

Growth, Development, and Survival of *Nosema pyrausta*-Infected European Corn Borers (Lepidoptera: Crambidae) Reared on Meridic Diet and Cry1Ab

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J. Econ. Entomol. 97(4): 1198-1201 (2004)

ABSTRACT Transgenic corn, *Zea mays* L., hybrids expressing crystal protein endotoxin genes from *Bacillus thuringiensis* Berliner are an increasingly popular tactic for managing the European corn borer, *Ostrinia nubilalis* (Hübner), in North America. *O. nubilalis* populations also are often vulnerable to the ubiquitous entomopathogenic microsporidium *Nosema pyrausta* (Paillot). We examined the effect of feeding meridic diet incorporated with purified Cry1Ab on growth, development, and survival of *Nosema*-infected and uninfected neonate *O. nubilalis*. Infected larvae developed more slowly than uninfected larvae. Increasing the concentration of Cry1Ab in diet reduced larval development, and this effect was amplified by microsporidiosis. Infected larvae weighed significantly less than uninfected larvae. The relationship among *Nosema* infection, Cry1Ab concentration, and larval weight was fitted to an exponential function. The LC_{50} of infected larvae was one-third that of uninfected larvae, indicating that infected larvae are more vulnerable to toxin. This work has implications for resistance management of *O. nubilalis* and demonstrates that it is important to determine whether *N. pyrausta* is present when testing susceptibility of larvae to transgenic corn hybrids.

KEY WORDS *Ostrinia nubilalis*, *Nosema pyrausta*, Bt, corn

THE EUROPEAN CORN BORER, *Ostrinia nubilalis* (Hübner), is estimated to have entered the United States from Europe between 1909 and 1914 (Smith 1920, Fracker and Fluke 1926). Since its arrival, *O. nubilalis* has established itself as a major pest of corn, *Zea mays* L. (Cyperales: Poaceae). Rice (1994) suggested that yield loss due to *O. nubilalis* may reach 81.5 bushels per hectare. Genetically modified corn hybrids that express crystal protein endotoxin genes from *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) (Bt) for the control of *O. nubilalis* have been available commercially since 1996 (Koziel et al. 1993) and are an increasingly popular management tactic (Pilcher et al. 2002). However, development of resistance by pestiferous Lepidoptera may reduce the duration of efficacious use of transgenic technology.

Nosema pyrausta (Paillot) (Microspora: Nosematidae) is probably the most chronically detrimental, naturally occurring pathogen of *O. nubilalis* in the United States (Lewis and Lynch 1978). A ubiquitous microsporidium, *N. pyrausta* reduces egg hatch, developmental rate, fecundity, and life span of *O. nubilalis* (Zimmack and Brindley 1957, Windels et al. 1976). Pierce et al. (2001) examined the interactions of Dipel ES (a spray formulation of Bt), *N. pyrausta*, and *O. nubilalis*, and they concluded that the susceptibility of *O. nubilalis* to Dipel ES increased when infected with *N. pyrausta*. Dipel ES, however, contains bacterial spores and several different endotoxins active against Lepidoptera, including

Cry1Aa, Cry1Ab, Cry1Ac, and others (Rukmini et al. 2000, Huang et al. 2002) that may maximize the toxicity of this Bt preparation to *O. nubilalis* (Mohd-Salleh and Lewis 1982). In contrast to insecticidal sprays for *O. nubilalis*, gene transfer technology currently uses one or a few genes, including the *cry1Ab* crystal protein endotoxin gene (Pilcher et al. 2002). It is, therefore, important to assess the interactions of *O. nubilalis*, *N. pyrausta*, and Cry1Ab. The objective of this research was to examine the effect of purified Cry1Ab on growth, development, and survival of *Nosema*-infected and uninfected *O. nubilalis*.

Materials and Methods

Insect and Pathogen Cultures. Adult *O. nubilalis* collected during the summer of 2002 from light traps were used to establish a colony at the Corn Insects and Crop Genetics Research Unit, Ames, IA. To ensure that the colony was *Nosema*-free, eggs were heat-treated (Raun 1961). Moths were reared following procedures similar to Guthrie et al. (1965).

N. pyrausta spores were isolated from field-collected *O. nubilalis* in 2002. Infected larvae were homogenized in a glass tissue grinder with 10× phosphate-buffered saline (PBS), and the homogenate was filtered through cheesecloth. Aureomycin (50 mg/ml solution) was added to solution to inhibit microbial growth. The concentration of spores was determined by using a hemocytometer (Levy, Horsham, PA) un-

der 400 \times phase contrast microscopy. The suspension was frozen at -20°C when not in use.

Purified trypsin activated Cry1Ab (HPLC chromatogram-demonstrated purity) was obtained from Dr. M. Carey (see Acknowledgments). The toxin was activated and isolated from a *cry1Ab* clone expressed in *Escherichia coli*. It was then desalted, freeze-dried, and shipped overnight to the USDA-ARS laboratory. When not in use, the toxin was stored in a -20°C freezer.

Experimental Design. A completely randomized design was used for this study, and treatments were replicated three to four times over time depending on treatment combination. Treatment design was a 2 by 8 factorial. The two levels of the first factor were *Nosema*-infected and uninfected larvae, respectively, and the eight levels of the second factor corresponded to concentrations of Cry1Ab in meridic diet. Thus, 16 treatment combinations (*Nosema* infection by toxin concentration in diet) were evaluated.

Diet Preparation. Standard meridic diet of wheat germ was prepared using methodology similar to Lewis and Lynch (1969) for each replication except Fumidil B (*Nosema* growth inhibitor) was omitted. A stock solution of Cry1Ab was serially diluted with PBS for each respective toxin concentration. Aliquots of diet were mixed with one dilution of purified Cry1Ab in a blender for ≈ 30 s, giving seven individual concentrations and a control with no Cry1Ab. The eight toxin concentrations used were 370, 185, 92, 46, 23, 11, 5, and 0 (control) ng/ml. About five milliliters of diet from each mixture was poured into 18.5-ml plastic diet cups (Fill Rite, Newark, NJ), and the diet cups were covered with a plastic sheet. The diet solidified at room temperature for ≈ 6 h. One-half of the diet cups at each concentration received 250 μl of *Nosema* spores (4.96×10^6 spores per milliliter) topically, and the *Nosema* treatments dried at room temperature for an additional 6–12 h before larvae were applied.

Dose-Response Bioassay. Only neonate larvae (<24 h posteclosion) were used. A single larva was placed onto the surface of diet within each diet cup. For each treatment combination, ≈ 20 larvae were tested; ≈ 320 larvae were used each replication. Diet cups were held in an environmentally controlled room at 27°C , a photoperiod of 24:0 (L:D) h, and 80% RH. After 10 d, growth, development, and mortality of the larvae were determined. At least 10 surviving larvae from each treatment were weighed and assessed for instar based on body length, prothoracic shield size (Dewitt and Stockdale 1983), and the number of head capsule exuvia found in each diet cup. Instar was used to quantify development, and weight was used to measure growth. Larvae were destructively sampled to confirm presence or absence of *Nosema* infection. For analyses that included mortality, living first instars were recorded as *dead* because they effectively have negligible contribution to plant damage and subsequent *O. nubilalis* populations.

Statistical Analyses. Distributions of the five instars for each level of infection were analyzed in an 8 by 5 contingency table for independence between Cry1Ab concentration in diet and larval development (CHISQ

option of the SAS procedure PROC FREQ, SAS Institute 1988). Instar data were pooled over replications because visual inspection of the data failed to indicate a trend temporally.

The influences of *Nosema* infection, toxin concentration, replication, and main-effect interactions on transformed larval weight were assessed by using analysis of variance (ANOVA), and means were separated using the Tukey multiple range test (PROC GLM, SAS Institute 1988). Weight data were transformed for ANOVA by using a natural logarithm to meet the assumptions of homogeneity of variance and normality. Additionally, the relationship among *Nosema* infection, toxin concentration, and larval weights was described by using the exponential function $Y = \beta_0 + \exp[\beta_1 - (\beta_2 * \text{TOXIN}) - (\beta_3 * \text{INFECTION})]$, where Y is larval weight; TOXIN is the concentration of Cry1Ab in diet; INFECTION is the presence or absence of *N. pyrausta*; and β_0 , β_1 , β_2 , and β_3 are estimated. The exponential function was fitted to the larval weight data by using nonlinear regression using the SAS procedure PROC NLIN (SAS Institute 1988).

Survival data were subjected to Probit analysis to calculate LC_{50} values for infected and uninfected larvae, respectively, on a logarithmic base 10 scale for each replication. The transformed LC_{50} values were subsequently analyzed by using ANOVA and the Tukey multiple range test (PROC GLM, SAS Institute 1988).

Results and Discussion

The results of the contingency table indicated that the number of uninfected larvae in each stadium was dependent on Cry1Ab concentration in diet ($\chi^2 = 822.09$, $\text{df} = 28$, $P < 0.01$; $n = 567$). Similarly, the number of infected larvae in each stadium was dependent on toxin concentration in diet ($\chi^2 = 796.37$, $\text{df} = 28$, $P < 0.01$; $n = 760$). However, for infected larvae, the effect was more pronounced and higher

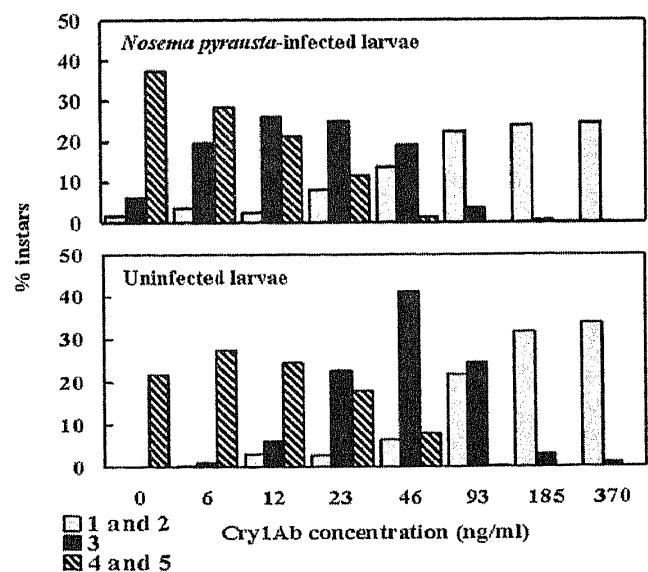


Fig. 1. Percentage of both first and second, third, and both fourth and fifth instars of *N. pyrausta*-infected and uninfected *O. nubilalis* at different concentrations of purified Cry1Ab in meridic diet after 10 d.

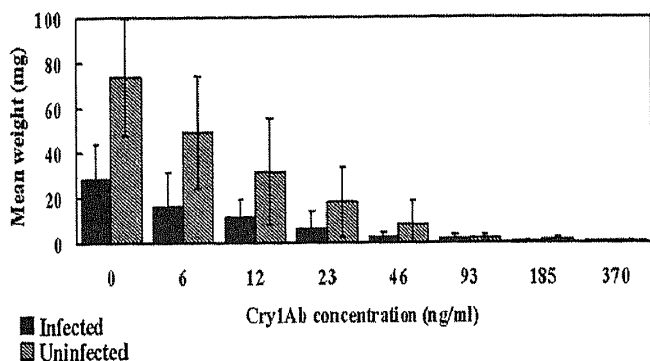


Fig. 2. Mean larval weights of *N. pyrausta*-infected and uninfected *O. nubilalis* fed different concentrations of purified Cry1Ab in meridic diet for 10 d.

numbers of larvae remained in earlier stadia. Overall, larval development was reduced when toxin concentration in diet increased, and the reduction in development was amplified by *Nosema* infection (Fig. 1).

The ANOVA indicated a significant relationship among transformed larval weight, *Nosema* infection, Cry1Ab concentration in diet, and replication ($F = 43.69$; $df = 53, 441$; $P < 0.01$). As Cry1Ab concentration increased, the difference between the weights of infected and uninfected larvae decreased (Fig. 2), which is demonstrated by the significant interaction term ($F = 2.53$; $df = 7, 441$; $P = 0.01$). The reduction in larval weight associated with Cry1Ab in diet was increased by *Nosema* infection. Although replication was significant in the model ($P < 0.01$), differences in larval weight among replications were not chronological, suggesting that the viability of the *Nosema* spores and the toxicity of Cry1Ab were not progressively changing temporally. Nonlinear regression analysis indicated a significant exponential relationship among larval weight, toxin concentration in diet, and infection ($F = 13.80$; $df = 4, 76$; $P < 0.01$; $R^2 = 0.61$) ($n = 495$). The overall model was larval weight = $3.9 + \exp[3.8 - (25437 * \text{TOXIN}) - (0.5 * \text{INFECTION})]$.

The Probit analysis used to obtain LC_{50} values for infected and uninfected *O. nubilalis* was significant (Table 1). The LC_{50} for infected larvae was significantly lower than that for uninfected larvae ($F = 8.48$; $df = 1, 5$; $P = 0.03$; Fig. 3). For infected larvae and uninfected larvae, the LC_{50} was 76 and 245 ng/ml, respectively. Thus, uninfected larvae were >3 times as tolerant of Cry1Ab in diet than infected larvae.

Dipel ES may be more pathogenic to *O. nubilalis* than purified Cry1Ab. When a corresponding LC_{50}

Table 1. Toxicity of Cry1Ab to *N. pyrausta*-infected and uninfected *O. nubilalis*

	<i>n</i>	Slope (SE) ^a	LC_{50} (95% FL) ^b	<i>df</i>	χ^2 ^c
Infected	684	-1.73 (0.19)	76 (58-101)	1	84.35
Uninfected	512	-1.68 (0.24)	245 (176-394)	1	51.11

FL, fiducial limit.

^a Transformed to logarithm base 10 scale.

^b Units are nanograms of toxin per milliliter of meridic diet.

^c $P < 0.01$ for all chi-square values.

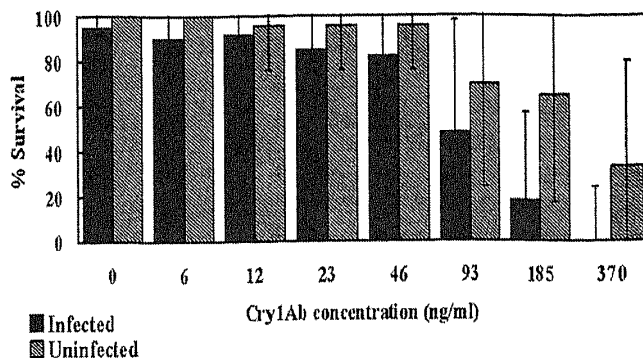


Fig. 3. Survival of *N. pyrausta*-infected and uninfected *O. nubilalis* fed different concentrations of purified Cry1Ab in meridic diet for 10 d.

calculated by Pierce et al. (2001) was compared with the LC_{50} for infected larvae derived from the current study, the LC_{50} associated with Dipel ES (≈ 0.01 ng/ml) was 7,600 times less than that of the LC_{50} associated with purified Cry1Ab (76 ng/ml). This suggests that larvae respond differently to Dipel ES than purified Cry1Ab. Furthermore, the differential response of larvae to purified Cry1Ab and Dipel ES demonstrates the synergism among endotoxins and bacterial spores (Mohd-Salleh and Lewis 1982). This observation, however, may be confounded with differences in the *Nosema* spores and larvae used and the methodology of diet preparation used in the two studies.

Our findings may have implications for management of *O. nubilalis* resistance to transgenic corn hybrids. *Nosema*-infected larvae that are resistant to transgenic corn hybrids may be overlooked during screening processes that rely on survivorship or growth, and such larvae could be misidentified. In such a situation, without intervention a resistance trait may be propagated through an insect population. Other pathogens—not examined in this study—may similarly cause an increase in errors when conducting bioassays.

Acknowledgments

We are indebted to Jean Dyer, Bob Gunnarson, and Miriam Lopez (Corn Insects and Crop Genetics Research Unit, USDA-ARS, Ames, IA), for technical assistance and to Marianne Carey (Department of Biochemistry, Case Western Reserve University, Cleveland, OH), for supplying Cry1Ab. We also thank Leellen Solter (Insect Pathology, Illinois Natural History Survey, Urbana, IL), Stefan Jaronski (Pest Management Research Unit, USDA-ARS Sidney, MT), and Blair Siegfried (Department of Entomology, University of Nebraska, Lincoln, NE) for critical reviews of this manuscript. Finally, we thank Thomas Sappington (Corn Insects and Crop Genetics Research Unit, USDA-ARS) for insightful suggestions. This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or a recommendation by the USDA for its use.

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Received 12 February 2004; accepted 1 March 2004.