NOTE

Survivorship of Field-Collected European Corn Borer (Lepidoptera: Crambidae) Larvae and Its Impact on Estimates of Resistance to *Bacillus thuringiensis* Berliner[1]

R. C. Venette, J. C. Luhman[2] and W. D. Hutchison[3]

USDA-APHIS, Department of Entomology, and Midwest Ecological Risk Assessment Center, 1980 Folwell Ave., University of Minnesota, St. Paul, MN 55108 USA

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Widespread production of transgenic corn, *Zea mays* (L.), engineered to express insecticidal crystal proteins (e.g., cry1Ab) from the bacterium *Bacillus thuringiensis* subsp. *kurstaki* Berliner (*Bt*), has generated concerns over targeted insects developing resistance (Gould, 1998, Annu. Rev. Entomol. 43: 701–726). The primary target for *Bt*-corn is *Ostrinia nubilalis* (Hübner), the European corn borer. Resistance management plans for *O. nubilalis* (Ostlie et al., 1997, *Bt* corn and European corn borer: long term success through resistance management, University of Minnesota, St. Paul; Vlachos et al., 1999, http://www.ncga.com/02profits/insectMgmtPlan/main.htm) assume that resistant individuals are rare (e.g., <1 in 1000 insects or 10^{-3}). To evaluate this assumption, sampling strategies must provide a high probability of detection and correct classification of larvae with resistance to *Bt*.

In a previous study, we used an in-field screen with *Bt*-sweet corn to detect resistant larvae; live *O. nubilalis* larvae (≥third instar) that had been feeding on *Bt* expressing tissue (independently confirmed with immunoassay) were operationally considered resistant (Venette et al., 2000, J. Econ. Entomol., in press). However, the National Corn Growers Association recently suggested more restrictive criteria to characterize resistance (Vlachos et al. 1999). Specifically, a larva collected from *Bt* expressing tissue is "resistant" if it also survives a discriminating concentration of an appropriate cry1A protein in a laboratory assay. In the event that a discriminating concentration is not available, a larva shall be considered resistant if its offspring satisfy the following criteria: (a) the concentration of cry1A protein required to kill 50% of the population (LC_{50}) is statistically greater than the historical LC_{50} for the popu-

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[2]Minnesota Department of Agriculture, 90 West Plato Boulevard, St. Paul, MN 55107.

[3]Department of Entomology & Midwest Ecological Risk Assessment Center, 1980 Folwell Ave., University of Minnesota, St. Paul, MN 55108.

lation; and (b) >30% of progeny survive and >25% of the leaf area is damaged in a laboratory bioassay using leaf tissue expressing the appropriate cry1A protein. Presumably field-collected larvae that do not survive the bioassay or produce offspring will not be considered resistant.

During a discriminating dose bioassay with larvae collected from *Bt* fields, mortality may be due to susceptibility to *Bt* toxins, mechanical damage incurred during larval collections, inappropriate environmental conditions, pathogens, parasitoids, or poor genetic fitness. Because *Bt*-resistant larvae are rare, controls to account for non-*Bt* related mortality are not practical. If we assume that larvae in transgenic and isogenic corn are equally likely to be infected, parasitized, or damaged during collection, the degree of non-*Bt* related mortality could be measured from larvae sampled from non-*Bt* corn. However, the incorporation of such information into an estimate of the frequency of resistance remains problematic. In this study, we report annual larval mortality for fifth-instar *O. nubilalis* collected from non-*Bt* field corn throughout Minnesota. Given estimates of larval mortality, we then provide probabilities for the number of larvae that are likely to survive to adulthood and explore how non-*Bt* related mortality may affect estimates of the frequency of resistance.

Between 1984 and 1997, annual surveys for *O. nubilalis* larvae were conducted in corn-producing counties throughout Minnesota. From each country, ~30 apparently healthy fifth-instar larvae were collected from 5 to 6 arbitrarily selected fields (~6 larvae/field). Stalks were selected randomly within 45 m from the edge of a field. Larvae from the 5 fields were pooled to form a single sample for the county. Larvae were placed in doubled paper bags inside a plastic bag to prevent escape and given pieces of corn stalk to allow boring. The sample was kept cool and returned to the lab for processing.

Larvae were removed from corn stalks using a knife and forceps. Any larva with emergent hyphae of *Beauveria bassiana* (Balsamo) Vuillemin or damaged during the extraction process was discarded. Larvae obviously infected with *B. bassiana* were eliminated to prevent contamination of other larvae. Discarded larvae were not included in statistical analyses. Remaining larvae were dipped in a 5% bleach solution (volume bleach: volume water) and placed individually in cups containing an agar-based medium (1.88% agar, 0.16% methyl paraben, 0.06% sorbic acid, and 98% water w/w). Larvae were stored for ~8 wks at 4°C, 80% relative humidity, and complete darkness to allow larvae to complete diapause. After completing diapause, larvae were inspected for disease, parasitism, or inviability. Status of larvae was recorded, and dead larvae were removed. Larvae were placed at 29°C, 80% relative humidity, and full light to break diaspause. Mortality due to diseases, parasites, or unknown causes was noted weekly. Infection by *B. bassiana* was confirmed by light microscopy. Emerging parasitoids were identified as *Macrocentrus grandii* Goidanich, *Eriborus terebrans* Gravenhorst, *Eumeae caesar* (Aldrich), *Sympiesis viridula* (Thomson), or other. The number of surviving adult *O. nubilalis* was recorded.

Data were transformed using ARSIN(SQRT(x)) and analyzed by one-way analysis of variance using PROC GLM with mean separation by Ryan-Einot-Gabriel-Welsch multiple range test (SAS Institute, 1995, SAS/STAT user's guide, Cary, NC). For probability analyses, we assume each larva represents an independent Bernoulli trial and allow the average probability of success (i.e., surviving to adulthood) to be rep-

resented by p; 1-p is average mortality. If N larvae are collected from the field, the probability that x larvae will survive to adulthood is given by:

$$p(x) = \binom{N}{x} p^x (1-p)^{N-x} \qquad \text{(Equation 1).}$$

For these analyses, we assume a maximum N of 4, which represents the maximum number of *O. nubilalis* larvae collected to date from large plots of *Bt* sweet corn (Andow and Hutchison, 1998, pp. 19–66, *In* M. Mellon and J. Rissler [eds.], Now or never: serious new plans to save a natural pest control, Union of Concerned Scientists, Cambridge, MA).

Mortality of field-collected, fifth-instar *O. nubilalis* was greatest in 1984 and 1991 when ~75% of all larvae died (Table 1). In these years, ~50% of larvae died due to unknown causes. Low rates of mortality were observed between 1987–89 and 1992–93 (Table 1). Across these periods, mortality averaged 27% which was still predominantly due to the effects of unknown causes. Infection by *Nosema pyrausta* (Paillot) may have caused some of the unknown deaths. Assays in 1994, 1995, and 1997, indicated that 27%, 39%, and 63%, respectively, of all *O. nubilalis* collected in this survey, including individuals that pupated or emerged as adults, were infected with *N. pyrausta* (D. Ragsdale, Univ. Minnesota, unpubl. data). Over the 11 years of this study, <10% of all larvae collected died due to parasitoids. On average, *M. grandii* accounted for 51.2 ± 7.6% ($\bar{x}$ ± SEM) of the deaths due to parasitism; *Eriborus terebrans,* 37.6 ± 7.4%; *Eumeae caesar,* 2.9 ± 1.2%; and *S. viridula,* 1.2 ± 0.5%.

As mortality due to parasitoids, pathogens, or other non-*Bt* related factors increases, the probability that all larvae from a small subsample (i.e., ≤4 individuals) will survive is reduced (Table 2). If mortality is ~30% (i.e., 70% survivorship) and ≥2 larvae are collected, we expect ≥1 deaths. If mortality increases to ~70%, we expect ≥2 deaths. As more larvae are collected, the probability that at least one larva will survive to an adult becomes greater (Table 2). These calculations rely on the assumption that larvae from *Bt* and non-*Bt* corn experience the same likelihood of dying due to infection, parasitism, mechanical damage, or other non-*Bt* related factors.

If a field-collected larva must survive to undergo a laboratory bioassay before it can be considered resistant, non-*Bt* related mortality will lower the estimated frequency of resistant individuals. Venette et al. (2000) estimated the frequency of resistance, E[f], using the equation E[f] = (S + 1)/(ML), where S is the number of resistant larvae found, M is the number of *Bt* plants examined, and L is the average density of resistant and susceptible larvae per non-*Bt* plant. If 4 resistant larvae (S) were observed in a sample of 1200 *Bt* plants (M) and an average of 3 larvae were observed per non-*Bt* plant (L), we expect the frequency of resistance to be 1.4 ± 10^{-3}. If larval survivorship from non-*Bt* corn averages 60% (Table 1), we expect only 2 to 3 of the resistant larvae will survive to adults for further laboratory bioassays (Table 2). Therefore, S is now 2, and if all other variables are the same, the frequency of resistance becomes 8.4×10^{-4}.

Appropriate doses of *Bt* to distinguish resistant from susceptible individuals have been established for neonates of certain lepidopteran pests (e.g., Sims et al., 1996, p. 229–242, *In* T. M. Brown [ed], Molecular genetics and evolution of pesticide resistance, American Chemical Society, Washington, DC; Marcon et al., 1999, J. Econ. Entomol. 92: 279–285). These doses do not apply to later-instars because larger susceptible insects exhibit greater tolerance to *Bt* (Davis and Coleman, 1997, J.

Table 1. Percent mortality (±SEM) of Minnesota field-collected, fifth-instar *Ostrinia nubilalis* caused by parasitoids or pathogens under standardized laboratory conditions

	% Mortality due to:							Counties	Total
Year	*M. grandii*	*E. terebrans*	*E. caesar*	*S. viridula*	*B. bassiana*	Unknown*	Total	(n)	Larvae
1984	1.6 ± 0.4ab	0.6 ± 0.3de	0.0 ± 0.0c	0.0 ± 0.0b	6.0 ± 1.9bc	64.1 ± 3.8a	72.3 ± 3.4a	34	1424
1987	7.7 ± 1.4a	1.6 ± 0.5bcde	0.0 ± 0.0c	0.2 ± 0.1ab	1.0 ± 0.5de	12.1 ± 1.9g	22.5 ± 2.3g	55	2302
1988	0.6 ± 0.3c	0.3 ± 0.1e	<0.1 ± **c	<0.1 ± **b	0.0 ± 0.0e	23.0 ± 2.0de	24.0 ± 2.0de	46	2241
1989	2.0 ± 0.5bc	0.8 ± 0.3de	0.4 ± 0.2ab	0.0 ± 0.0b	0.0 ± 0.0e	25.8 ± 1.4cde	29.1 ± 1.5cde	65	2166
1990	6.8 ± 1.3ab	0.9 ± 0.2cde	<0.1 ± 0.1bc	0.1 ± **ab	0.0 ± 0.0e	28.7 ± 1.4cd	36.5 ± 1.8c	69	2284
1991	0.3 ± 0.1c	0.7 ± 0.2de	0.0 ± 0.0c	0.0 ± 0.0b	35.0 ± 3.1a	41.9 ± 2.8b	77.9 ± 1.9a	65	2046
1992	5.4 ± 1.2ab	4.0 ± 0.8ab	<0.1 ± **c	<0.1 ± **b	8.9 ± 2.2bc	11.1 ± 1.3g	29.7 ± 2.4cde	63	990
1993	3.3 ± 0.9bc	5.0 ± 1.0ab	<0.1 ± **c	<0.1 ± **b	0.8 ± 0.4de	18.9 ± 2.1f	28.2 ± 2.3de	69	1016
1994	4.5 ± 0.8ab	4.6 ± 0.9a	0.5 ± 0.2a	0.3 ± 0.1ab	2.8 ± 0.9de	18.5 ± 1.5ef	31.2 ± 1.8cd	73	2131
1995	4.4 ± 0.8ab	3.1 ± 0.7abcd	0.5 ± 0.2a	0.0 ± 0.0b	4.0 ± 0.9cd	23.8 ± 1.6de	36.5 ± 1.8c	71	1772
1997	7.9 ± 1.7a	3.4 ± 3.4abc	0.4 ± 0.2ab	0.4 ± 0.1a	11.5 ± 1.8b	33.7 ± 1.9bc	57.3 ± 1.9b	73	2148
Mean	4.0 ± 0.8	2.3 ± 0.5	0.2 ± 0.1	0.1 ± **	6.4 ± 3.1	27.4 ± 4.6	40.5 ± 5.9	683	20520

Percentages within a column followed by the same letter are not statistically different ($P>0.05$) as determined by Ryan-Einot-Gabriel-Welsch multiple range test.
* Includes larvae that apparently died due to unidentified bacterial or fungal pathogens or dead larvae with normal shape and coloration.
** SEM<0.1.

Table 2. Effect of average survivorship of fifth-instar *Ostrinia nubilalis* larvae on the probability that field-collected individuals will survive to adults

Average survivorship	Collected larvae (N^*)	Probability of having ≥0 surviving larvae				
		Number of survivors (x)				
		0	1	2	3	4
0.70	1	0.30	0.70			
('87–'90, '92–'95)	2	0.09**	0.42	0.49		
	3	0.03	0.19	0.44	0.34	
	4	0.01	0.08	0.27	0.41	0.24
0.60	1	0.40	0.60			
(All years)	2	0.16	0.48	0.36		
	3	0.06	0.29	0.43	0.22	
	4	0.03	0.15	0.35	0.35	0.13
0.30	1	0.70	0.30			
('84, '91, '97)	2	0.49	0.42	0.09		
	3	0.34	0.44	0.19	0.03	
	4	0.24	0.41	0.27	0.08	0.01

* Variables N and x relate to Equation 1, described in the text.

** Example: When average survivorship is 70% and 2 larvae are collected from a field, there is a 9% probability that no fifth instar larvae will survive to adulthood.

Kansas Entomol. Soc. 70: 31–38; VanFrankenhuyzen et al., 1997, J. Econ. Entomol. 90: 560–565). In-field identification currently requires that a larva be ≥ third instar and actively feeding on *Bt*-expressing tissue (confirmed by immunoassay) to be considered resistant (Venette et al. 2000). It is improbable that all larvae identified as resistant in the field will survive to adulthood for additional laboratory analysis (Table 2). Furthermore, not all survivors will successfully mate and produce viable offspring (R. C. Venette, unpubl. data).

A larva is definitively resistant if it is collected from *Bt* tissue and it, or its offspring, can survive an appropriate bioassay. However, a larva collected from *Bt* tissue that does not survive to undergo bioassay is not necessarily susceptible. Accounting for the effects of non-*Bt* related mortality on resistance frequency estimates is difficult because resistant larvae are likely to be scarce. If additional laboratory confirmation is required before a larva can be considered resistant, the levels and risks of *Bt* resistance in targeted pest populations will be underestimated.

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