) secondary plastic container (sweater box) consisting of several kernels of 3-d-old isoline sprouted corn. The delis were transferred to the sweater boxes after Bt exposure for generations 7–10. All exposure and larval containers were maintained at 25°C and 60% RH in complete darkness.

After 10 d, the sweater boxes were transferred to emergence containers. Each emergence box was 1 by 1 by 0.3 m deep; made of galvanized sheet metal; and partially subdivided horizontally, allowing for a dark (bottom) and light chamber (top). The top subdivision permitted ambient light via a ≈15-cm-diameter, glass-covered aperture. A second ≈15-cm top aperture was fitted with a mesh cloth sleeve, allowing access to emerging adults for collection. These emergence containers were placed in rooms maintained at 25°C and 60% RH and with constant light. Control lines reared on MON863 isoline were randomly culled each generation to ≈200 individuals to match densities for MON863 exposed lines. For each line, emerging adults were collected and placed in screen mesh mating cages as described above.

Bioassay. Beginning with generation 4 and continuing through generation 10, selection response was tracked every other generation by evaluating survivorship from neonate larva to adults for the moderate selected and control lines on MON863 and isoline corn. The bioassay analysis for these lines followed the normal western corn rootworm rearing procedure where ≈500–1,000 eggs each were transferred to four deli containers for each line containing 3 d old sprouted MON863 corn for 14 d. The larvae and root mat from two deli containers then were transferred to a sweater box of 3-d-old MON863 sprouted corn for at least 10 additional days and then transferred to emergence containers. All exposure and larval containers were maintained at 25°C and 60% RH in complete darkness. For each line, emerging adults were collected and placed in screen mesh mating cages as described above.

For evaluating survivorship of moderately selected and control lines on isoline corn, only one deli was used from each of the lines and these were combined for their respective group. In generation 4, those control beetles emerging off of the MON863 were used to create two new Bt resistant experimental lines called intense selected lines. In subsequent generations, exposure in these lines was held constant at 14 d (Fig. 1). In generation 10, we conducted a more comprehensive analysis of survivorship by increasing the number of delis for each line to 20. Here, we evaluated the three moderate selected, two intense selected, and two control lines on MON863 and isoline corn.

All estimates of survivorship were adjusted for egg viability. Egg viability was estimated by plating three 100 subsamples per line on moist filter paper placed in lid-sealed 11- by 11- by 2.5-cm plastic containers and maintained at 25°C and 60% RH in complete darkness for 21 d. Dishes were observed weekly for egg hatch. Eggs that were discolored or physically damaged were removed and not included in determining viability (Boetel and Fuller 1997). Proportion survivorship was calculated as adults emerged ÷ (total number of eggs × percentage of egg hatch).

After arcsine transformation for normalization of proportion data (Sokal and Rohlf 1995, Zar 2009), the control and moderate selected lines were tested for differences in survivorship on MON863 and isoline corn separately using unpaired *t*-tests for generations 4, 6, and 8 combined. We used a single-factor analysis of variance (ANOVA) to test for differences in survivorship of the control, moderate, and intense selected lines on MON863 and isoline corn for generation 10.

Realized Heritabilities. The heritabilities of survivorship on MON863 were estimated as realized heritabilities (h^2) for the control, moderate, and intense selected lines by using the equation $h^2 = R/S$, where R represents the response to selection and S represents the selection differential (Falconer 1989). R is the change in mean phenotypic value between the offspring of the selected parents and the entire parental generation before selection (Falconer 1989, Visscher et al. 2008). S is the difference in mean phe-

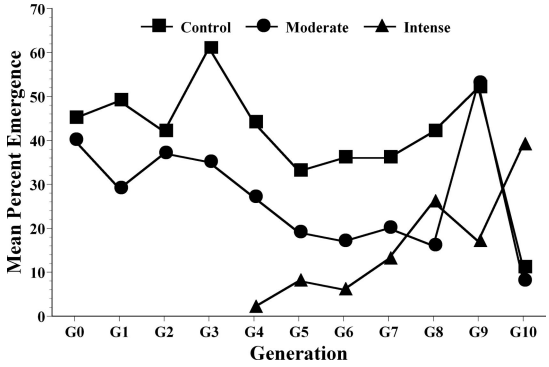

Fig. 2. Mean percentage of emergence for moderate, intense, and control lines for generations 0–10.

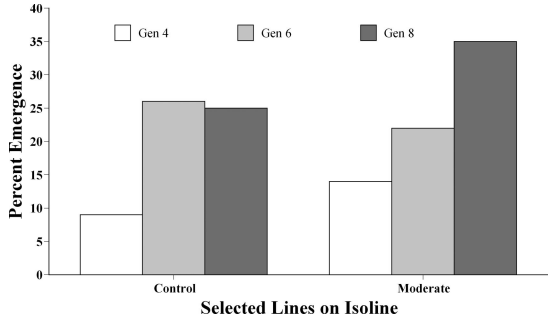

Fig. 3. Percentage of emergence for control and moderate selected lines reared on MON863 isoline corn through first, second, and third instars for generations 4, 6, and 8.

notypic value between the individuals selected as parents and the average phenotypic value of the entire parental generation before applying selection (Falconer 1989, Visscher et al. 2008). R values were calculated for generations 6, 8, and 10 based upon the average survivorship in each line for each of these generations. Values of S were obtained for generations 6, 8, and 10 by taking average mortality (where mortality = 1 − survivorship) from the previous n-2 generation. One exception occurred. Because the intense selected line was initiated from the control line after generation 4, mortality from the control line in generation 4 was used as a proxy for S_{Gen6} intense selected line. Per generation values for S were summed across generations 6, 8, and 10 to yield cumulative values for each of these three lines. Then, using a linear model (sensu Falconer 1989) across generations 6, 8, and 10, values of R were regressed upon $S_{cumulative}$ for each line, and the slope of each was taken. Final h^2 values were calculated for the moderate and intense selected lines after subtracting the slope of the control line from each of the moderate and intense selected lines to account for laboratory domestication, an important consideration when estimating parameters associated with selection experiments (Hill and Caballero 1992).

Results

Over the course of the experiment, mean emergence among all lines ranged from 2.0% for the intensely selected lines to 61% for control lines (Fig. 2). Although our goal of 20% survivorship for the moderate selected lines was slightly higher in the first few generations, as the Cry3Bb1 exposure time increased (Fig. 1), mean emergence decreased to near 20% for several generations (Fig. 2). Mean emergence from the moderate selected lines remained below the control lines throughout much of the selection process until generation 9 at which time both had >50% mean emergence. The moderate selected and control lines were terminated early in generation 10. Mean emergence for the intense selected lines increased very slowly initially and then began increasing at a higher rate in subsequent generations (Fig. 2).

Collectively for generations 4, 6, and 8, there was no difference in emergence between the moderate selected and control lines when reared on isoline corn ($t = -0.45$, df = 4, $P = 0.68$) (Fig. 3). However, when reared on MON863 corn, emergence for generations 4, 6, and 8 combined was significantly greater for the moderate selected lines than for the control lines ($t = -3.96$, df = 12, $P = 0.002$) (Fig. 4). In generation 10, we found significant differences in mean emergence among the moderate selected, intense selected, and control lines ($F = 6.6$; df = 5, 8; $P = 0.01$), which was due primarily to the low emergence of the control lines on MON863 corn (Fig. 5). Overall, three major trends were observed in these bioassays. First, survivorship for all lines increased over time. This trend was evident regardless of whether the lines were under selection, and whether they were evaluated on MON863 or its isoline, suggesting that all lines experienced adaptation to the laboratory rearing environment. Second, survivorship was consistently better on MON863 isoline corn compared with MON863 corn. Third, moderate selected and intense selected lines displayed >4 times average adult survivorship on MON863 than control lines by the last generation of selection.

Response to selection steadily increased in both the moderate selected and intense selected lines between

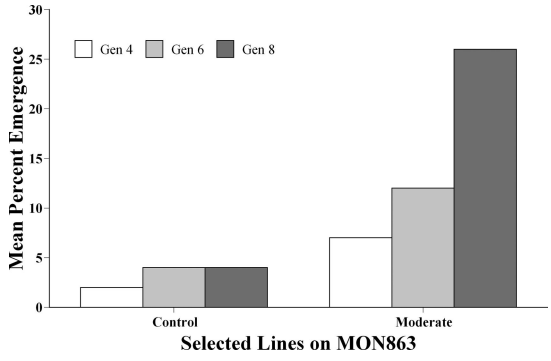

Fig. 4. Mean percentage of emergence for control and moderate selected lines reared on MON863 corn through first, second, and third instars for generations 4, 6, and 8.

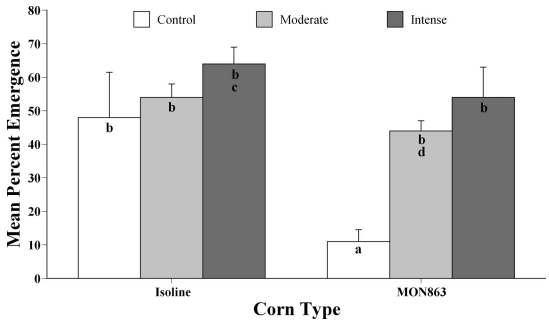

Fig. 5. Mean ± SEM percentage of emergence for control, moderate, and intense selected lines reared on MON863 and isoline corn through first, second, and third instars for generation 10. Bars with different letters are significantly different ($P < 0.05$; Fisher's protected least significant difference test).

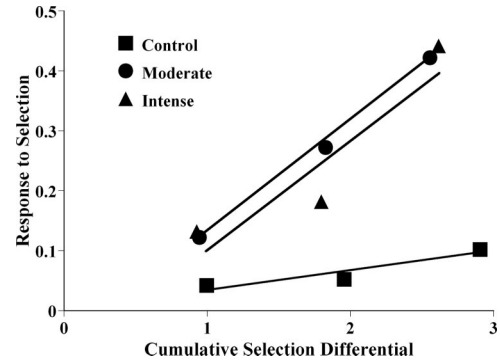

Fig. 6. Regression analysis for control, moderate, and intense selected lines based upon data from generations 6, 8, and 10.

generation 6 and generation 10 despite steady decreases in selection differentials (Table 1). Slopes of line-specific linear regression analyses based upon generations 6–10 (Fig. 6) were very similar across moderate selected (0.19) and intense selected (0.18) lines, whereas by comparison, the slope of the control line was much lower (0.03). Estimates of h^2 were likewise very similar among the moderate selected ($h^2 = 0.16$) and intense selected ($h^2 = 0.15$) lines.

Discussion

Both the moderate and intense selected lines displayed significantly increased survivorship to a fixed quantity of Bt and therefore qualify as Bt resistant colonies (Tabashnik 1994). Our data show that increases in average rates of emergence can develop quickly when western corn rootworm populations are continuously exposed to the Bt selection pressures imposed by MON863. Indeed, average rates of emergence increased six-fold over the final six generations in the moderate selected line and ≈3.4-fold over the final four generations in the intense selected line. By the conclusion of the study, both selected lines displayed approximately four-fold increases in emergence compared with controls. These results are consistent with previous observations of rapid pesticide resistance development within western corn rootworm populations exposed to more traditional chemical pesticides (Metcalf 1983, Meinke et al. 1998) and are also in accord with Meihls et al. (2008) who developed resistance to MON863 in greenhouse-reared western corn rootworm populations over multiple generations. Concordance of rapid resistance development among-studies indicates that development of Cry3Bb1 resistant western corn rootworm populations is routinely possible, whereas discordance (lack of selection response in some experiments) could suggest either intricate environmental (Raymond et al. 2005) or population (Raymond et al. 2007) level controls.

Table 1. Selected lines by generation used to estimate the realized heritability of resistance to the Cry3Bb1 protein

Selected line	Generation	Selection differential	Response to selection	Slope
Control	6	0.98	0.04	
Moderate		0.93	0.12	
Intense		0.91	0.13	
Control	8	0.96	0.05	
Moderate		0.88	0.27	
Intense		0.87	0.18	
Control	10	0.95	0.10	0.03
Moderate		0.73	0.42	0.19
Intense		0.82	0.44	0.18

Values include the selection differential (S), response to selection (R), and slope of regressing R on $S_{cumulative}$.

Because we were able to produce Bt-resistant lines via two selection regimes with different selection pressures, the acquisition of Bt resistance within western corn rootworm seems to be inducible not only over a small number of generations but also with different selection intensities. These findings highlight the utility of the IRM strategy currently in place to manage western corn rootworm populations in the United States. The IRM for Bt corn mandated by the U.S. EPA is based upon a high dose, structured refuge model (Glaser and Matten 2003). Under this model, infusion of Bt susceptible alleles from individuals migrating from non-Bt corn refuges is expected to prevent resistance from developing in Bt-cornfields. Non-Bt corn refuges are required to be planted in proximity to Bt corn to promote matings between Bt-susceptible individuals and Bt-resistant individuals emerging from Bt fields. Heterozygous offspring resulting from crosses between resistant and susceptible individuals would be expected to be susceptible to Cry toxins if resistance is recessive, thus preventing or slowing Bt resistance (Bates et al. 2005). Although extrapolating laboratory results to field environs must be done with caution (Tabashnik 1992), results from our study suggest that the presence of non-Bt refuges may indeed play a crucial role in delaying development of Bt resistance within western corn rootworm field populations.

Estimates of realized h^2 values based upon survivorship data recorded over the final five generations of selection were nearly identical among the moderate selected ($h^2 = 0.16$) and intense selected ($h^2 = 0.15$) lines. The low heritabilities indicate that genetic variation accounts for only a small proportion of the total variation for emergence rates on Bt corn (resistance) compared with environmental variation. Our heritability estimates are within the range of those reported for laboratory populations of diamondback moth, *Plutella xylostella* (L.); tobacco budworm, *Helicoverpa virescens* (F.); and Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Tabashnik 1992, 1994) and are also consistent with late-generation heritability estimates reported for European corn borer, *Ostrinia nubialis* (Hübner) (Huang et al. 1999). Furthermore, our heritability estimates are in accord with h^2 estimates for western corn rootworm laboratory populations reared for tolerance to event DAS-59122-7, a variety of Bt corn expressing Cry34Ab1 and Cry35Ab1 proteins (Lefko et al. 2008). Consistently low heritability estimates across independent studies and a wide variety of insect taxa indicates that Bt resistance seems to be a trait heavily influenced by environmentally based variation relative to genetically based variation, a conclusion that may be extremely useful for IRM managers who oversee and regulate policies designed to maximize the longevity of Bt corn (e.g., structured refuges; Tabashnik et al. 2003, Murphy et al. 2010).

Bt resistance research within western corn rootworm has lagged well behind analogous pursuits in other pest insects, most notably dipterans and lepidopterans (Gassmann et al. 2009). The considerable financial investments and high levels of rearing expertise required for creation of Bt resistant lines tend to prohibit their routine production. Nevertheless, development of resistant lines has been critical to understanding the biochemical and genetic mechanisms responsible for Bt resistance in western corn rootworm as well as within a variety of other pest species (Gassmann et al. 2009, Sayed et al. 2010). These lines are an important early step toward characterizing the biological nature of Bt resistance in diabroticite beetles and also provide data that can assist in the optimization of IRM plans for Bt corn in the United States.

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