Chapter 23

Monitoring Strategies for Early Detection of Lepidoptera Resistance to *Bacillus thuringiensis* Insecticidal Proteins

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We discuss assay approaches for monitoring the sensitivity of Lepidoptera to *Bacillus thuringiensis* (Bt) insecticidal proteins and compare the relative sensitivity of larval feeding bioassays in which, respectively, mortality or growth inhibition were scored. *Heliothis virescens* (F.) and *Helicoverpa zea* (Boddie), major lepidopteran pests targeted for control by transgenic cotton, were used for assay comparison. Larval growth inhibition assays using sublethal CryIA(c) protein concentrations were considerably more sensitive than dose-response mortality assays. Growth inhibition assays were easy to set-up and read, and could readily deliver a diagnostic dose allowing for visual discrimination of resistant from susceptible phenotypes. The ability of a larval growth assay, combined with a diagnostic dose, to unambiguously separate resistant from susceptible insects was validated using a CryIA(c) protein resistant strain of *H. virescens* and F_1 hybrids derived by crossing the resistant strain to a susceptible *H. virescens* strain.

Threat of Insect Resistance to Transgenic Plants Producing Bt-Proteins

Transgenic plant technology has the potential to provide significant improvements in crop protection and benefit to growers. The "first generation" of transgenic plant products produce *Bacillus thuringiensis* (Bt)-derived insecticidal proteins that are pest-specific, environmentally safe, and extremely effective. Unfortunately, the long-term success and maintenance of transgenic plant effectiveness is threatened by the development of insect resistance (*1,2*). For example, many geographically isolated populations of the diamondback moth, *Plutella xylostella* (L.), have already developed field resistance to microbial preparations of Bt which has led to control failures (*2*). Several other species of Lepidoptera and Coleoptera that are now controlled by transgenic crops can develop high levels of resistance to Bt proteins under laboratory selection (*2*-4, Luttrell, R., Mississippi State University, unpublished data). Consequently, the most controversial issues accompanying the introduction of Bt-producing transgenic crops have centered around the potential for insect resistance and the

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preemptive implementation of appropriate resistance management procedures (*1,2*).

Current resistance management theory suggests that a useful resistance management strategy for a transgenic crop involves combining an "optimal" dose of insecticidal protein with a refuge of non-transgenic plants (*5*). If resistance is a recessive or partially recessive trait, then most heterozygous individuals will be unable to survive an optimal dose (also referred to as a "high" dose) of the insecticidal protein. Genetic analysis of Bt-resistant Lepidoptera strains (*Plodia interpunctella* (Hübner), *P. xylostella, H. virescens*) generally supports the assumption that Bt-resistance is a partially (or incompletely) recessive character (*2,4*). The number of genetic elements contributing to resistance is less clear, even for a relatively well-studied species such as *H. virescens* (*4,6*). Genes for resistance will initially be rare in populations and homozygous resistant individuals, with the greatest potential for survival on transgenic plants, will initially be extremely rare compared to heterozygotes (*4,7*). Adequate refuges and successful production of susceptible insects will increase the probability that any resistant homozygote will mate with a susceptible individual to produce heterozygous progeny. This "assumed recessive trait + optimal dose + refuge" resistance management strategy is the cornerstone of several first-generation insect-control plant products.

Resistance Monitoring

An important, but sometimes neglected, component of all preemptive Bt resistance management strategies involves the simultaneous implementation of an efficient resistance monitoring program. Data from appropriate monitoring programs helps us to evaluate the effectiveness of resistance management strategies and permits early detection of resistant phenoypes. Under favorable circumstances, this would allow remedial measures to be implemented prior to control failures (*8*). Historically, the development and implementation of significant resistance monitoring procedures for chemical insecticides has followed, rather than preceded, the initial occurrence of control failures. The availability of field-derived resistant phenoypes permitted studies on level (magnitude) of resistance, genetics of resistance, and resistance mode of action. In addition, practical resistance monitoring assays were developed, validated using resistant insect strains, and used to study subsequent changes in insecticide susceptibility within resistant and non-resistant populations. Studies on *H. virescens* and *Helicoverpa armigera* (Hübner) populations resistant to pyrethroid insecticides represent good examples of this approach (*9,10*).

Diagnostic Doses

Resistance to conventional chemical and microbial insecticides has typically been monitored and compared using the log-dose probit mortality responses of insect strains. This approach allows calculation of a resistance ratio (the LD_{50} or LC_{50} of the field test strain divided by the LD_{50} or LC_{50} of a reference susceptible strain) and statistical comparison of the LD_{50}s and slopes of the probit regression lines (*11,12*). Similarly, "baseline" susceptibility studies on insects targeted for control

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by transgenic plants have generated LC_{50}s and slope estimates for different populations exposed to the specific Bt protein incorporated into a suitable diet matrix (*13-15,* Diveley, G., University of Maryland, unpublished data). However, LC_{50}s and slope estimates, although suitable for distinguishing resistant phenotypes at a high frequency, are not adequately sensitive for detecting resistance when the incidence of resistance is low, e.g. 10^{-3} - 10^{-4} (*7*). Diagnostic doses (i. e. doses that unambiguously discriminate between resistant and susceptible phenotypes) are a more efficient means of finding resistant phenotypes because all individuals tested provide useful data (*7,8*).

Assay Description

The dose-mortality response assays and larval growth inhibition assays that we compared were initiated in a similar manner. Approximately 24 mL of a liquid agar-based insect diet (*16,17*) with 20% of the water omitted was added to a 6 mL sample of test liquid (distilled water containing a dose of the CryIA(c) protein). Treated diet was blended using a Vortex mixer, poured into 96-well insect assay trays (Jarold Mfg. Co., St. Louis, MO), and allowed to cool and harden. Each well of the assay tray had a 2.0 mL capacity and contained 1.0 - 1.5 mL of treated diet. One 1st instar *H. virescens* or *H. zea* larva was added to each well. The wells were then covered with Mylar plastic and ventilated with a single insect pin hole. Assays were incubated at 28 ± 2°C and evaluated after 7 days by scoring the number of survivors (individuals showing movement when probed with a needle) per concentration or by weighing larvae in groups of 10 - 48 and calculating the mean larval weight. The dose-response function of treatments was fit using either probit analysis (mortality data) or non-linear regression analysis for larval weight data (*18*). The non-linear logistic model used was: $\text{weight} = W_0 / [(1 + (\text{concentration} / EC_{50})^B]$ where W_0 is the expected control weight, concentration is the amount of CryIA(c) protein per mL of diet, EC_{50} is the effective concentration of CryIA(c) protein that is expected to reduce larval weight by 50%, and B is the logistic function slope parameter (*19*). For calculation of the EC_{99} values and 95% CIs, the modified equation used was: $\text{weight} = W_0 / [(1 + (100\text{-}1) (\text{concentration} / EC_{99})^B]$.

Dose Mortality Response Evaluation

We re-evaluated the data of Stone and Sims (*14*) by examining the combined dose mortality responses of 12 strains of *H. virescens* and 15 strains of *H. zea* to purified 63 kDa (trypsin-activated) CryIA(c) protein. There was initial indication of significant differences in CryIA(c) protein susceptibility among population samples from distinct geographic locations. However, the present analysis combined all data to examine the potential of a single dose, using mortality as an endpoint, to discriminate between resistant and susceptible individuals over a significant proportion of each species' distribution. The results are presented in Figures 1 and 2. Each data point represents an assay determining the percent mortality response of 24 - 48 larvae exposed to the indicated dose. The total number of assays contributing to the analyses for *H. virescens* and *H. zea* were 234 and 456 respectively. The data sets were evaluated by probit analysis to estimate

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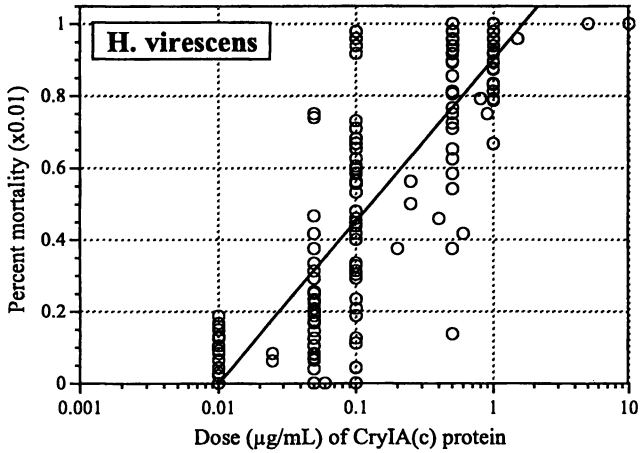

Figure 1. Mortality response of *Heliothis virescens* larvae to purified 63-kD CryIA(c) protein.

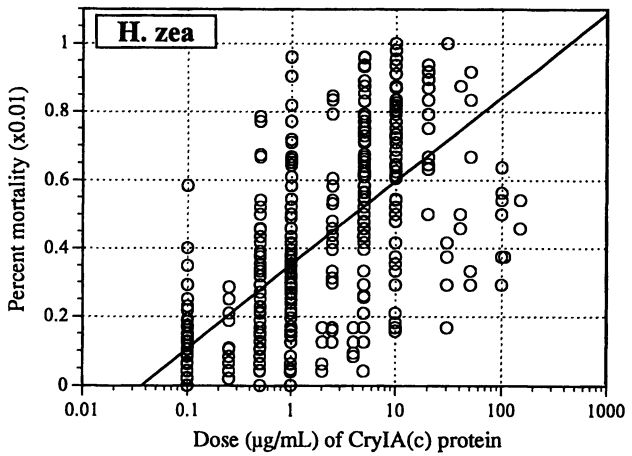

Figure 2. Mortality response of *Helicoverpa zea* larvae to purified 63-kD CryIA(c) protein.

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LC_{99} values and 95% confidence limits. LC_{99} estimates for the 63 kDa protein were 3.3 μg/mL (95% CI = 2.3 - 5.3) for *H. virescens* and 6661 μg/mL (95% CI = 1003 - 2.12 x 10^5) for *H. zea*. Because transgenic cotton produces the non-activated, full-length CryIA(c) protein (~ 130 kD) that is approximately 2X the molecular weight of the trypsin-resistant core, the LC_{99} estimates for the full-length CryIA(c) protein are 6.6 μg/mL for *H. virescens* and 13322 μg/mL for *H. zea*. *H. zea* clearly is significantly less sensitive to the CryIA(c) protein than *H. virescens* and would require a very high concentration if the LC_{99} was used as a possible diagnostic dose.

Growth Inhibition Response Evaluation

Growth inhibition of larvae in response to purified full-length CryIA(c) protein was studied using *H. virescens* and *H. zea* from the USDA, Stoneville, MS laboratory colonies and two additional *H. zea* colonies initiated from Brooksville, MS. The results are presented in Figures 3 and 4. Each data point represents one assay determining the mean larval weight (mg) of a sample of 10 - 32 larvae in response to the indicated dose. The total number of assays contributing to the analyses for *H. virescens* and *H. zea* were 178 and 173 respectively. The data set for each species was fit by nonlinear regression to estimate EC_{99} values, i. e. the concentrations required to reduce larval weight to 1% that of the mean control weight, and 95% confidence intervals. EC_{99} values were 0.058 μg/mL (0.030 - 0.086) for *H. virescens* and 28.8 μg/mL (-7.4 - 65.1) for *H. zea*. These estimates are considerably lower (114-fold less for *H. virescens*, 463-fold less for *H. zea*) than the corresponding LC_{99} estimates for the full-length CryIA(c) protein.

Diagnostic Doses and Resistance Monitoring

The CryIA(c) EC_{99} diagnostic doses indicated for *H. virescens* (0.058 μg/mL) and *H. zea* (28.8 μg/mL) provide reasonable starting points for the dose-setting process. For *H. zea*, the EC_{98} (6.6 μg/mL, 0.1 - 13.0) might be more practical because it provides adequate discrimination (stunting) of susceptible larvae at a much lower concentration. In general, the lowest test concentration providing the requisite degree of larval growth inhibition should be selected. Final diagnostic doses are probably best achieved empirically by testing populations from across the geographic range of each species against 1 or 2 doses that bracket the doses proposed here. This multi-population dose-setting procedure was used to establish discriminating doses of microbial Bt products against Australian *Helicoverpa armigera* and *Helicoverpa punctigera* (*20*). A possible monitoring approach for obtaining initial information on the intensity of resistance would be to simultaneously use more than one diagnostic dose (see *21*) although a sequential testing procedure for *H. virescens and H. zea* would probably be more economical. In our studies, essentially all healthy larvae of both species tested on control diet were 3rd - 5th instars and weighed ≥ 10 mg (usually ≥ 100 mg) after 7 days. Therefore, it seems most practical to set the final diagnostic dose at a concentration preventing all, or most, susceptible larvae from reaching 3rd instar. Due to variability in larval growth rates, this criteria would involve concentrations producing a mean larval weight of 1.0 mg or less. Above this weight, a significant

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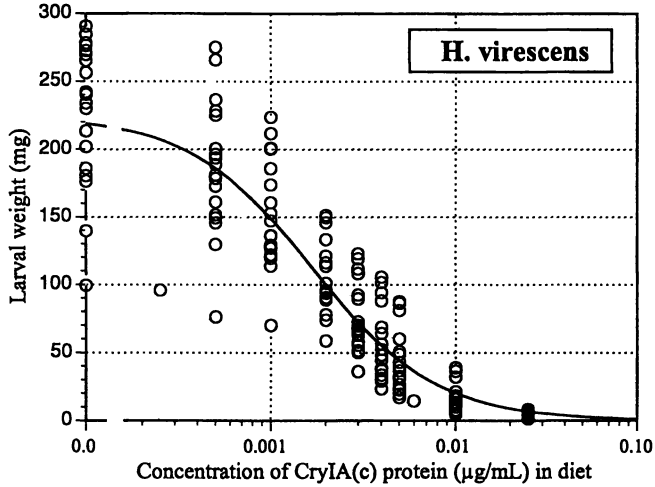

Figure 3. Growth inhibition of *Heliothis virescens* larvae in response to purified 130-kD CryIA(c) protein.

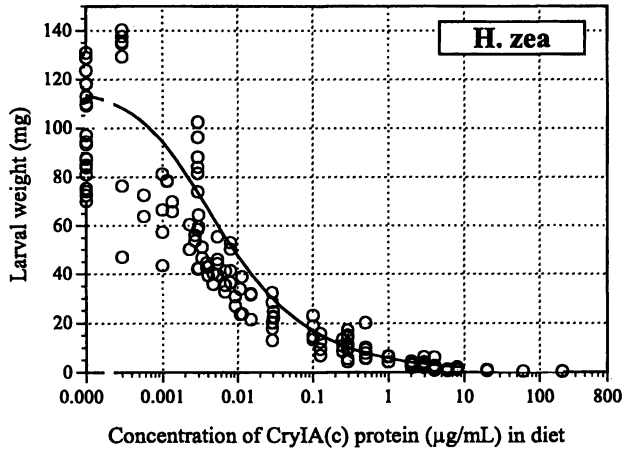

Figure 4. Growth inhibition of *Helicoverpa zea* larvae in response to purified 130 kD CryIA(c) protein.

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percentage of susceptible larvae might still reach 3rd instar (Figure 5) and the incidence of false positives would be greater. We note that the proposed diagnostic EC_{99} concentrations for CryIA(c) protein will result in some larval mortality for both *H. virescens and H. zea* (*14,22*, Figs. 1 and 2). This would not reduce the efficiency of the growth assay because both dead and stunted larvae would be correctly classified as susceptible.

Additional Sampling Considerations

Species Identification. The two species involved, *H. virescens* and *H. zea*, are not equally susceptible to the CryIA(c) protein and require significantly different diagnostic doses. Larvae will therefore need to be identified before being placed on the appropriate test diet concentration. In contrast, *H. armigera* and *Helicoverpa punctigera* (Wallengren) in Australia have approximately equal susceptibility and can potentially be monitored using a single dose (*20*).

Subtle morphological differences between eggs of *H. virescens* and *H. zea* are not sufficiently consistent to provide reliable field identification (*23,24*). Species identification would require hatching and additional larval development. Larval characteristics do not allow reliable species discrimination between *H. zea* and *H. virescens* before the 3rd instar. The 3rd and later instars of *H. virescens* have a large retinaculum ("tooth") on the inner side of the mandible and short spines present on tubercles located on the dorsum of the 8th abdominal segment whereas *H. zea* larvae lack both the retinaculum and the spines (*25*). In addition to morphological characters for species identification, immunoassay test kits are currently being developed for differentiating between *H. zea* and *H. virescens*. Similar test kits (LepTon) based on monoclonal antibodies to species-specific lipophorins have been developed by Abbott Labs to reliably distinguish eggs and larvae of *H. armigera* from *H. punctigera*. Unfortunately, insects sampled and killed for species identification are unavailable for bioassay.

Sampling. Collection of eggs from host plants requires much effort but may fail to provide adequate sample material. Larvae could be collected on non-transgenic cotton but since both *H. zea* and *H. virescens* are polyphagous, other preferred host plants should not be overlooked. For example, in some locations sampling *H. zea* larvae from maize and *H. virescens* larvae from soybean or tobacco might be more efficient than collecting larvae from non-transgenic cotton. Collected larvae could complete development on artificial insect diet and the resulting adults mated. Individual pair, rather than mass, matings would be preferable to maximize the effective population sample size. Light trapping might be the best solution for ease of collecting sample material. Light trapping techniques are well-developed and could potentially supply all of the females needed to assess population sensitivity (*26*). Adult females would be collected in light traps, identified to species by wing scale pattern and color, and held for oviposition.

Sample Locations. Limited resources will obviously require that the number of locations sampled be restricted to a small subset of the possibilities. *H. zea* and *H. virescens* are major problems on 4 to 6 million acres of cotton in the United States and transgenic cottons could eventually be grown on a large percentage of this area.

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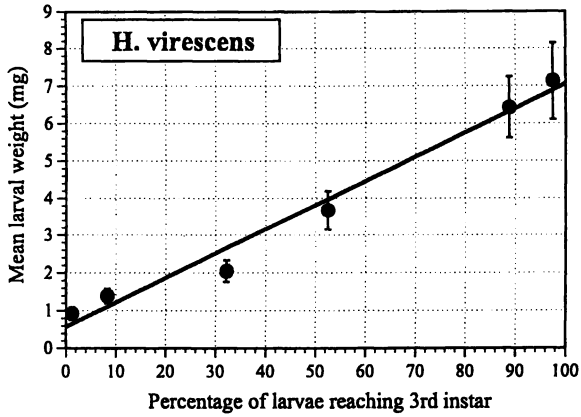

Figure 5. Relationship between *H. virescens* larval weight and percentage of individuals reaching 3rd instar on insect diet.

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Sales data could help identify acreage with transgenic plants on both a state and county basis which could, in turn, be used to prioritize sampling locations. In addition to routine monitoring, intensive scouting of transgenic cotton fields might identify situations where numbers of larvae and feeding damage on transgenic plants is unusually high. After verifying that the transgenic plants involved are producing Bt protein, larvae should be collected, identified, and subsequently tested for Bt protein susceptibility.

Bioassay Techniques and Source(s) of Bt Protein Added to Diet. We found that lyophilized plant tissue containing a known concentration of CryIA(c) protein could readily be added to insect diet to provide a diagnostic dose for *H. virescens*. Because *H. zea* requires a significantly greater diagnostic dose concentration, plant powder cannot be used because of growth inhibition effects caused by gossypol and other cotton allelochemicals. Therefore, purified or partially purified protein would be required. This requirement might be met by using commercial CryIA(c)-containing microbial products such as MVP (*4*) or other transgenic microbes expressing only the CryIA(c) protein. We have used a low gelling point agar (Serva Feinbiochemica GmbH & Co. KG, Heidelberg), workable at temperatures of from 50 to 55°C, to avoid denaturing proteins. An inexpensive, soybean-based, premixed diet (Southland Products, Lake Village, AK), has been convenient for testing *H. virescens, H. zea* and many other species of Lepidoptera.

Validation of the Diagnostic Dose Against CryIA(c) -Resistant *H. virescens*

Larvae from a North Carolina strain of *H. virescens* (YHD2) selected for > 1000-fold resistance to CryIA(c) protein (*4*) were used to validate the concept of a diagnostic dose in combination with a larval growth inhibition assay. CryIA(c) protein, within a lyophilized transgenic cotton leaf tissue matrix, was incorporated into insect diet at concentrations of 4, 20, 60, and 80 mg/mL. The concentrations of active CryIA(c) protein in these diets were determined to be approximately 0.24, 1.20, 3.6, and 4.8 ug/mL respectively by insect bioassay and ELISA (*19*). Diets containing appropriate concentrations of leaf tissue from non-transgenic C312 cotton were used as controls for weight comparisons. The results showed that resistant YHD2 larvae developed at a significantly faster rate on all CryIA(c) concentrations compared to larvae from a non-selected susceptible laboratory strain (YDK) (Fig. 6). The mean weight of presumptive heterozygotes for the resistance trait (i.e. YHD2 x YDK and YDK x YHD2) can be distinguished from the mean weight of YDK larvae reared on diet treated with 4 mg/mL of transgenic leaf powder (Fig. 7). However, a detailed analysis of individual growth rates (4) indicated that a significant proportion of susceptible YDK larvae grew at the same rate as presumptive heterozygotes. Nevertheless, the sensitivity of the growth assay and the potential for detecting any resistant heterozygotes significantly increases the probability of detecting resistance while it is still rare (*7*).

Discussion

Larval growth inhibition assays are considerably more sensitive than corresponding dose-mortality assays for detecting incipient changes in *H. virescens* and *H. zea*

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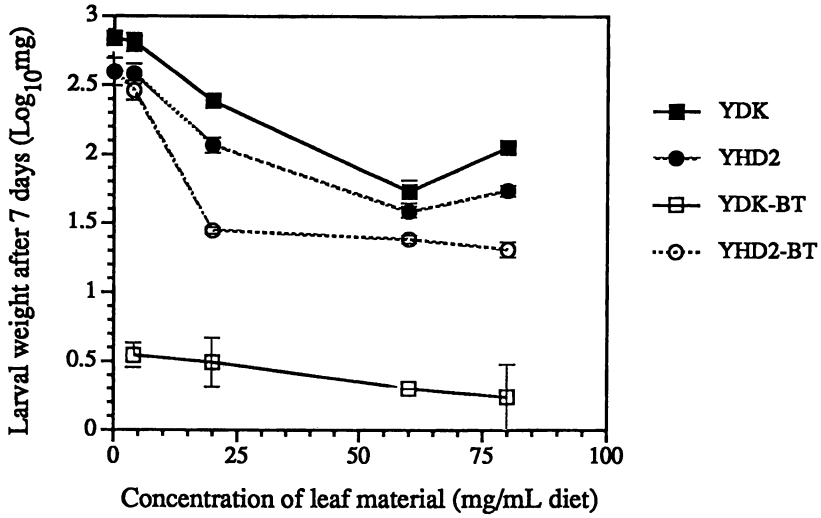

Figure 6. Effect of CryIA(c) protein, in transgenic cotton leaf tissue, on weight gain (± 1 SEM) of susceptible (YDK) and resistant (YHD2) *H. virescens* larvae.

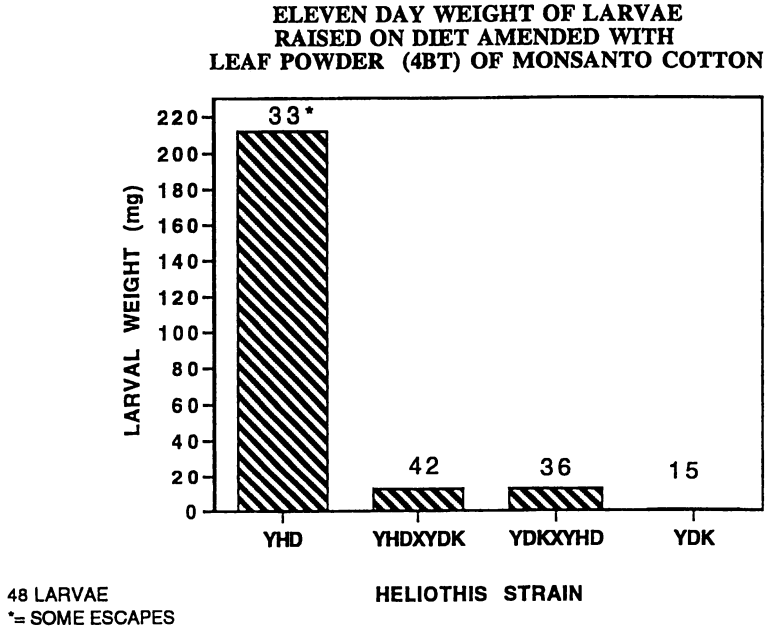

Figure 7. Growth response of CryIA(c) resistant, susceptible and reciprocal F1 hybrid *H. virescens* larvae to a discriminating dose (4 mg dry transgenic cotton tissue per mL diet) of CryIA(c) protein.

susceptibility to the Bt CryIA(c) protein. Since both assay types require ingestion of the insecticidal protein mixed into an insect diet matrix, little additional effort is required to set-up and score growth inhibition tests. Size differences between 1-2nd instar vs. 3rd instar larvae are usually obvious but chances for error in interpretation can be minimized by concurrently testing sample larvae on control diet to provide a direct size comparison. Diagnostic doses, used in combination with larval growth inhibition, are likely to be the most efficient means of tracking population susceptibility, especially when the assay can detect the decreased susceptibility present in resistant heterozygotes. Davidson (*27*) and Georghiou and Taylor (*28*) recognized the importance of diagnostic doses in testing for insecticide resistance and Roush and Miller (*7*) explored the genetic and logistical implications of using diagnostic doses. We estimated diagnostic doses for *H. virescens* and *H. zea* empirically, and suggest that the most practical approach for dose validation is to use individuals sampled from numerous populations within the geographic range of each species.

Dulmage and Martinez (29) were among the first to report that sublethal concentrations of Bt spore-crystal preparations in insect diet inhibit larval growth of *H. virescens*. More recently, Sims and Berberich (*19*) and J. Greenplate (Monsanto Co., unpublished data) demonstrated that extremely low concentrations (< 1 ng protein/mL of diet) of purified CryIA(b) and CryIA(c) proteins can be detected using a *H. virescens* larval growth inhibition assay. Gould et al. (*4*) described the use of a chronic exposure assay in which neonate *H. virescens* larvae were exposed to a sublethal concentration of CryIA(c) protein. This assay allowed differentiation of CryIA(c)-resistant and CryIA(c)-susceptible larvae based on larval weight. Growth inhibition assays are presently being evaluated for monitoring other cotton pest species such as the pink bollworm, *Pectinophora gossypiella* (Saunders) (Watson, T., University of Arizona, unpublished data), and might also be useful for monitoring Lepidoptera species with previously documented Bt resistance (*2*). For example, McGaughey and Beeman (*30*) suggested that the use of a mortality diagnostic dose for monitoring resistance in Indianmeal moth, *Plodia interpunctella* (Hübner), would not be effective until the resistance gene reached high levels. Accordingly, Halliday and Burnham (*31*) demonstrated that the greatest probability of identifying resistance in an Indianmeal moth population would occur with large sample sizes (~ 2000) and a high resistance gene frequency (0.05 to 0.10). Both the required sample size and minimum detectable gene frequency could be reduced by using a more sensitive assay technique coupled with a diagnostic dose. Another application of growth assays could be the analysis of allelic frequencies of resistance prior to field release of transgenic plants. One possible way to do this is to screen populations for individuals surviving to 3rd instar on an approximate EC_{99} concentration. Following transfer to fresh diet, completion of development, and adult mating, resulting progeny would be tested against an appropriate diagnostic dose for the presence of genetic factors having major effects on susceptibility. This approach, based on larval growth, might also be a more useful method for obtaining resistant insect strains compared to selection based on larval survival.

The highly vagile nature of adult *H. virescens* and *H. zea* makes it difficult to interpret estimates of interpopulation variation in Bt susceptibility (*32*). Interpopulation variation in susceptibility (*14*) may therefore reflect non-genetic

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variation or sampling error rather than genetically fixed geographic differences. For example, repeated bioassays determining LC_{50} and LC_{99} values for Bt proteins against larvae from single strains of the Colorado potato beetle and diamondback moth have demonstrated variability similar to that reported among geographic strains (*14,33*). The maximum LC_{50} and LC_{99} toxicity ratios (highest LC_{50} or LC_{99} divided by the lowest values) for Colorado potato beetle were 12.8 and >150 respectively. Comparable LC_{50} and LC_{99} toxicity ratios for diamondback moth were 3.7 and 10.2 respectively. More than 50% of the LC_{99} estimates differed significantly from the standard minimum value due to within-strain variability alone.

A practical, but often difficult, goal of resistance monitoring is to determine the relationship between laboratory-derived assay results and field control (*34-36*). Strains of insects selected, under laboratory conditions, for resistance to microbial or purified Bt protein preparations often remain susceptible to similar proteins when they are produced in transgenic plants. For example, a Colorado potato beetle (*Leptinotarsa decemlineata*) strain selected for > 60-fold resistance to microbial *B. thuringiensis tenebrionis* (*3*) could not survive as larvae nor reproduce as adults when fed on transgenic potato (*5*). Similarly, a strain of *H. virescens* highly resistant to purified CryIA(c) protein developed slowly on commercial transgenic cotton foliage (Gould, F., North Carolina State University, unpublished data). Conversely, field-selected, apparently homozygous Bt-resistant diamondback moths completed development on transgenic broccoli expressing CryIA(c) protein but F1 heterozygotes, produced by crossing the resistant line with a susceptible strain, did not complete development (*37,38*). Assays on existing laboratory-selected resistant strains might overestimate the potential field importance of these types of resistance. We conclude that it is critical to determine the relationship between resistance intensity and the ability of resistant phenotypes to develop on, and cause damage to, transgenic plant tissues. Without this information, the significance of various levels of resistance, as quantified on insect diet, will be unclear and it will be more difficult to suggest appropriate modifications, in response to resistance, to existing management programs.

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Brown; Molecular Genetics and Evolution of Pesticide Resistance
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.