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# Development of Diagnostic Concentrations for Monitoring *Bacillus thuringiensis* Resistance in European Corn Borer (Lepidoptera: Crambidae)

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**ABSTRACT** Two candidate diagnostic concentrations of the Cry1Ab and Cry1Ac toxins from *Bacillus thuringiensis* corresponding to the $LC_{99}$ and $EC_{99}$ (effective concentration that causes 99% growth inhibition) for European corn borer, *Ostrinia nubilalis* (Hübner), were determined based on previously obtained baseline data. Validation experiments using field-collected European corn borer populations from across North America showed that for Cry1Ab, a concentration corresponding to the upper limit of the 95% confidence interval of the $LC_{99}$, produced mortality >99% for all populations tested. However, for Cry1Ac, adjustments and further validation are probably necessary. Development of *B. thuringiensis* resistance monitoring programs that rely on diagnostic techniques are discussed.

**KEY WORDS** European corn borer, *Bacillus thuringiensis*, resistance monitoring

IT IS BECOMING increasingly apparent that transgenic corn plants that express toxins from the bacterium, *Bacillus thuringiensis*, will become an integral component of production systems (Fischhoff 1996). The associated risk for *B. thuringiensis* resistance development in target pests, such as European corn borer, *Ostrinia nubilalis* (Hübner), will severely limit the benefits of employing *B. thuringiensis* corn as a new pest management option (Ostlie et al. 1997). However, resistance management strategies that minimize selection pressures may help to maintain the viability of this environmentally sound and economically important pest management option (Gould 1996, Roush 1996).

One of the most critical components of resistance management strategies is the ability to effectively monitor the development of resistance in pest populations (Dennehey 1987). The ability to detect resistance is necessary to (1) determine whether control failures are caused by the presence of resistant insects or some other factor that affects product performance; (2) assess the extent and distribution of resistant populations; and (3) test the effectiveness of management programs designed to reduce the frequency of resistant individuals (Halliday and Burnham 1990).

Insecticide resistance detection has traditionally involved complete dose–response tests requiring 4–5 doses of insecticide that produce 10–90% mortality. Resistance is then expressed by the ratio of the $LD_{50}$ or $LD_{90}$ of the resistant strain divided by that of the susceptible strain. Such techniques have been adequate for documenting resistance that has reached high levels, but are insensitive to small changes in the frequency of resistant individuals, particularly when resistance is first appearing (Halliday and Burnham 1990). An alternative to traditional dose-mortality testing involves the use of diagnostic or discriminating doses (ffrench-Constant and Roush 1990). These techniques offer the advantage of being more efficient for detecting low frequencies of resistance because all individuals are tested at an appropriate dose and none are wasted on lower or higher doses where percentage mortality is not informative. Additionally, diagnostic tests require fewer individuals and much less time than complete dose-mortality tests, and can be used to bioassay a much greater number of populations (Halliday and Burnham 1990).

In situations where resistance has not been fully documented or where an insecticide has yet to elicit sufficient selection pressures, a dose that kills 99% of susceptible individuals ($LD_{99}$) has been proposed for diagnostic bioassays (ffrench-Constant and Roush 1990). This approach provides a compromise between allowing few susceptible survivors but does not risk killing as many resistant individuals as a higher dose might. A problem with this approach is the choice of a susceptible population from which to derive the $LD_{99}$. A great deal of variation can be expected between relatively unexposed field populations and even laboratory populations can vary significantly from generation to generation (Robertson et al. 1995). A solution to this problem is to determine the $LD_{99}$ from

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**Table 1. European corn borers field-collected in the summer of 1996 for validation of candidate diagnostic *B. thuringiensis* concentrations**

| Collection site | Life stage collected | No. adults used to initiate colony |
|---|---|---|
| Aurora, NE | Larvae/Adults (772) | 639 |
| Mead, NE | Larvae/Adults (1,166) | 980 |
| Rosemount, MN | Larvae (1,000) | 212 |
| Ames, IA | Egg masses (30) | 210 |
| Bloomington, IL | Egg masses (36) | 128 |
| West Laffayette, IN | Egg masses (24) | 236 |
| Selinsgrove, PA | Egg masses (100) | 225 |
| Plymouth, NC | Egg masses (180) | 750 |

Numbers in parentheses represent sample size of field collection.

**Table 2. Combined analyses of European corn borer susceptibility to Cry1Ab and Cry1Ac based on mortality and larval growth inhibition**

| Toxin | *n* | Slope ± SE[a] | $EC_{99}$ (95% CI)[a] | $LC_{99}$ (95% CI)[b] |
|---|---|---|---|---|
| Cry1Ab | 22,574 | 1.92 ± 0.04 | 20.0 (13.9–32.1) | 67.8 (56.7–83.7) |
| Cry1Ac | 16,744 | 1.93 ± 0.03 | 1.7 (1.3–2.0) | 5.8 (4.7–7.3) |

Baseline data from 18 field populations (Marçon et al. 1999) combined in overall analysis. $LC_{99}$ (concentration that causes 99% mortality) and 95% confidence intervals expressed as ng toxin/$cm^2$.

[a] Data were analyzed by probit analysis (Finney 1971) using POLO-PC (LeOra Software 1987).

[b] Larval weights were transformed to percent growth inhibition relative to the controls and these data analyzed by nonlinear regression fitted to a probit model (Marçon et al. 1999). $EC_{99}$ (concentration that causes 99% growth inhibition) and 95% confidence intervals expressed as ng toxin/$cm^2$.

field populations before the wide commercial introduction of the specific pesticide.

Recently, we developed an extensive data base for *B. thuringiensis* susceptibility among geographically distinct European corn borer populations. In 1993 and 1994, we obtained dose-mortality data for 11 different populations from across the state of Nebraska (Siegfried et al. 1995). Although there was considerable variation in response to *B. thuringiensis* (based on mortality data), our results indicate that such variation is not the result of previous selection because there was as much variation between generations of the same population as there was between populations (Siegfried et al. 1995). In 1995, *O. nubilalis* were collected from eight states (Colorado, Nebraska, Iowa, Illinois, Indiana, Missouri, Pennsylvania, and Tennessee) and from areas representing the various pheromone strains and voltinism ecotypes (Marçon et al. 1999). This study represented a much broader sampling of corn borer populations, although similar levels of variation in both mortality and larval growth inhibition were observed.

The objective of the current study was to develop a diagnostic technique for monitoring *B. thuringiensis* resistance in field populations of *O. nubilalis.* Candidate concentrations for diagnostic bioassays were derived from the baseline susceptibility study conducted previously (Marçon et al. 1999). Validation of these diagnostic concentrations involved field collections of *O. nubilalis* from areas representing the major voltine ecotypes and pheromone races, and evaluating their progeny at both the $LC_{99}$ and $EC_{99}$ calculated from baseline data.

## Materials and Methods

**Insects.** Adults, egg masses, or larvae were field-collected from seven states across the United States (Table 1). Nebraska was represented by two different populations, Aurora and Mead, collected at sites ≈150 km apart. Most collection sites represented the bivoltine ecotype and *Z* pheromone race. Populations from North Carolina were collected in areas where the multivoltine *E* pheromone race predominates, and the Pennsylvania collection originated from an area where the different races occur sympatrically (Mason et al. 1996). Field-collected larvae or neonates from field-collected egg masses were immediately placed on artificial diet in individual containers to minimize spread of diseases. These larvae were then reared to adult and allowed to mate using standardized rearing techniques (Lewis and Lynch 1969) as described previously (Marçon et al. 1999). Egg masses from the mated females were collected and allowed to hatch. Neonates ($F_1$ generation) obtained from field-collected parents were used for subsequent bioassays. Collection sites and sample sizes are presented in Table 1.

**Designation of Candidate Diagnostic Concentrations.** Baseline susceptibility of *O. nubilalis* to both the Cry1Ab and Cry1Ac *B. thuringiensis* toxins was previously described (Marçon et al. 1999), and although significant differences among populations were detected for both toxins, these differences apparently reflect natural variation in the response of *O. nubilalis* rather than prior selection pressure. Therefore, these data were reanalyzed by combining data sets of all *O. nubilalis* populations tested to increase sample size and minimize confidence intervals, thus providing a more precise estimate of the $LC_{99}$ (Robertson et al. 1984). Mortality data were analyzed by probit analysis (Finney 1971) using POLO-PC (LeOra Software 1987). A summary of the combined probit analysis for the two toxins is presented in Table 2. The upper 95% confidence limit of each overall $LC_{99}$ estimate was chosen as a starting point in establishing a diagnostic concentration. This represented a compromise between frequent misclassification of an unexposed population as resistant, while minimizing the risk of killing resistant individuals at a higher concentration, resulting in decreased sensitivity of the technique.

To increase the likelihood of choosing a useful candidate diagnostic concentration, overall $EC_{99}$ (effective concentration causing 99% growth inhibition) values for Cry1Ab and Cry1Ac were also estimated from combined baseline growth inhibition data collected on the same populations. Larval weights were transformed to percent growth inhibition relative to the controls and these data were analyzed by nonlinear regression (SAS PROC NLIN, SAS Institute 1997) fitted to a probit model (Table 2) (Marçon et al. 1999). Again, the upper 95% CL of each overall $EC_{99}$ estimate

was chosen as a candidate diagnostic concentration. As shown by Roush and Miller (1986), even a small error in the estimation of the $LC_{99}$ (or $EC_{99}$) can cause considerable error in estimation of resistance frequencies using diagnostic concentrations. Thus, final diagnostic concentrations can only be recommended after empirical validation by testing diverse populations from across the geographic range of *O. nubilalis.*

**Bioassay.** All bioassays were conducted by exposing neonates (<24 h after hatching) to treated artificial diet (Marçon et al. 1999). Bioassays involved surface treatment of single wells of artificial diet. The rearing diet developed for *Heliothis virescens* (F.) (King et al. 1985) and adapted for *O. nubilalis* (Marçon et al. 1999) was used instead of rearing diet because it was relatively inexpensive, required less time for preparation, and larval growth was comparable to growth on the standard rearing diet through the first several instars (B.D.S., unpublished data).

Bioassays were performed in 128-well trays (each well was 16 mm diameter by 16 mm high; CD International, Pitman, NJ). Approximately 1 ml of diet was dispensed into each well and allowed to solidify. Each well was treated with 30 $\mu$l of the appropriate toxin concentration prepared in 0.1% Triton-X 100 nonionic detergent to obtain uniform spreading onto the diet surface. Control treatments consisted of diet treated with 0.1% Triton-X 100 only. Wells were allowed to air dry for 1 h, and one neonate was transferred into each well. Wells then were covered with vented lids (CD International), and trays were held at 27°C, 80% RH, and a 24-h scotophase. Mortality and individual larval weights were recorded 7 d later. When mortality was recorded, larvae that had not grown beyond first instar and were ≤0.1 mg in weight were considered to be dead. As a result, the criterion for "mortality" used in this study accounts for both severe growth inhibition and death.

Two *B. thuringiensis* toxins were tested, Cry1Ab and Cry1Ac. Purified Cry1Ab was obtained from the *B. thuringiensis kurstaki* HD1–9 strain, which produces only the Cry1Ab protein. This material was provided by Ciba Seeds Inc. (Research Triangle Park, NC). The crystal protein preparation was obtained by density gradient centrifugation and contained ≈98% crystal protein, as determined by phase contrast microscopy. Cry1Ac was obtained from the commercial *B. thuringiensis kurstaki* MVP Bioinsecticide formulation (Mycogen, San Diego, CA), which contains a modified Cry1Ac protein produced by a recombinant strain of the bacterium *P. fluorescens.* MVP Bioinsecticide contains only the Cry1Ac toxin and does not contain spores or any other potentially toxic ingredients (Gould et al. 1995). Nevertheless, a washing procedure was used to eliminate most inert ingredients present in the formulation. This procedure involved centrifugation of 1 ml-batches of commercial product at 10,000 × *g* for 30 s. The supernatant was discarded and the pellet was resuspended in double-distilled water to 1 ml total volume. This material was centrifuged again for 30 s, the supernatant was removed, and the pellet was resuspended to 1 ml in double-distilled water. The resulting preparation was used as a stock solution for all bioassays with Cry1Ac.

Egg masses collected during a given 24-h period were held in plastic petri dishes, provided with filter paper moistened with sterile water to prevent desiccation, and incubated at 27°C until hatching. Neonates hatching within a 12-h period were selected at random and placed in individual wells treated at concentrations corresponding to the estimated $LC_{99}$ or $EC_{99}$. Control treatments consisted of wells treated with 0.1% Triton-X 100 only. For most populations, three replicates of 112 larvae were tested and the entire experiment was repeated on three separate dates (total of nine replications). For each group of 112 larvae challenged with a diagnostic concentration (1 replication), 16 larvae served as controls. Approximately 1,000 individual larvae (9 replications) were evaluated from each $F_1$ population and weights of surviving larvae were recorded 7 d later.

**Analysis.** Differences from the expected mortality rate (i.e., 99%) were determined by a two-sided Z-test at the 95% confidence level as described by Roush and Miller (1986). Percent growth inhibition relative to the controls was calculated separately for each replication (the percent growth inhibition of each group of 112 larvae was calculated based on the average weight of the 16 control larvae tested simultaneously). Differences from the expected growth inhibition rate (i.e., 99%) were determined by a two-sided *t*-test (Snedecor and Cochran 1989).

## Results

**Cry1Ab.** The estimated $EC_{99}$ (32 ng [AI]/$cm^2$) resulted in larval growth inhibition rates that were between 99 and 100% for all populations tested (Table 3). The observed growth inhibition was significantly >99% for all but two of the populations (Iowa and Illinois), suggesting that the estimated $EC_{99}$ was slightly higher than the average for all *O. nubilalis* populations. However, given the variation in response to *B. thuringiensis* previously identified from baseline susceptibility studies (Siegfried et al. 1995, Marçon et al. 1999), it is not unexpected to see variation in percent growth inhibition among the populations tested. None of the populations exhibited growth inhibition significantly <99%, supporting the assumption that the *O. nubilalis* is susceptible to the *B. thuringiensis* Cry1Ab toxin across the geographical regions tested.

The estimated $EC_{99}$ produced mortality that was significantly <99% for all populations tested (i.e., 32 ng [AI]/$cm^2$ was not high enough to produce 99% mortality in susceptible populations). This result was not unexpected but is important because it helps to define a lower limit on the range of possible diagnostic concentrations (i.e., $LC_{99}$ >32 ng [AI]/$cm^2$). In four of the eight populations tested at the $LC_{99}$ (84 ng [AI]/$cm^2$), the observed mortality was not significantly different from the expected value (99%). Additionally, the observed mortality was not <99% for any of the populations tested, which again supports overall susceptibility of the *O. nubilalis* to the Cry1Ab toxin.

**Table 3. Validation of candidate diagnostic *B. thuringiensis* concentrations using Cry1Ab and Cry1Ac from *B. thuringiensis* against European corn borer neonate larvae**

| Population | *n* | % growth inhibition | % mortality | |
|---|---|---|---|---|
| | | at $EC_{99}$ | at $EC_{99}$ | at $LC_{99}$ |
| Cry1Ab | | 32 ng/cm$^2$ | 32 ng/cm$^2$ | 84 ng/cm$^2$ |
| Minnesota | 672 | 100.00* | 97.49** | 100.00* |
| Aurora, NE | 976 | 99.93* | 98.29** | 99.08 |
| Mead, NE | 1,120 | 99.68* | 97.71** | 99.51 |
| Iowa | 1,008 | 99.36 | 95.71** | 99.79** |
| Illinois | 1,008 | 99.48 | 96.61** | 99.61 |
| Indiana | 1,008 | 99.87* | 98.02** | 99.90* |
| Pennsylvania | 1,008 | 99.74* | 95.24** | 100.00** |
| North Carolina | 1,008 | 99.87* | 97.52** | 99.31 |
| Cry1Ac | | 2 ng/cm$^2$ | 2 ng/cm$^2$ | 7 ng/cm$^2$ |
| Minnesota | 1,008 | 99.52 | 97.49** | 98.98 |
| Aurora, NE | 1,008 | 99.80* | 97.80** | 99.92** |
| Mead, NE | 784 | 99.83* | 98.42 | 99.72** |
| Concord, NE | 672 | 99.17 | 95.83** | 99.87** |
| Pennsylvania | 1,008 | 98.80 | 85.62** | 97.12** |
| North Carolina | 1,008 | 99.31 | 88.47** | 97.73** |

*, Observed response significantly different from the expected value (99%). Significance of differences ($P < 0.05$) determined by a two-sided *t*-test on percent growth inhibition means (Snedecor and Cochran 1989). **, Observed response significantly different from the expected value (99%). Significance of differences ($P < 0.05$) determined by a two-sided Z-test on survivorship counts, according to the following equation: $Z = [|s-ng|-0.5]/[ng(1-g)]^{1/2}$, where $s$ is the observed number of survivors, $n$ is the sample size, and $g$ is the expected fraction of survivors (0.01) in a susceptible population (Snedecor and Cochran 1967, cited by Roush and Miller 1986).

These experiments clearly indicate that the $LC_{99}$ for susceptible field populations challenged with Cry1Ab is higher than 32 ng (AI)/cm$^2$ but not >84 ng (AI)/cm$^2$.

**Cry1Ac.** The observed growth inhibition at the estimated $EC_{99}$ (2 ng [AI]/cm$^2$) was significantly >99% for only two of the six populations tested (Aurora and Mead, NE) (Table 3). As was observed with Cry1Ab, the mortality produced by the estimated $EC_{99}$ was significantly lower than 99% for five of the six populations tested, and therefore sets a lower limit for the $LC_{99}$ ($LC_{99}$ >2 ng [AI]/cm$^2$). At the $LC_{99}$ (7 ng [AI]/cm$^2$), the observed mortality was significantly lower than the expected value for two populations (Pennsylvania and North Carolina) and significantly higher for the three Nebraska populations. The observed mortality of *O. nubilalis* from Minnesota did not differ significantly from the expected 99% level. These results show that the estimated $LC_{99}$ was slightly elevated for the Nebraska populations (more susceptible than average) and slightly low for the populations from Pennsylvania and North Carolina (more tolerant than average). Overall, these experiments indicate slightly greater variability in response to Cry1Ac than to Cry1Ab. Nevertheless, it is obvious that the Cry1Ac $LC_{99}$ for susceptible *O. nubilalis* field populations is >2 ng (AI)/cm$^2$. The upper response limit, however, is not as clearly defined and may be slightly >7 ng (AI)/cm$^2$ for some populations in the upper range of the expected natural variation in *B. thuringiensis* susceptibility.

## Discussion

In validation experiments using the estimated $EC_{99}$ and $LC_{99}$ as candidate diagnostic concentrations, significant differences among populations were observed for the two *B. thuringiensis* proteins evaluated. However, the overall results support the assumption that *O. nubilalis* is susceptible to both Cry1Ab and Cry1Ac *B. thuringiensis* toxins throughout much of the geographic range of the populations tested. The estimated $EC_{99}$ was reasonably accurate (all populations responding between 99 and 100%). However, this concentration may not necessarily be useful as a diagnostic concentration because of the requirement for determining larval weights, a procedure incompatible with large scale monitoring programs. The informative aspect of evaluating the lower candidate diagnostic concentration (equivalent to the estimated $EC_{99}$) for mortality was the identification of a lower limit of the true $LC_{99}$, which may help refine the diagnostic concentration in future studies.

It is important to emphasize that the mortality criteria used in this study accounted not only for mortality but also for extreme growth inhibition (larvae <0.1 mg scored as dead). Therefore, this bioassay technique should be more sensitive for identifying the sublethal effects of *B. thuringiensis* proteins than a standard mortality assay. Because of the simplicity in scoring dead and stunted larvae (as opposed to recording larval weights), the use of this response criterion coupled with the estimated $LC_{99}$ as a diagnostic concentration represents a more appropriate initial approach for estimating *O. nubilalis* susceptibility levels.

The estimated Cry1Ac $LC_{99}$ resulted in mortality <99% in two of the six populations evaluated, suggesting that a higher concentration may be necessary to achieve at least 99% response in all susceptible populations. Further studies will be required to refine this diagnostic concentration. In contrast, the estimated Cry1Ab $LC_{99}$ resulted in responses between 99 and 100% in all populations tested, suggesting that for many *O. nubilalis* populations, the actual Cry1Ab $LC_{99}$ may be slightly lower than the concentration proposed here. However, given the high levels of expression of *B. thuringiensis* toxins in transgenic corn (Koziel et al. 1993, Armstrong et al. 1995), it is likely that this concentration ($LC_{99}$) is several times lower than that expressed by transgenic *B. thuringiensis* corn plants. Therefore, the Cry1Ab concentration proposed here should not misclassify resistant individuals as susceptible and should be suitable for *B. thuringiensis* resistance detection.

In a similar study, Sims et al. (1996) developed a diagnostic bioassay to be used in monitoring *B. thuringiensis* resistance to transgenic *B. thuringiensis* cotton in *Heliothis virescens* and *Helicoverpa zea* (Boddie). The response criterion they used was similar to the "mortality" response used in the present investigation and involved classifying individual larvae as susceptible to the Cry1Ac toxin if they were stunted (<1.0 mg) or dead after 7 d of exposure to the toxin

incorporated into a diet matrix. This bioassay coupled with a diagnostic concentration was found to be more sensitive than a standard mortality bioassay because it better discriminated between susceptible and resistant phenotypes. Currently, resistant strains of *O. nubilalis* are being developed (Lang et al. 1996, Huang et al. 1997, Bolin 1998) and when available they will be useful for testing the power of this diagnostic technique to discriminate between resistant and susceptible populations. Future validation studies should include not only comparisons with resistant *O. nubilalis* strains, but also comparisons with expression levels in transgenic corn varieties.

Because of the expected increased use of *B. thuringiensis*-based products in the next decade and the associated risk of *B. thuringiensis* resistance development in target pests, it is essential that effective resistance monitoring tools be developed (Andow et al. 1998). Ideally, an efficient and reliable bioassay should be developed, such as the glass vial technique used for monitoring pyrethroid resistance in *Helicoverpa zea* and *Heliothis virescens* (Plapp et al. 1990). For *B. thuringiensis* resistance monitoring, however, this type of bioassay is not appropriate because toxic *B. thuringiensis* proteins are active only upon ingestion by the insect, are more labile than chemical insecticides, and are not soluble in organic solvents (Stone and Sims 1993). Additionally, because of their unique mode of action, *B. thuringiensis* toxins require a longer period for response to be evaluated (usually 7 d).

The bioassay described in the current study provides consistent data that can be used to monitor the susceptibility of *O. nubilalis* field populations to *B. thuringiensis* as they become exposed to transgenic *B. thuringiensis* corn. However, the effectiveness of the proposed methodology relies greatly on standardized procedures, including rearing field-collected insects, maintaining test insects in a controlled environment for the time of the bioassay (7 d), and the requirements for standardization in diet preparation and treatment, and training of personnel for evaluating the results (Bolin et al. 1998). Therefore, it is likely that centralized monitoring efforts will provide more reliable information regarding *O. nubilalis* susceptibility. Corn is grown across most of North America and it will be cost-prohibitive to effectively monitor all corn-producing areas. Therefore, it will be essential to identify and prioritize sampling areas where transgenic *B. thuringiensis* corn is planted and to scout *B. thuringiensis* corn fields for unexpected *O. nubilalis* damage. Additionally, the diagnostic concentrations presented here could be used in tandem with other monitoring approaches, such as the $F_2$ screen (Andow et al. 1998) and in field monitoring efforts that employ sweet corn as a trap crop (W.D.H., unpublished data). Such a multi-tiered monitoring strategy may be more efficient at identifying localized areas with potential *B. thuringiensis* resistance problems, and thereby facilitate containment procedures before resistance becomes widespread.

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