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Effect of *Bacillus thuringiensis* Cry3Bb1 Protein on the Feeding Behavior and Longevity of Adult Western Corn Rootworms (Coleoptera: Chrysomelidae)

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ABSTRACT The first transgenic corn hybrids expressing the *Bacillus thuringiensis* (Bt) Cry3Bb1 protein to control corn rootworm (*Diabrotica* spp.) larvae were registered for commercial use in 2003. This study was conducted to investigate the effect of Cry3Bb1 protein in combination with a cucurbitacin bait on adult feeding and longevity of both organophosphate-resistant and -susceptible western corn rootworms, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae). In choice and no-choice tests, possible repellency to the Bt protein was quantified by comparing beetle consumption of cellulose disks treated with three concentrations of Bt in combination with a feeding stimulant (Invite EC) to disks treated with stimulant alone. A lethal-time assay also was conducted to examine survival of beetles exposed to Bt protein in their diet. Results from these assays indicate that adult rootworms are not significantly deterred by the presence of Cry3Bb1 on the treated discs and that ingestion of toxin does not adversely affect adult longevity.

KEY WORDS western corn rootworm, transgenics, Cry3Bb1, behavior, survival

The first transgenic corn, *Zea mays* L., hybrids targeted to control corn rootworms (*Diabrotica* spp.) were registered for commercial use in 2003 (EPA 2003). Event MON863 hybrids express the Cry3Bb1 protein from *Bacillus thuringiensis* (Bt). Corn expressing the Cry3Bb1 protein is intended to protect against larval injury from the western corn rootworm, *Diabrotica virgifera virgifera* LeConte; northern corn rootworm, *Diabrotica barberi* Smith & Lawrence; and Mexican corn rootworm, *Diabrotica virgifera zea* Kryan & Smith that all feed on and injure the roots. Although larval feeding on roots is the primary mechanism for exposure, concentrations of Cry3Bb1 protein (micrograms of protein per gram of fresh weight tissue) have been reported to range from 30 to 93 in the leaf, from 49 to 86 in grain, from 30 to 93 in pollen, and from 3.2 to 66 in roots (EPA 2003). Whole-plant expression of the protein suggests that adults will be exposed to the toxin through their feeding on the aboveground portions of the plant. Very little information is available, however, on how adult exposure to the protein might influence subsequent behavior and fitness. This information could be particularly important for event MON863, because the protein is expressed at a low-to-moderate dose, and significant

beetle emergence (17–62%) has been reported from these hybrids (EPA 2003). Although Cry3Bb1 has been suggested to have no effect on rootworm adults (Vaughn et al. 2005), there is little quantitative information available on potential effects on adults.

In this report, we describe results from a set of experiments designed to document the effect of purified Cry3Bb1 protein on adult rootworm feeding and longevity. The protein was combined with a known feeding stimulant and arrestant for diabroticite beetles derived from a cucurbitacin bait (Schroder et al. 1998) to ensure consumption of the toxin and provide a means to evaluate repellency of the toxin in the presence of the arrestant and feeding stimulant.

Materials and Methods

Insect Populations. Beetles from two western corn rootworm populations (Gresham and Whitlock) were obtained from the USDA Northern Grain Insect Research Laboratory in Brookings, SD. Both colonies were initiated from at least 500 field-collected beetles and reared in the laboratory for at least five or six generations with at least 200,000 eggs collected at each generation. Previous studies had characterized the Gresham population (York County, Nebraska) as resistant both to methyl-parathion and carbaryl (Meinke et al. 1998), whereas the Whitlock population (South Dakota) was highly susceptible to both (L.J.M., unpublished). Beetles were maintained in

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Plexiglas cages at 22–25°C on a diet of fresh sweet corn ears and lettuce, *Lactuca sativa* L. until the assays were conducted.

Test Substance. Purified Cry3Bb1 was obtained as a stock solution (4 mg/ml) of protein in Na₂CO₃ buffer, pH 10.3, from Monsanto Co. (St. Louis, MO) and stored at –80°C until use. Protein dilutions for the assays were prepared in Invite EC (Florida Food Products Agrochemicals, Eustis, FL) that had previously been diluted 10-fold in double distilled H₂O and chilled on ice. Invite is a semiochemical (cucurbitacin)-based bait formulated as an emulsifiable concentrate containing Hawkesbury watermelon juice, *Citrullus vulgaris* Schrad. Because cucurbitacins are known to stimulate compulsive feeding and arrestant responses in diabroticite beetles (Metcalf and Lampman 1989), Cry3Bb1 was diluted with Invite to ensure consumption of the Bt protein by the beetles.

Feeding Behavior Bioassays. No-choice and choice feeding assays were conducted to determine whether Cry3Bb1 inhibited beetle feeding and attraction to the Invite formulation. Beetles were 10–16 and 12–18 d old when the no-choice and choice experiments, respectively, were conducted. Four treatments were evaluated, consisting of Invite alone, Invite + 10 µg Bt/g disk, Invite + 100 µg Bt/g disk, and Invite + 1000 µg Bt/g disk. The treatments of 10 and 100 µg Bt/g disk were chosen to represent the range of Cry3Bb1 reported to be expressed in the aboveground corn tissues (EPA 2003). The 1,000-µg treatment, ≈10-fold higher than levels of Cry3Bb1 reported in aboveground corn tissues, was included to determine whether any behavioral response could be detected. Each treatment was replicated 10 times. For each treatment, 20 µl of toxin/Invite solution was applied to the surface of a 13-mm-diameter cellulose membrane disk (pore size 0.45 µm, Pall Life Sciences, East Hills, NY) as described by Parimi et al. (2003). Air-dried disks were positioned on minuten pins above water-moistened filter paper in 9-cm-diameter petri dishes with a paraffin wax bottom. For no-choice tests, a single disk was placed in the center of each petri dish with two beetles of the same sex and population. For the choice test, two disks (one with Invite and one with Invite + Bt) were arranged on opposite ends of each petri dish that each contained a single beetle. Petri dishes were placed in a growth chamber with constant lighting at a temperature of 22°C. After 8 and 48 h of feeding, disks from the no choice and choice tests, respectively, were removed, and their surface areas were measured with a LI-COR 3000 leaf area meter (LI-COR, Lincoln NE).

Lethal-Time Bioassay. Rootworm adults, 15–21 d old, were confined in individual wells of bioassay trays (CD International, Pitman, NJ) containing 0.25 ml of 1% agar, 1% agar containing Invite, or Invite + Bt protein at two concentrations (10 and 100 µg Bt/g diet). All treatments contained 0.32% methyl paraben and 0.12% sorbic acid dissolved in water to minimize microbial contamination. For the treatments requiring Invite, 10% of the water used to prepare the agar was replaced with Invite. Preparation required heating the

agar to a boil, cooling to 60°C, and thoroughly mixing the Invite and purified Cry3Bb1 protein into solution. Immediately after mixing, 0.25 ml of diet was dispensed into each well and allowed to solidify. Individual beetles were confined in each well immediately after the agar solidified and covered with an adhesive lid (CD International). In total, 16 beetles were assayed per treatment for each population by gender combination. Beetle mortality, determined as the inability to move when prodded, was recorded daily until all beetles were dead. Trays were maintained in a growth chamber at 22°C and photoperiod of 16:8 (L:D) h.

Individual beetles exposed to the Invite/Cry3Bb1 mixture within the wells were tested for the presence of Cry3Bb1 in whole body homogenates by using methods adapted from Spencer et al. (2003). Beetles that had died during a 24-h period were collected and stored at –20°C. Individual beetles were homogenized in 1.5-ml microfuge tubes in 200 µl of distilled water and a gene check strip (EnviroLogix, Portland, ME) was inserted into the sample. The strips were read at 30 min and confirmed at 24 h to determine whether the Cry3Bb toxin was detected in the sample. Tests conducted on beetles exposed to either agar or to the Invite/agar treatments were never positive for the Cry3Bb toxin.

Statistical Analyses. For the choice data, a consumption index (CI) was calculated for each treatment, where CI equals the surface area of the Invite control disk (*I*) minus the surface area of the Invite + Bt (*Bt*)-treated disk divided by *I* and represents the percentage of Bt-treated disk area consumed relative to the Invite control [CI = (*I* – *Bt*)/*I* × 100]. Consumption indices for treatments in the no-choice test were also calculated; however, the average area of the unfed control disk was substituted for *I* in the above-mentioned equation. For both the choice and no-choice tests, analysis of variance (ANOVA) was used to test for treatment effects on CI; multiple pairwise comparisons of treatment means were performed using the Tukey–Kramer honestly significant difference test in SAS (SAS Institute 2001).

For the lethal time assay, mortality rates of beetles exposed to each diet treatment were described with a logistic model by using the probit procedure in SAS (SAS Institute 2001). Goodness-of-fit of the regression lines were determined from the χ^2 test statistics (lack of fit indicated by significant χ^2 value). Within a population and gender, the significance of differences among LT₅₀ and LT₉₀ values was indicated by non-overlapping 95% fiducial limits (FL) (Savin et al. 1977).

Results and Discussion

Choice Test. Results of the choice disc consumption rates are presented in Fig. 1. No significant differences between populations ($n = 120$, $P = 0.06$), sexes ($n = 120$, $P = 0.17$) or Invite versus Invite + Bt ($n = 120$, $P = 0.46$) were apparent (Mann–Whitney *U* test; data not shown), so data were pooled for subsequent anal-

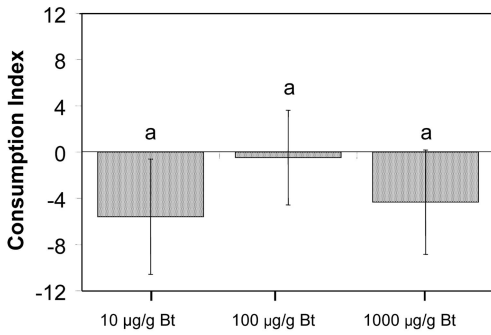


Fig. 1. Mean (\pm SE) consumption indices of disks treated with three concentrations of purified Cry3Bb1 protein combined with Invite by western corn rootworm adults in a choice assay. Consumption index indicates percentage of Bt-treated disc area consumed relative to discs treated with Invite alone ($n = 40$).

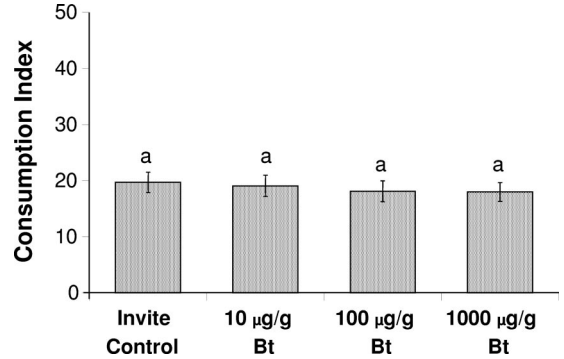


Fig. 2. Mean (\pm SE) consumption indices of disks treated with Invite alone (Control) and three concentrations of purified Cry3Bb1 protein combined with Invite by western corn rootworm adults in a no-choice assay. Consumption index represents percentage of treated disk area consumed relative to the mean area of unfed, control disks ($n = 40$).

ysis. Although the mean consumption index for the Bt treatments indicated a slight preference (values slightly less than zero) for disks treated with Invite versus disks treated with Invite + Bt, there were no significant differences ($F = 0.34$; $df = 2, 115$; $P = 0.71$) among the three Bt concentrations tested (Fig. 1).

No-Choice Test. Results from ANOVA indicated no significant differences in disk areas consumed between populations ($F = 2.22$; $df = 1, 145$; $P = 0.14$) or sexes ($F = 1.34$; $df = 1, 145$; $P = 0.25$), and data were pooled for subsequent analyses. There were no significant differences ($F = 0.21$; $df = 3, 145$; $P = 0.89$) in the mean consumption index among the four treatments, indicating that in a no-choice situation, beetles fed equally on disks treated with Invite + purified Cry3Bb1 protein compared with disks treated with Invite alone (Fig. 2).

Lethal-Time Assay. In general, the exposure to the Cry3Bb1 toxin did not seem to negatively impact sur-

vival time (Table 1). Results of assays to detect the presence of the Cry3Bb1 toxin in individual beetles by using gene check strips indicated that although toxin was not detected in every individual, a high percentage (>50%) tested positive for the presence of toxin even at the lowest concentration (data not shown), thereby confirming exposure to the toxin. The LT_{50} values for both populations were generally shortest for those individuals fed agar alone, although the effect was more pronounced in males. The presence of Invite seemed to enhance the nutritional value of the agar as LT values were generally longer for both populations (Table 1). The presence of Cry3Bb1 toxin at both concentrations further enhanced survival time for males of both populations, suggesting that the toxin had been used as a source of nutrition and was clearly not having a significant detrimental effect. However, results for females were not consistent across treat-

Table 1. Susceptibility of male and female adult western corn rootworm to purified Cry3Bb1 Bt protein incorporated at two concentrations (10 and 100 µg Bt/g diet) into agar-based diet ($n = 16$)

Pop ^a	Diet treatment	Slope (\pm SE)	LT_{50} (95% FL) ^b	LT_{90} (95% FL) ^b	χ^2 ^c	df	$P > \chi^2$
Gresham male	Agar	1.3 (0.2)	4.9 (4.54–5.44)	6.6 (6.09–7.58)	2.15	26	1.00
	Invite EC	0.3 (0.02)	9.9 (8.89–10.90)	18.2 (16.71–20.06)	6.22	28	1.00
	10 Bt	0.2 (0.02)	16.5 (15.28–17.73)	28.1 (25.92–30.95)	13.94	28	0.98
	100 Bt	0.3 (0.03)	20.1 (19.11–21.17)	28.3 (26.58–30.78)	5.06	20	0.99
Gresham female	Agar	0.5 (0.05)	14.4 (13.65–15.05)	18.6 (17.64–19.89)	5.05	26	1.00
	Invite EC	0.2 (0.02)	22.4 (21.14–23.69)	34.2 (31.89–37.25)	17.21	30	0.97
	10 Bt	0.2 (0.02)	20.3 (19.22–21.50)	30.8 (28.89–33.26)	16.90	31	0.98
	100 Bt	0.2 (0.01)	15.9 (14.66–17.17)	28.5 (26.39–31.20)	7.06	32	1.00
Whitlock male	Agar	0.9 (0.13)	7.1 (6.57–7.64)	9.5 (8.79–10.54)	2.94	30	1.00
	Invite EC	0.2 (0.02)	10.8 (9.77–11.86)	19.7 (18.26–21.68)	14.41	33	0.99
	10 Bt	0.3 (0.03)	15.3 (14.34–16.25)	22.9 (21.53–24.69)	10.36	28	0.99
	100 Bt	0.2 (0.02)	16.1 (14.98–17.11)	24.9 (23.35–26.97)	16.30	26	0.93
Whitlock female	Agar	0.6 (0.06)	12.1 (11.39–12.75)	15.9 (15.05–17.25)	6.24	25	0.99
	Invite EC	0.3 (0.02)	11.3 (10.22–12.29)	19.6 (18.11–21.48)	7.34	28	1.00
	10 Bt	0.4 (0.04)	16.8 (16.02–17.55)	21.8 (20.78–23.27)	8.86	24	0.99
	100 Bt	0.2 (0.02)	11.5 (10.38–12.58)	21.3 (19.72–23.46)	14.55	31	0.99

^a Individual beetles were confined in wells of bioassay trays containing 250 µl of an agar-based diet treated with agar alone, Invite EC, or Invite EC+ purified Cry3Bb1 protein at two concentrations (10 and 100 µg Bt/g diet).

^b Lethal time (days) of adult western corn rootworms when confined on one of four agar-based diet treatments with 95% FLs at the 50% (LT_{50}) and 90% (LT_{90}) levels of probit mortality.

^c Chi-square goodness-of-fit statistics as determined by fitting the logistic model with the probit procedure in SAS (SAS Institute 1999).

ments and populations. There were no apparent reductions in survival time of females exposed to Invite + Cry3Bb1 relative to survival on agar alone for either population. However, for Gresham females exposed to the higher concentration of toxin, the LT_{50} was similar to agar alone and significantly lower than the agar + Invite treatment. In a no-choice situation, consumption of high concentrations (100 $\mu\text{g/g}$ diet) of Cry3Bb1 seemed to reduce adult female longevity in the Gresham population. Differences between populations may indicate that such effects are dependent on the reproductive and physiological condition at the time of the assay. However, in general there does not seem to be a strong negative impact of the protein on adult longevity in any of the comparisons.

Within the context of beetle age structure included in each assay, the consumption of purified Cry3Bb1 Bt protein at concentrations comparable with those reported for aboveground tissues of transgenic corn plants in combination with the feeding stimulant Invite EC did not significantly influence feeding behavior or relative longevity of western corn rootworm adults. It seems likely that physiological differences between adult rootworms and larvae, such as differences in gut pH and the presence of activating/degrading proteases, are likely to contribute to the apparent lack of susceptibility in adult rootworms.

These data suggest that western corn rootworm adults will exhibit similar feeding behaviors when encountering transgenic (expressing Cry3Bb Bt protein) or nontransgenic corn plants in the field and that it is unlikely that Cry3Bb Bt protein in transgenic plants will have significant effects on adults that might impact resistance management recommendations. For example, if the Cry3Bb1 protein were repellent to adult corn rootworms, there may be a decrease in the number of beetles from refuge fields entering the transgenic field. This would decrease the effectiveness of the refuge acres. These results support the general lack of fitness effects observed by Al-Deeb and Wilde (2005) for adult rootworms that were maintained on Cry3Bb1-expressing corn plants as larvae or adults. However, similar experiments that compare response to Cry3Bb1-expressing plant tissue with nonexpressing tissue should be conducted over the entire life cycle to confirm the lack of response to adult corn rootworms.

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