Evaluation of Tolerance to Bacillus thuringiensis Toxins Among Laboratory-Reared Western Bean Cutworm (Lepidoptera: Noctuidae)

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ABSTRACT  The western bean cutworm, Striacosta albicosta (Smith) (Lepidoptera: Noctuidae), is a destructive insect pest of dry beans and corn within its native range of western Nebraska and eastern Colorado. However, since the initiation of an eastward range expansion of S. albicosta in the late 1990s, economic damage has been observed in the Midwest, and the species has now reached the Atlantic Coast and Quebec. Economic damage to corn occurs by larval feeding on ears, which is not controlled by commercial transgenic hybrids that express Bacillus thuringiensis (Bt) Cry1Ab, but partial control is observed by corn varieties that express Cry1 F toxins. Inadequate protocols for laboratory rearing of S. albicosta have hindered controlled efficacy experimentation in the laboratory and field. We report an S. albicosta rearing methodology used to maintain a laboratory colony for 12 continuous generations. Rearing procedures were adapted for Bt toxin diet-overlay assays, revealing that S. albicosta larvae exposed to Bt toxin for 14 d were insensitive to Cry1Ab concentrations up to 25,000 ng/cm². In contrast, neonates exposed to Cry1 F toxin at ≥250 ng/cm², showed reduced developmental rates, with estimated effective concentrations of EC₅₀ = 1.187.5 ng/cm² and EC₉₅ = 10,000.5 ng/cm². The ability to mass produce this pest insect will enhance fundamental research, including evaluation of control tactics and toxin susceptibility.

KEY WORDS  Bacillus thuringiensis, western bean cutworm, transgenic corn, toxin, dose–response assay

Larvae of the western bean cutworm, Striacosta albicosta (Smith) (Lepidoptera: Noctuidae), cause feeding damage to crops of dry beans (Phaseolus vulgaris L.) and corn (Zea mays L.) grown in parts of the United States. This moth is native to the Great Plains and Intermountain West of the United States (Keaster 1999, Michel et al. 2010), and economic damage was historically most common in Colorado, Idaho, and Nebraska (Hoerner 1948; Hagen 1963, 1976). Western bean cutworm recently has undergone an eastward range expansion with economic damage to corn documented in Minnesota and northwestern Iowa during 1999 and 2000, respectively (O’Rourke and Hutchinson 2000, Rice 2000). Although previously present in South Dakota, economic damage to corn was reported for the first time in eastern South Dakota in 2000 (Catangui and Berg 2006). S. albicosta reached Missouri and Illinois by 2004 (Dorhout and Rice 2004, Rice et al. 2004), Wisconsin in 2005 (Cullen and Jyoutika 2008), and Indiana, Michigan, and Ohio by 2006 (Pope 2007, Rice and Pilcher 2007, DiFonzo and Hammond 2008). Currently, S. albicosta is present in regions as far east as Pennsylvania, New York, Quebec, Delaware, and New Jersey (Baute 2009, Tooker and Fleischer 2010, Ingerson–Mahar 2012, Whalen 2012). S. albicosta overwinters as a prepupa in the soil with best survival in sandy soils (Michel et al. 2010, Hutchinson et al. 2011), and individuals pupate in the spring (Hoerner 1948, Douglass et al. 1957, Hagen 1962, Michel et al. 2010). Adults usually emerge beginning in late June or early July in Iowa (Rice 2006), and subsequently oviposit on suitable host plants (Hagen 1962, Blickenstaff and Jolley 1982). Feeding damage caused by early instar S. albicosta primarily occurs to P. vulgaris leaves and flower buds, but later instars burrow into young bean pods. Analogously, early instars feed on reproductive and leaf tissue of corn before moving into ears to complete development (Hagen 1962, Michel et al. 2010). In contrast to larvae of some other noctuids such as the corn earworm, Helicoverpa zea (Boddie), late instar S. albicosta are not cannibalistic, and multiple larvae are often observed in the same corn ear. The resulting damage to corn ear tips and husks impacts the quality of grain and increases crop susceptibilities to mold and fungal infections including aflatoxin (Hagen 1962).

Applications of foliar pyrethroids provide limited control of feeding damage after larvae have entered ear husks (Michel et al. 2010). Since the mid-1990s,
transgenic corn hybrids that express toxin proteins derived from the Gram-positive soil bacterium Bacillus thuringiensis (Bt) (Berliner) have been commercialized for the control of lepidopteran pests. The Cry1Ab toxin from Bt subspecies kurstaki, Cry1 F from Bt subspecies atsacai, and Vip3A from Bt strain AB424 significantly suppress larval growth in some lepidopteran species (Estruch et al. 1996, Burkness et al. 2010), but many lepidopteran species are naturally tolerant or lack susceptibility to certain toxins. Larval feeding injury by S. albicosta occurs on hybrids that express the Cry1Ab toxin under field conditions, and larvae show no significant decreases in developmental rates compared with those infesting non-Bt hybrids (Catangui and Berg 2006, Eichenseer et al. 2008). Although Cry1 F-expressing Bt corn hybrids provide control of S. albicosta, the species shows some natural tolerance (Eichenseer et al. 2008). For example, larvae were observed causing damage to 24% of ears from hybrids that expressed the Cry1 F toxin in an unpublished study from Iowa, although the extent of kernel damage within the ears was low (Rice 2007).

Artificial infestation of crops using laboratory-reared larvae or eggs has been a staple of entomological field trial research, and provides consistent and controlled insect pressures that are comparable across treatments and replicates (Dent 1991, Smith et al. 1993). Highly variable and unpredictable natural field infestations cause difficulties during the evaluation of existing and experimental S. albicosta control tactics, and the current lack of a laboratory-rearing procedure has hindered the investigation of insecticide field efficacy (Eichenseer et al. 2008). In addition, laboratory experimentation to estimate S. albicosta larval tolerance to Bt toxins, characterization of mortality rates when exposed to natural enemies, and any number of behavior and ecology experiments are not possible without effective artificial-rearing protocols.

Herein, an experimental approach was taken to develop and optimize S. albicosta laboratory-rearing procedures, including pathogen suppression, and environmental cues required for optimal oviposition. We adapted these laboratory-rearing protocols for experiments to estimate the effective concentration (EC) of the Bt toxins Cry1Ab and Cry1 F against larvae using diet overlays. These data, heretofore impossible to obtain because of the lack of adequate rearing methodology, will be important for the development and evaluation of field tactics to protect corn from S. albicosta feeding damage.

**Materials and Methods**

**Rearing.** Western bean cutworm egg masses were initially collected from commercial cornfields, by cutting out a portion of the leaf around the mass, near Grant, NE (courtesy of Appel Crop Consulting Inc., Grant, NE) on 23 July 2009, and shipped overnight to the U.S. Department of Agriculture–Agricultural Research Service (USDA–ARS), Corn Insects & Crop Genetics Research Unit (CICGRU) laboratory in Ames, IA. Emerged larvae were used to initiate a culture of S. albicosta at the CICGRU laboratory. Additional S. albicosta individuals were collected from cornfields near Grant, NE, in July of 2010 and 2011, and were combined with laboratory colony individuals in attempts to maintain genetic diversity.

Procedures used to rear S. albicosta in the laboratory were modified from those originally developed for Agrotis ipsilon (Hufnagel) (Reese et al. 1972), and are presented in brief here. More detailed explanations of the steps in this protocol are available in the online rearing guide (Supp. File 1 [online only]). Laboratory colony-produced egg masses were removed from P. vulgaris plants by cutting ~2 cm of leaf tissue surrounding each, and were placed egg mass side up on a shallow tray lined with moist paper towels. Egg masses were incubated at a constant temperature of 26.6°C, 70–80% relative humidity (RH), and a photoperiod of 16:8 (L:D) h cycle using GE Ecolux Plant and Aquarium wide spectrum fluorescent and Sylvania F40 Sunstick bulbs. In all, 47 g of agar (Moorhead Co, Van Nuys, CA) was diluted in 1.9 liters of tap water and dissolved by heating to 90°C in a microwave for ~10 min, then homogenized in a blender until the temperature of the solution decreased to ≤59°C. Black Cutworm Dry Mix (212.0 g), General Lepidopteran Dry Mix (144.0 g) (BioServ, Frenchtown, NJ), and Fumidil B (2.5 g) (Dadant and Sons, Hamilton, IA) were added to and blended with the agar solution. This diet was then immediately poured into 26-cm-diameter by 9-cm-high plastic rearing dishes, allowed to solidify ≥4 h, and then cut into ~0.6- by 2.5-cm strips. Each strip was placed into a 4.7-cm-diameter Fisher-brand Petri dish (Cat. no. 09-720-502). Once neonates started to hatch, an egg-free edge of an individual leaf cutting containing an S. albicosta egg mass(s) was placed under a diet strip, and incubated at 26.6°C, 70–80% RH, and a photoperiod of 16:8 (L:D) h until larvae reached second instar.

For older larvae, the S. albicosta diet was prepared as described previously, but cut into ~1.9- by 12.7-cm strips. In total, four or five strips were placed into 26- by 9-cm plastic dishes, infested with ~100 2nd-instar S. albicosta per dish transferred by small camel hair brush. The lid on each dish had a ~10-cm-diameter hole covered with 0.55-mm brass mesh to allow air exchange. Larvae were reared at 26.6°C, 70–80% RH, and a photoperiod of 16:8 (L:D) h through fifth-instar. Final (sixth) instars were transferred with feather-tipped forceps to individual 1.0-oz (29.6-ml) opaque jelly cups (Fabri-Kal, Kalamazoo, MI) with ~7.5 ml of the S. albicosta diet (prepared as described earlier), and incubated at 26.6°C, 70–80% RH, and a photoperiod of 16:8 (L:D) h.

After ~20 d, S. albicosta prepupae were transferred individually to 1-oz (29.6-ml) opaque jelly cups using feather-tipped forceps. The cups contained 10 ml of a 2:1 mix of Premium Sand (Quikrete, Kansas City, KS) to dry play sand (Quickrete) moistened with ~50 ml of tap water. These prepupal S. albicosta were provided with a photoperiod of 16:8 (L:D) h cycle and incubated at 26.9-20.0°C (L:D) with 70–80% RH.
Prepupation occurs in a burrow in the sand, and there the prepupa enters diapause that lasts ~3 mo.

Adult emergence was monitored daily beginning 1 wk before its expected initiation. At this time, pinto bean, *P. vulgaris*, seeds were planted in 17.8-cm pots containing germination mix (The Scotts Company LLC, Marysville, OH), and incubated at 26.9°C and constant light. *P. vulgaris* seeds were planted twice weekly throughout the adult emergence period to ensure availability of optimal plant maturities throughout the oviposition period. One-week-old plants were placed into 55.9- by 61.0-cm cages constructed from 0.11-mm copper wire mesh, into which ~100 moths were transferred without direct physical handling. An adult diet consisting of a 5% sucrose and 0.2% ascorbic acid solution was provided in the cages throughout the mating and oviposition periods. Egg masses were collected from *P. vulgaris* leaves as described earlier.

Larvae were reared as described previously from neonate through sixth-instar. Immediately after each molt, head capsule width and body length (mandible to anus) were measured from 14 individuals using an ocular micrometer calibrated using a Monostat Mechanical Caliper (Accurate Sales, Brooklyn, NY). Mean and variance statistics were calculated and graphed using Microsoft Excel (Microsoft Corp., Redmond, WA), as were the correlation between body length and head capsule width.

**Cry Toxin Bioassay.** Diet for use in Cry toxin bioassays of *S. albicosta* was prepared as described earlier, except that it was blended for 5 min to homogenize ingredients. One milliliter of diet was dispensed into cells of a 128-cell assay tray (BioServ, Frenchtown, NJ; item no. BAW128). Any diet with air bubbles was removed and diet realiquoted into the same cell. Diet was allowed to cool for 4 h at room temperature. Trypsinized native Cry1Ab or native Cry1Fa toxin (nontransgenic versions of the proteins were purchased from Dr. M.P. Carey at Case-Western University) was diluted to concentrations of 0.03–833.33 ng/µl in 0.1× Triton-X (Dow Chemical Co, Midland, MI). In all, 30 µl was dispensed in the center of cells containing 1.0 ml artificial diet, and distributed over the diet surface and allowed to dry 1 h as described by Marçon et al. (2000). Final overlay concentrations of 1, 5, 10, 50, 75, 100, 250, 500, 750, 1,000, 2,500, 5,000, 10,000, 12,500, 15,000, and 25,000 ng/cm² in 0.1× Triton X (Sigma) were placed at 26.6°C, 70–80% RH, and a photoperiod of 16:8 (L:D) h. Using a camel hair brush, neonates <6 h old that had not previously fed on artificial diet or leaf material were transferred to the Cry1Ab and Cry1 F toxin overlayed cells. Sixteen neonates were included per replicate, and three replicates were performed for each toxin concentration. After a 14-d incubation, the instar was determined by head capsule width. A control treatment of 0.1× Triton-X was replicated three times. The median concentration of Cry1 F toxin required to delay *S. albicosta* development to 50% of that observed among control larvae at day 14 was estimated using the PROC

| Instar | Larval body length | Larval head capsule width |
|--------|--------------------|--------------------------|
|        | Range (mm)         | Mean (mm)                |
| 1      | 2.0–2.9            | 2.5 ± 0.2                |
| 2      | 3.3–4.5            | 3.8 ± 0.5                |
| 3      | 6.5–8.2            | 7.7 ± 0.5                |
| 4      | 15.0–17.0          | 15.3 ± 3.5               |
| 5      | 17.0–22.0          | 19.3 ± 1.8               |
| 6      | 28.0–34.0          | 29.1 ± 3.4               |

Range and mean (±SE) body length and head capsule width of instars immediately after molt.

**PROBIT** procedure of SAS 9.2 (SAS Institute, Cary, NC).

**Results and Discussion**

The reason or reasons behind the initiation of the eastward range expansion of *S. albicosta* out of the Great Plains in the late 1990s remain unknown, but likely involve a complex interaction of biotic and competitive factors within the agroecosystem (Hutchison et al. 2011). In recently colonized regions east of the Missouri River, *S. albicosta* generally has been a sporadic yet occasionally severe pest on corn and dry bean crops, and research interest and efforts have increased dramatically. It has proven a difficult insect to rear in the laboratory (e.g., Antonelli 1974, Doyle 1994, Dorhout 2007), and no methods for successful continuous rearing have been reported. Lack of an adequate rearing methodology has limited the ability to conduct controlled field and laboratory experiments to guide development and evaluation of control tactics (Eichenseer et al. 2008).

The accurate estimation of larval instar is important for assessing larval growth rates under treatment regimes, and would be useful for evaluation of methods designed to control crop feeding damage. Antonelli (1974) developed a larval instar estimation protocol by measuring head capsule width and body lengths of *S. albicosta* across the six instars when reared on an artificial diet. The estimates by Antonelli (1974) are summarized in Table 2 of Michel et al. (2010), and are different than those obtained in the current study. We measured head capsule width and body length in larvae raised on our artificial diet immediately after each molt to a new instar. Larvae grown in laboratory culture advanced from egg hatch (neonate) to sixth-instar over a 28-d period, which is similar to that described from egg hatch (neonate) to sixth-instar across the six instars when reared on an artificial diet immediately after each molt to a new instar. Larvae raised in laboratory culture advanced from egg hatch (neonate) to sixth-instar over a 28-d period, which is similar to that reported by DiFonzo (2009) for the earliest third of a field population in Michigan reaching sixth instar. Development rates are dependent on degree-days (or heat units) and may be influenced by diet, but little information is available (Michel et al. 2010). Heat units needed for development on artificial diet are reported in the rearing manual (Supp. File 1 [online only]). Head capsule width and body length of larvae reared using the methods herein ranged from 0.33 ± 0.03 to 3.49 ± 0.38 mm and 2.5 ± 0.2 to 29.1 ± 3.4 mm, respectively, across instars (Table 1). A strong linear correlation was observed between body length and
head capsule width across larval instars ($R^2 = 0.9749$; Fig. 1). Compared with Antonelli (1974), measures of body lengths and head capsule widths within our study were less at third through sixth-instars (Table 1). The measures are comparable, but differences may be a result of differences in rearing method or time point at which measurements were taken. Specifically, our measures were taken immediately after each molt when head capsules were unscleritized. In contrast, Antonelli (1974) provided no indication of precise timing of body or head capsule measurements, such that the increased measures might have resulted from growth that occurred within each instar.

The 14-d exposure of neonate *S. albicosta* larvae to 25,000 ng Cry1Aa, Cry1Ab, or Cry1Ac toxin/cm$^2$ in our diet overlay bioassays resulted in 4.0% mortality, which was not significantly different than the 2.4% mortality observed on nontoxin control diet (equality of variances F-statistic = 1.0; $P$ value = 0.50; remaining data not shown). Our results indicate that there was no differential response or developmental delay when *S. albicosta* larvae were exposed to $\approx 25,000$ ng/cm$^2$ of Cry1Aa, Cry1Ab, or Cry1Ac toxin, which agree with field observations that Cry1Ab-expressing transgenic events show no significant reductions in *S. albicosta* feeding damage (Helms and Wedberg 1976, O’Rourke and Hutchison 2000, Catangu and Berg 2006, Eichenseer et al. 2008). Larvae of the noctuid corn pest, *A. ipsilon*, also show insensitivity toward Cry1A toxins in laboratory bioassays (de Maagd et al. 2003). Furthermore, $\approx 15$–25% of *Helicoverpa* sp. larvae survive on transgenic plants expressing Cry1A toxins in the United States (Gould et al. 1994, Burkness et al. 2001, Storer et al. 2001, Horner and Dively 2003, Daly and Buntin 2005) and China (Li et al. 2007). These data suggest that noctuids, including *S. albicosta*, may have high levels of native insensitivity for certain Cry1A toxins, and highlights the need for laboratory methods to evaluate these traits.

Corn hybrids derived from the transgenic Event TC1507 expressing the *Bt* toxin Cry1F were commercialized in the early 2000s, and are labeled for the control of noctuid pests, including *S. albicosta*. In contrast to our analysis of native Cry1Ab diet overlay results, the exposure of *S. albicosta* neonates to increasing levels of native Cry1F toxin resulted in a phenotypic response. Measurable effects on *S. albicosta* growth rates were not observed at levels <25 ng Cry1F/cm$^2$, but the mortality was 46.9% at 2,500 ng Cry1F/cm$^2$ (data not shown). Because 100% mortality was never achieved, lethal concentration (LC) estimates could not be calculated. However, estimates of EC$_{50}$ and EC$_{95}$ for growth inhibition when *S. albicosta* were exposed to Cry1F for 14 d were $\approx 731.1$ ng/cm$^2$ and $\approx 10,000.5$ ng/cm$^2$, respectively (Fig. 2). Larval development was determined by the mean head capsule width as described previously (Table 1; Fig. 1), and showed a strong negative correlation with Log$_{10}$(ng Cry1F/cm$^2$) across increasing Cry1F toxin levels ($R^2 = 0.8734$ across effective range of 250–25,000 ng/cm$^2$; Fig. 2). The estimated EC values for *S. albicosta* were $\approx 100$-fold higher than levels required to evoke similar mortalities among Cry1F-resistant *Ostrinia nubilalis* (Hubner) larvae (Pereira et al. 2008). It should be noted that the toxins used in this study may not be identical in amino acid sequence or insecticidal activity compared with the same toxin expressed by transgenic corn. Regardless, bioassay results that showed no insecticidal activity of Cry1A and partial growth inhibition by Cry1F are analogous to observations on transgenic corn, and suggest that dose–response assays with activated native Bt toxins may be effective in estimating *S. albicosta* susceptibilities.

A high level of variance in *S. albicosta* head capsule width, and thus instar, was observed at each level of Cry1F toxin (Fig. 2). Specifically, the variance about
the mean head capsule width at each Cry1F toxin exposure level ranged from 0.29 to 0.47 mm for neonates (n = 11), second (n = 1), third (n = 3), and fourth instars (n = 2) that survived the 25,000 ng Cry1F/cm² dose (remaining data not shown). An analogous level of population variation was observed in Bt toxin tolerance among A. ipsilon larvae, which required a ~42.5-fold range in toxin concentration to elicit larval mortality (de Maagd et al. 2003). High phenotypic variation among larvae in Cry1F toxin dose-response assays could be a consequence of use of a heterogeneous population of S. albicosta larvae, which is expected to display a greater range of susceptibility than a selected strain. Nonetheless, our results suggest that wild populations of S. albicosta harbor genetic variation in larval capacity to feed and develop when exposed to Cry1F toxin. Although the toxin is only on the surface of the diet, neonates cannot escape ingestion by burrowing, because they must eat their way through it. Diet overlay bioassays are routinely used for quantifying susceptibility to Bt toxins (e.g., Tabashnik et al. 2011), and have been used to select for resistant strains in lepidopteran species, indicating that this method is effective for evaluating efficacy of Bt toxins. Furthermore, these laboratory results are consistent with field reports of some S. albicosta feeding damage to corn ears of Cry1F-expressing hybrids (Rice 2007, Eichenseer et al. 2008).

Our data are the first reported for S. albicosta susceptbility (developmental delay) to Cry toxins using laboratory dose-response bioassays, and were made possible by the development of laboratory-rearing procedures. Dose-response assay results suggest that larval S. albicosta tolerance to Cry1F toxin is relatively high, even among neonates, compared with other lepidopteran species (Pereira et al. 2008). It is possible that tolerance may be greater in later instars as observed for other Lepidoptera (Halcomb et al. 1996, Henneberry et al. 2001), and is deserving of future testing. Dose-response assay results are difficult to directly correlate with field instances of feeding damage, but are consistent with field reports of some S. albicosta damage to corn ears of Cry1F-expressing hybrids (Rice 2007, Eichenseer et al. 2008). Regardless of any differences between on-plant and diet overlay assays, the latter are more useful for studying larval tolerance, because one can limit the confounding variance in mortality levels caused by environmental conditions or predators. With the ability to rear S. albicosta in the laboratory, it may be possible to select strains with varying levels of Cry1F toxin susceptibilities, which could in turn be used to investigate the genetic basis of resistance.

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