Ten Years of Bt Resistance Monitoring in the European Corn Borer:

What We Know, What We Don't Know, and What We Can Do Better

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The European corn borer (ECB), *Ostrinia nubilalis* Hübner, is one of the most destructive pests of corn in the United States. It is a cosmopolitan species, originally distributed in Europe and from there introduced into America, where it has now spread to most of southern Canada and the United States east of the Rocky Mountains. Larval feeding on corn plants causes physiological disruption of plant growth and structural damage. Chemical pesticides are effective against ECB but generally result in poor control because of the narrow application windows on large plants and the tunneling behavior of the insect that provides refuge from pesticide exposure (Mason et al. 1996).

Transgenic corn plants that express proteins from *Bacillus thuringiensis* (Bt) are an integral component of maize production systems, providing highly effective in-plant protection from feeding damage by ECB. Transgenic corn hybrids expressing the Cry1Ab insecticidal protein from Bt to control ECB have been used commercially in North America since 1996, and hybrids expressing Cry1F Bt protein have been available since 2003. In 2005, Bt corn was planted on 35% of U.S. corn acreage, exceeding 50% in some states (e.g., Nebraska, South Dakota) with even higher levels of adoption in certain counties (USDA NASS 2005; the 2005 planting numbers include Bt corn for corn rootworm management and stacked Bt corn for corn rootworm and corn borer control).

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Although genetically altered plants producing their own protective insecticides provide an important new approach to insect control, the concern is that a large-scale introduction of insecticide-containing crops would rapidly lead to the development of resistance to Bt within pest populations (Andow and Hutchison 1998, ILSI 1998, Ferré and Van Rie 2002, Shelton et al. 2002, Glaser and Matten 2003). The effect of insects developing resistance to corn hybrids expressing a Bt protein would be the loss of an economical and environmentally safe management option for insect control.

Resistance Monitoring

The ability to effectively detect the evolution of insecticide resistance before a control failure is an integral component of resistance management strategies for transgenic plants that express Bt toxins and a regulatory requirement for registering Bt-expressing corn hybrids in the United States (EPA 1998, 2002; ILSI 1998). Monitoring focuses on key target pest species in which loss of sensitivity would significantly affect the utility of the technology. Resistance detection for conventional insecticides traditionally uses dose–response tests with 4–5 doses or concentrations of insecticide that produce 10–90% mortality. Resistance levels are then estimated by the ratio of the LD_{50} or LD_{90} of a suspected resistant strain divided by that of a susceptible strain. Such techniques are adequate for documenting resistance that has reached high levels, but are generally insensitive to small changes in resistance allele frequency, particularly

when resistance is first appearing (Halliday and Burnham 1990).

An alternative to traditional dose–mortality testing uses diagnostic or discriminating doses. These techniques are more efficient for detecting low frequencies of resistance because all individuals are tested at an appropriate dose, and none are wasted on lower and higher doses where percentage of mortality is uninformative (ffrench-Constant and Roush 1990). These tests also require fewer individuals and less time than complete dose–response tests, and can be used to bioassay many more populations (Halliday and Burnham 1990).

One limitation, however, is that individuals heterozygous for a recessive resistance allele have a susceptible phenotype (they will not survive the discriminating dosage), which potentially leads to lower detection sensitivity for resistance alleles. When allele frequencies are low (i.e., before selection pressure), resistance alleles are most frequently found as heterozygotes. Because recessive alleles in heterozyogtes will be missed by the diagnostic methods, reliable detection of allele frequencies $<10^{-2}$ is impractical.

Sensitivity of detection is better if resistance is not completely recessive because a dose that discriminates between RS and SS genotypes theoretically can be developed (e.g., Beeman 1983), but distinguishing among all three genotypes (SS, RS, RR) requires two discriminating doses. The resistance to Bt toxins that has been identified in ECB populations through laboratory selection (Huang et al. 1999, Alves et al. 2006, Pereira 2006) has not been completely recessive; this suggests that the diagnostic concentration assays may be a suitable tool for detecting Bt-resistant alleles in this species. Thorough characterization of resistance-associated traits will be necessary to estimate the sensitivity of diagnostic assays more accurately.

Other approaches to monitoring have been proposed that offer the potential for increased sensitivity. Molecular diagnostics derived from identifying specific resistance-conferring mutations have the advantage of being amenable to high throughput screening, but they are dependent on the identification and characterization of a resistant allele (e.g., Morin et al. 2004, Tabashnik et al. 2005). Moreover, identifying a particular resistant allele may represent only one of several possible resistance mechanisms, and molecular diagnostics that detect a single mechanism may be insensitive to resistance caused by another (unknown) mechanism.

Another approach, commonly referred to as the F_2 screen (Andow and Alstad 1998), has the advantage of potentially detecting recessive alleles for resistance in a heterozygous state. This methodology involves collecting large numbers of individuals from the field and establishing single-female family lines. The offspring of each collected female are inbred within family lines. The offspring of these matings (i.e., the F_2 of the collected generation) are then screened at a discriminating concentration for tolerance to the toxin. The inbreeding process allows potentially heterozygous offspring of the collected females to mate with each other, generating a significant and easily detectible fraction of homozygous resistant offspring. The frequency of the resistance allele in the sampled population can be estimated by back-calculation of the frequency of family lines containing a resistant allele.

The main limitation of this method is that it is labor intensive and the rearing requirements are expensive. Moreover, its sensitivity is limited by the number of sibling families that can be obtained from a single collection. To date, this method has only been used to estimate resistant allele frequencies in individual ECB populations (Andow et al. 2000, Bourget et al. 2003, Stodola and Andow 2004, Stodola et al. 2006); differences among geographically distinct populations or potential changes in frequency over time have not been assessed. This technique has been used to estimate an upper limit for Bt-resistant allele frequencies, which is important for assessing the utility of resistance management strategies.

Regardless of the method chosen for monitoring resistance, accurate and reliable bioassay methods are an essential component of resistance detection and characterization. Given our current understanding of the mechanisms and genetics of corn borer resistance to Bt toxins, all proposed methodologies have limitations. The most comprehensive and systematic data set has been generated by using a bioassay-based approach that combines diagnostic concentration bioassays with population response curves to assess Cry1Ab susceptibility among ECB populations.

In this article, we report results of this standardized bioassay-based approach to Bt resistance monitoring in ECB that has been used in the United States since the introduction of Cry1Ab-expressing corn hybrids in 1996. Standardized methodologies, historical trends in susceptibility, and limitations of the technique are discussed.

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Bioassay Methods and Baseline Susceptibility

As previously described, a major component of resistance management strategies involves resistance-monitoring programs that are capable of early detection of resistance and make it possible to implement appropriate mitigation decisions in a timely manner (Dennehy 1987). The first steps in implementing such programs include developing appropriate bioassay techniques and estimating baseline susceptibility to the Bt protein among populations across the geographic range of the target species. Baseline data for ECB susceptibility to the Cry1Ab toxin were generated in 1995 before commercial release of transgenic hybrids (Marçon et al. 1999).

The methodology used to assess susceptibility of ECB populations to Bt toxins was developed for initial baseline studies and has remained relatively constant since 1995. All bioassays are conducted by exposing neonates (< 24 h after hatching) to treated artificial diet. This treatment methodology involves application of Bt protein to single wells of artificial

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diet. The rearing diet developed for *Heliothis virescens* (F.) (King et al. 1985) and adapted for ECB (Marçon et al. 1999) has been used throughout the past 10 yr because it is relatively inexpensive and easy to prepare, and larval growth is comparable to growth on the standard rearing diet (BDS, unpublished). The response criteria used to assess mortality include severe growth inhibition and death. When mortality is recorded, larvae that have not grown beyond first instar and weigh ≤0.1 mg after 7 d of exposure are considered to be dead. In addition to recording mortality, larval mass is determined for each concentration to determine an EC_{50} (concentration that causes 50% growth inhibition) for each population.

The technique of surface-treating artificial diet has been criticized because of the potential for inconsistencies in the diet surface that might result in non-uniform treatment and inconsistent exposure of larvae. However, we believe that strict quality control through visual inspection of each diet well has minimized these potential inconsistencies. Side-by-side comparisons of surface treatment with uniform diet incorporation (Table 1) were conducted using a Cry1Ab-susceptible ECB colony that has been reared continuously in the laboratory for >70 generations in the absence of selection and a Cry1Ab-resistant colony selected by chronic exposure to Cry1Ab incorporated into rearing diet (Siqueira et al. 2004).

Results of these assays suggest that there are no major differences between these techniques in precision of lethal concentration estimates (i.e., similar 95% confidence intervals), in slopes and standard errors of the probit regressions, and in estimates of resistance ratios. Similar comparisons of the two methods with ECB populations from Germany indicated similar trends (Saeglitz et al. 2006). However, the surface treatment method requires ~10-fold less Bt protein to generate a response curve (Table 1). Given the costs associated with protein preparation, instability of Bt proteins, and limitations in the amount that can be produced, the advantages of this method outweigh the possible increased uniformity of exposure that may be associated with incorporating the protein in rearing diet.

Although considerable variation in response to Cry1Ab was detected during initial baseline studies (Marçon et al. 1999), these results indicated that such variation was not the result of previous selection because there was as much variation between generations of the same population as there was among populations. Intrapopulation variation in response to chemical and microbial insecticides is a common phenomenon when any bioassay is repeated (Robertson et al. 1995). Therefore, estimating the level of intra- and interpopulation variation in susceptibility that is naturally present is prerequisite to detecting biologically important changes.

Diagnostic Bioassays

To identify a diagnostic concentration for monitoring, baseline data obtained before commercial release were pooled to increase sample size and to increase the precision of the estimated diagnostic concentrations (Marçon et al. 2000). An overall LC_{99} was calculated from the baseline data and the upper end of the 95% confidence interval for this overall LC_{99} was chosen as a diagnostic concentration and validated with field populations. At this concentration, >1% survival is regarded as statistically (though not necessarily biologically) significant. This concentration has been used since 1996 in conjunction with complete dose–response assessments to assess susceptibility of ECB populations. Methods for diet treatment and exposure of neonate larvae have remained the same as described earlier. For each population established, the diagnostic assay is repeated three times with 112 insects per replicate on each of two dates.

Population Sampling

During the initial baseline assessment and in the subsequent 5 yr, the focus on sampling ECB was to obtain as many populations as possible from across the geographic distribution in the United States, without considering market penetration or sample size. In 2000, the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) was formed, and a meeting of academic, government, regulatory and corporate scientists convened to discuss alternatives to the former sampling strategy. The recommendations of this

Table 1. Comparison of bioassay results using exposure to Cry1Ab incorporated into artificial diet vs. surface treatment

Strain	*n*	Slope ± SE	LC_{50} (95% FL)[a]	χ^2 (df)	Resistance ratio[b] (95% CI)[b]	Toxin Used/ Bioassay (µg) [c]
			Incorporated diet			
Susceptible (S)	278	1.2 ± 0.2	30.8 (14.0 – 53.3)	2.7 (4)	—	53.4
Resistant (R)	325	1.7 ± 0.3	1353.4 (853.5 – 1913.9)	1.8 (4)	43.9 (20.8 – 92.7)	975
			Surface treatment			
Susceptible (S)	273	4.2 ± 0.7	0.4 (0.3 – 0.4)	1.0 (4)	—	0.44
Resistant (R)	299	1.9 ± 0.2	10.6 (6.5 – 17.0)	6.1 (4)	29.6 (21.1 – 41.4)	5.58

[a] LC50 in ng/ml for surface treatment and ng/cm2 for surface treatment (95% Fducial Limits)
[b] 95% confidence intervals calculated according to Robertson and Priesler (1995)
[c] Based on 3 replications with 7 concentrations/bioassays

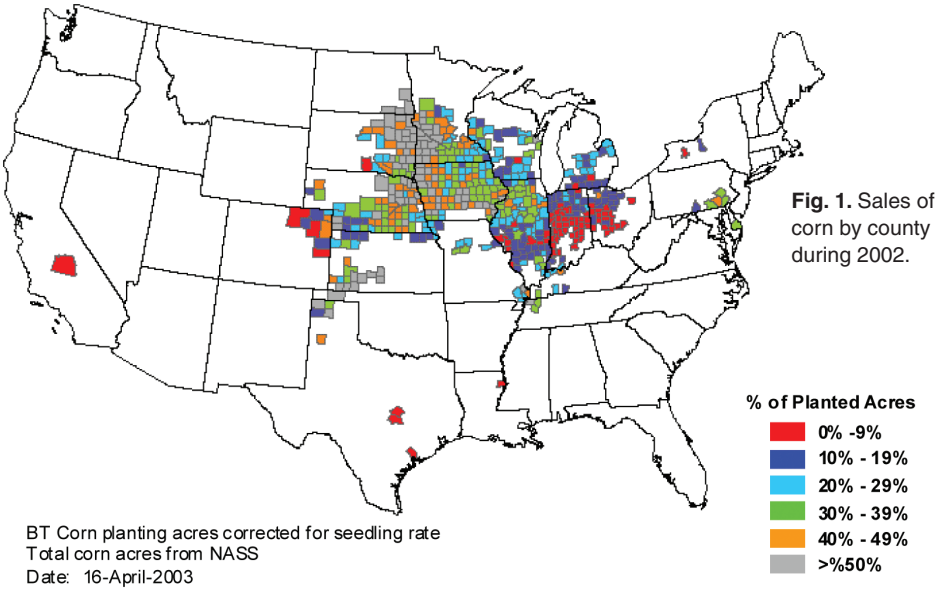

Fig. 1. Sales of Bt corn by county during 2002.

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group included focusing collections in regions where selection pressure was believed to be highest based on market penetration and insecticide application practices in non-Bt corn. Bt resistance is expected to evolve most rapidly in areas where Bt corn deployment is highest, and where non-Bt corn is treated with insecticides to manage target pests, thereby reducing the size of the nonselected population.

County Bt corn sales data from 1999 were compiled for the ABSTC by Fulfillment Systems (Monticello, MN) and used to create a Bt corn penetration map. The map was subsequently updated with 2002 sales data (Fig. 1). The map was used to determine three regions of monitoring focus for ECB, encompassing the areas of greatest adoption and/or insecticide use. The population monitoring program has focused on these regions since 2002. Currently, the regions are as follows: Region 1 (southwest Minnesota, eastern South Dakota, southeast North Dakota, eastern Nebraska, and northwest Iowa) contains counties with relatively high Bt corn penetration. Because the northern portion of this region may contain univoltine ECB populations, one ECB collection site targets those populations. Region 2 (southwest Kansas and the Texas–Oklahoma panhandle) contains counties with relatively high penetration of Bt corn and a history of insecticide use for corn borers. Region 3 (central to southeastern Iowa, north-central Illinois) includes counties with relatively high Bt corn penetration. Because of the location of the monitoring program in Nebraska, two additional sites for Nebraska have been included in the program since its inception.

The selection of sample sites within a region is driven by biological factors. The target pest population needs to be large enough to provide sufficient numbers of healthy individuals for collection. In addition, to ensure a representative sample of the local population, collections are made some distance (> ½ mile if possible) from the nearest Bt cornfield. Because of timing limitations and insect availability, we have tried to be flexible in the timing of collections and have used all stages of development.

To provide sufficient detection sensitivity, a goal of 200 larvae, 200 adults, 100 mated females, or 100 egg masses was established for each population. This provides at least 400 insect genomes per population (2/insect, or 4/mated female, or 4/egg mass). If collection of additional individuals is efficient (such as in adult traps), then the sensitivity of the testing is increased, so collection of larger samples is encouraged. Occasionally, small population sizes have limited the number of insects that can be collected, and a minimum population size of 50 larvae, 50 adults, 25 mated females, or 25 egg masses has been considered a valid sample for testing.

Ten-Year Summary

Over the past 10 yr, annual assessments of Cry1Ab susceptibility among geographically distinct ECB populations have involved diagnostic bioassays and concentration–response determinations. Comparing the concentration–response assays in 1995 with 2005 (Table 2), the results appear very consistent, although there was a general trend toward narrower 95% confidence intervals for LC_{50}s and EC_{50}s, and smaller standard errors for the slope of the response curve. These results suggest strongly that the precision of the estimates has improved, which reflects the consistency of techniques used over this 10-year period. Additionally, in all years that susceptibility determinations have taken place, there has been a consistent level of variation between the most susceptible and most tolerant populations based on LC_{50} or EC_{50} values (Fig. 2).

Table 2. Comparative responses of 16 field-collected populations of ECB to Cry1Ab protein.

Year	Number of populations	Mean LC_{50}[a] ± SE	Mean 95% FL[b] ± SE	Mean Slope ± SE
1995	16	4.49 ± 1.55	2.31 ± 1.11	2.11 ± 0.41
2005	16	2.20 ± 0.23	1.34 ± 0.19	2.29 ± 0.08

[a] ng/cm²
[b] Fiducial Limit

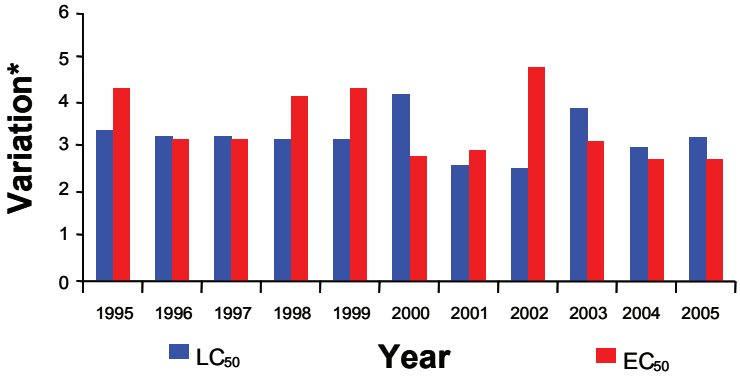

Fig. 2. Variability in response to Cry1Ab among ECB field populations from 1995 to 2005. *Variation expressed as the ratio of the highest to the lowest LC_{50} (Lethal Concentration that causes 50% mortality) and EC_{50} (Effective Concentration that causes 50% growth inhibition) for each year of monitoring.

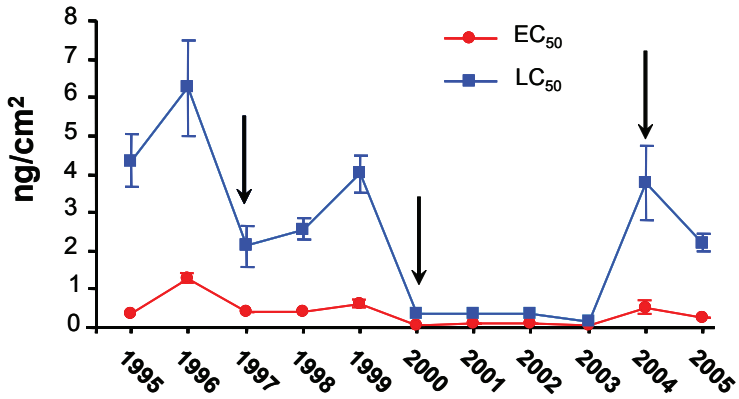

Fig. 3. Variation in susceptibility based on mortality (LC_{50}) and growth inhibition (EC_{50}) for European corn borer populations. Arrows indicate years in which a new source of toxin was used in the bioassays.

Although establishing response curves for each population every year is more costly and is limited in sensitivity, these determinations provide a measure of population variation that would not have been available from diagnostic concentrations alone. These response curves also provide a basis for assessing toxicity of different Cry1Ab preparations. The mean susceptibility of 10–15 geographically distinct populations has varied considerably between different batches and formulations of Cry1Ab, which is reflected in year-to-year variation in LC_{50} and EC_{50} estimates (Fig. 3). Differences in methods of purification, trypsin activation or formulation all seem to have had significant impacts on toxicity. These results emphasize the need for consistency in toxin preparation and methods for quantifying and standardizing concentrations of toxins.

Although determinations of complete concentration–response curves are time consuming and relatively insensitive to changes in resistance allele frequencies, they have provided the only means of identifying differences in the inherent toxicity of different Cry1Ab preparations. As a consequence of such variation, it has been necessary to validate new toxin preparations by conducting replicated side-by-side bioassays of different preparations against a standard susceptible laboratory colony. These comparisons have provided a means to maintain consistency in evaluation of ECB susceptibility and to standardize the Cry1Ab concentration used in diagnostic bioassays when new sources of protein are introduced.

Results of diagnostic bioassays have also been consistent from year to year. In the 10 yr that Cry1Ab susceptibility has been assessed and in more than 150 populations that have been assayed, only one population did not exhibit mortality >99% in the diagnostic bioassays. In 2001, a collection from Kandiyohi County, MN, exhibited significantly lower mortality at the diagnostic concentration. As a consequence, a set of additional tests was initiated to determine whether there was heritable resistance among survivors of the diagnostic concentration, quantify the magnitude of resistance, and to determine the level of survival on transgenic plants expressing Cry1Ab.

Results of these tests indicated that significant Cry1Ab resistance had been isolated from the Kandiyohi population. After pooling the survivors of the initial diagnostic bioassay and rearing for two generations, mortality was <10% at the diagnostic Cry1Ab concentration (Table 3). Additional bioassays were then conducted to measure survival on Cry1Ab-expressing leaf tissues and on whole plants. Increased survival was observed on 1-cm-diam leaf discs cut from whorl stage plants expressing Cry1Ab, although surviving larvae were significantly smaller than those developing on isoline plants (Fig. 4). Those individuals that survived for 4 d on leaf discs were again pooled and reared for three successive generations and tested on vegetative stage plants grown in the greenhouse. The results of these tests showed conclusively that, even though the colony exhibited high levels of resistance in diet bioassays and could feed on Cry1Ab-expressing leaf tissue, there was no evidence of feeding or survival on whole plants expressing Cry1Ab. Furthermore, in each year since 2001, additional collections obtained from the same area have not shown unusual survival at the diagnostic concentration.

These results illustrate the steps that are taken to confirm and characterize any Bt-resistant alleles that are detected and how we assess the implications for product performance. They demonstrate the sensitivity of the current monitoring efforts for identifying resistance in field populations; these methods clearly are capable of detecting even relatively low-level resistance alleles that exist at low frequencies.

Table 3. Results of diagnostic bioassays for populations from Kandiyohi County, MN

Population	Generation (Year)	*n*	Mortality % ± SE	*P*
Kandiyohi Co., MN	F_1 (2001)	896	98.4 ± 0.4	0.0637
S-Kandi [a]	F_3 (2001)	672	99.9 ± 0.1	0.9210
R-Kandi [b]	F_3 (2001)	644	8.2 ± 1.1	0.0001

[a] Derived from the original collection and maintained in the absence of selection.

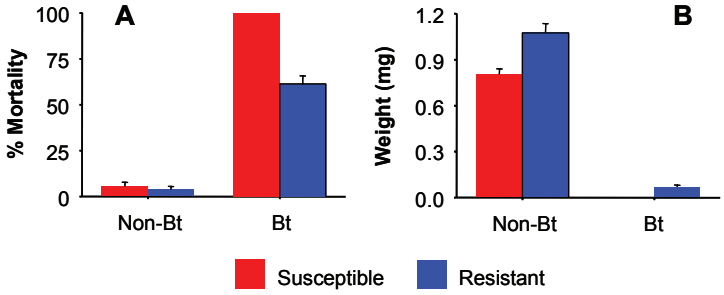

Fig. 4. Mean mortality (A) and larval weight (B) (±SE; $n = 128$) of neonate European corn borer larvae exposed to corn leaf disks from Cry1Ab expressing (Bt) and isoline (non-Bt) plants for 4 d.

Conclusions

Laboratory-based monitoring programs require consistent methodology over time (sampling strategy, laboratory methods, and toxin standardization) to produce a meaningful dataset and detect changes in susceptibility. Based on the combined results of concentration-mortality assays and diagnostic bioassays employing the Cry1Ab protein from Bt, we have not seen a detectable change in Cry1Ab susceptibility among ECB populations in the United States resulting from the introduction of transgenic corn. The methods used are sufficiently sensitive to detect a low frequency of insects with incomplete resistance, indicating that if resistance were evolving in the field, it is likely to have been detected. As we saw with the 2001 collection from Kandiyohi County, MN, the field relevance of any unusual findings in the program must be investigated in detail before the need for mitigation can be determined.

However, there are limitations with regard to sensitivity of current resistance detection tools, and it cannot be ruled out that the frequency of resistance alleles has increased in response to selection but remains below the limits of detection. In particular, the sensitivity of the methods to detect fully recessive resistant alleles is limited compared with other techniques. However, such resistance is expected to evolve only slowly (Gould 1998), and high detection sensitivity for recessive alleles in a monitoring program is not as important as it is for more dominant resistance alleles.

Laboratory-based testing is just one component in a comprehensive program of monitoring. The practicalities of a program based on field sampling limit the geography that can be investigated in any year. This program is more likely to detect changes in susceptibility occurring over a broad area, rather than localized hot spots. Therefore, the ABSTC Bt corn-monitoring program also involves investigating reports of unexpected damage to Bt cornfields to determine whether localized resistance may be a cause. If resistance is involved, actions can be taken to limit the survival of resistant insects and slow or prevent their spread.

To be useful, a resistance-monitoring program must be conducted within the context of the goal to maximize the effectiveness of the technology through time. The program presented here and the recognition of the potential limitations of this approach provide a foundation for continued monitoring and a model for developing similar programs elsewhere. Monitoring programs must embrace the need for efficiency and be linked to some action plan that realistically would be implemented to preserve the technology should resistance development be identified. Improved methods of detection that increase sensitivity should continually be explored and developed when appropriate. Regardless of the method chosen, accurate and consistent bioassay methods are imperative to resistance identification and quantification.

Acknowledgments

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