Industry Perspectives on Insect Resistance Monitoring for Transgenic Insect-Protected Crops

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Purpose of Insect Resistance Monitoring

Insect resistance management (IRM) is based on the premise that resistance will develop in an insect population with continuous use of any insecticidal product, including transgenic insect-protected crops such as *Bt* cotton or maize. For technology providers, resistance monitoring is a means of detecting when a decrease in susceptibility occurs in a target insect pest population to the insecticide or *Bt* protein of interest. Populations are monitored to measure changes in the frequency or level of resistance arising as a result of product use, with the primary goal being to detect resistance early enough to deploy mitigation measures that maintain future product value to customers and extend product life.

Properties of a Successful Resistance Monitoring Program

For greatest effectiveness in monitoring for insect resistance to transgenic crops, a monitoring program should meet each of the following criteria:

Relevant: The monitoring program should focus on changes in susceptibility that are biologically-relevant and have the potential to cause unacceptable economic damage in the field when using the product of interest. The change in susceptibility should be genetically based and cause increased fitness (survival to adult, reproduction) of the target pest on the transgenic crop. Interpretation of field relevance of resistance requires field or greenhouse studies demonstrating enhanced target pest survival on transgenic insecticidal plants and not be based solely on laboratory-based bioassays.

Sensitive: The monitoring program should be sufficiently sensitive to detect an increase in resistance frequency or resistance level prior to widespread field failure. Identifying such a change allows implementation of appropriate remedial actions as necessary to delay or ameliorate the effects of the resistance.

Stable: Resistance monitoring is a multi-year activity, potentially lasting for the life of the product, and is intended to identify susceptibility changes over time. Therefore, monitoring methods should remain as consistent as possible to allow for comparisons across years. Where changes are warranted or needed, such as in the source of the protein used in bioassays, studies should be conducted to bridge the new elements of the protocols to those being replaced. Bioassay results tend to be dependent on precise environmental conditions and should be conducted under highly controlled conditions to improve the likelihood of successfully identifying changes in insect populations. Routine testing of a susceptible laboratory colony is essential to differentiate variation resulting from methodology versus that resulting from pest resistance.

Scalable: Because resistance monitoring programs are likely to be extensive, involving multiple insect collections each year with increasing potential for field investigations as products mature, the collection, rearing and bioassay procedures should be amenable to relatively high throughput.

Transferable: A long-term and potentially large-scale monitoring program requires that the protocol be easily conducted by multiple laboratories, either simultaneously or sequentially. Differences among laboratories may impact bioassay results and affect overall conclusions.

Validated: Protocols to be used for large-scale monitoring programs should be validated for their ability to detect field-relevant resistance at an appropriate frequency. Insect colonies that have been artificially selected for field-relevant resistance can be useful in verifying the suitability of an assay.

Cost-effective: Resistance monitoring programs should not be so expensive and time consuming that they cannot reasonably be implemented.

What to Monitor

Monitoring should focus on those target pest populations that are at greatest risk for resistance development and for which the potential economic consequences of resistance are greatest. The pest(s) of interest should be “primary” in that they dominate a geographic cropping system and typically cause economic levels of crop damage under average population levels. Primary insect pests are typically those pests for which an insect resistance management plan has been designed. Secondary insect pests are more isolated in geographic impact and do not cause economic damage under average population levels. Only those primary insect pests that are normally controlled by the transgenic insect-protected crop should be candidate species for monitoring. If a transgenic insect-protected crop provides substantial value to growers and the development of resistance in a primary pest population would cause significant economic consequences, then that pest should be monitored for resistance to the product.

An additional consideration is that not all species are amenable to laboratory testing within the monitoring program. For some key primary pest or secondary pest species, there are not reliable collection, rearing and maintenance protocols. For such species, monitoring may consist only of field observations and potentially short-term bioassays of individuals collected directly from the field.

Where to Monitor

Resistance monitoring efforts should be focused on areas where the probability of resistance emerging with the use of a particular transgenic insect-protection trait is greatest. Historical literature and databases, together with entomological expertise at universities, private, and/or governmental organizations, can assist with this effort. Additionally, predictive modeling can be employed to help identify regions of greatest resistance risk, given what is known or can be assumed about the pest biology, pest x crop interactions, product dose, pest resistance genetics, and expected grower adoption of resistance management practices. In many cases, resistance monitoring regions for similar products can be developed collaboratively across the industry allowing coordination of resistance monitoring efforts.

The locations where monitoring should be targeted can change over time with adoption of the technology and other changes in agronomic practices.

Monitoring Approaches

a) Population screening

Insect resistance evolution in the field can vary depending on several factors including: genetics of resistance, insect biology (movement and behavior), and selection pressure based on product use. It may develop from one central focus or point source and expand over time or develop more broadly across a population through a more gradual increase in resistant allele frequency.

Population screening, whereby insect collections are made each year at locations that are intended to be representative of the population in the area, is most relevant for detecting a broad increase in resistant allele frequency. This approach is most suitable for species that are highly dispersive as adults and for which shifts in susceptibility are likely to occur over wide areas. For less mobile species in which resistance is expected to be more localized, programs that involve random collections are unlikely to include locations where resistance is developing.

The goal of population screening is to detect decreases in susceptibility to the insecticidal trait or increases in resistance allele frequency before major changes in product field performance occur. Ideally, detection occurs early enough in the evolution of resistance that case-specific remediation plans can be designed and implemented based on the characteristics of the identified resistance.

Screening consists of sampling insect populations from areas where the resistance risk or level of concern is greatest, such as areas of high adoption of the transgenic crop and high pest abundance. Each collection should be large enough to represent the general population and provide a reasonable opportunity to detect resistance alleles that may be present before they become common. The number of populations sampled should capture the natural variation prevalent within a targeted region.

Because the intention of the screening approach is to characterize the susceptibility of the larger insect population, it is important that the insects collected are representative of that population. For traits that routinely allow some level of survival of susceptible insects, collections of larvae, pupae or emergent adults should be made at some distance from a field

that contains the insect protection trait of interest (e.g. in a non-traited field or in an alternative host crop). Collections of eggs or ovipositing adults (i.e. stages that are not under active selection) can be made from traited or untraited host crops. The insect-protected crop in a field is expected to disproportionately remove insects that are most sensitive to the trait, raising both the mean fitness of the remaining insects and the frequency of resistant individuals above those of the larger population they are intended to represent. Therefore larvae, pupae and emergent adults in an insect-protected crop field are not representative of the larger population that is intended to be characterized in random screening-based monitoring.

b) In-field monitoring

In-field monitoring of the performance of the insect protection trait is complementary to, and in some cases more useful than, random screening. This is generally accomplished by the growers of the crops themselves who have an interest in tracking the performance of the traits and work with their technology provider if the performance of the insect control trait does not meet their expectations. Growers should be instructed to report unusual target pest survival to their technology provider to provide a broad understanding of product performance and allow early detection of changes in performance that may be related to resistance. This can also be accomplished through planting of sentinel plots by the technology provider or through networks of Extension agents, crop consultants, and public sector researchers. In all cases, crops are observed for evidence of unexpected damage based on prior experience with the crop as well as knowledge of the local pest population pressure.

When using this approach, careful consideration needs to be taken of the expected performance of the insect protection trait. For “high dose” products that rarely if ever support target pest survival to the adult stage or sustain observable target pest damage (for example, current *Bt* corn products targeting European corn borer), any indication of target pest survival or damage warrants investigation for potential resistance. For “less than high dose” products that routinely allow a small portion of the target pests to survive and cause plant damage (for example, current *Bt* corn products targeting corn rootworm), the level of survival and damage observed needs to be contextualized with the local pest pressure and environmental conditions before resistance is suspected as contributing to the observed damage.

If unexpected damage is observed that is not commensurate with pest pressure and environmental conditions, the local target pest population should be sampled. (For pests where there is not a clear threshold of unexpected damage, the decision to sample should err on the side of caution, particularly in the early years of commercialization of the insect protection trait, so that suspected incidents of resistance are not missed.) At a minimum, pest sampling should occur in the damaged field so that the survivors in the field can be studied in the laboratory. Additional samples from nearby non-protected crops can provide information about the susceptibility of the broader population in the area. For traits that routinely allow target pest survival, additional samples from fields that contain the same trait but that do not show unexpected damage can provide a useful comparator to understand the normal shift in response of a susceptible population to the trait.

Characterization of Field Collections

The insect collections made in a screening-based monitoring program or in response to unexpected damage should be characterized for potential resistance. Different approaches

can be used to accomplish this taking into account the properties of the trait (e.g. high dose or less than high dose), the availability of suitable artificial diets that allow feeding over a time period that is relevant to field exposure duration, the availability of purified insecticidal protein, the expected mechanism of resistance (e.g. physiological or behavioral), the suitability of on-plant bioassays, and whether known resistance alleles have been identified. Bioassays can be devised (a) to measure the mean fitness of the insects in the presence of the insecticidal agent, (b) to estimate the frequency of resistant individuals in the collection (phenotypic), or (c) to estimate the frequency of resistance alleles in the collection. In all cases, the bioassays need to be capable of determining a statistically and biologically significant change in a collection compared with baseline collections, contemporaneous field collections, and/or relevant laboratory susceptible colonies.

Concentration-response bioassays can be used to estimate population fitness measures, such as the LC_{50} (concentration required to cause 50% mortality during a fixed exposure duration), GI_{50} (concentration required to cause 50% growth inhibition during a fixed exposure duration), or MIC_{50} (concentration required to inhibit molting to a specific instar of 50% of the larvae during a fixed exposure duration). The concentration-response endpoint used should be related to the effect of the transgenic crop on the insects in the field. For example, mortality may be most appropriate for species that are highly sensitive to the trait, while sublethal endpoints may be most appropriate for species that can feed and survive on the crop for the duration that the bioassay runs.

Discriminating or diagnostic concentration bioassays can be used to estimate the frequency of putative resistant individuals by exposing larvae to a single concentration of the insecticidal agent that is known to cause a consistent response in susceptible individuals. Such a concentration could be the upper 95% confidence limit of the LC_{99} or MIC_{99} based on baseline collections. In these cases, survival or molting of greater than 1% of the tested insects indicates the potential presence of resistant individuals. For highly susceptible pests, use of a second, 10-fold higher, discriminating concentration provides valuable insight regarding the intensity of resistance, and thus the potential to confer ability to survive on Bt plants. Again the endpoint (mortality or sublethal effect) needs to be relevant to the effects of the trait in the field over the duration of the bioassay.

A discriminating or diagnostic concentration can also be useful for estimating the frequency of recessive resistance alleles using the F2 screen approach or, if a field-relevant homozygous resistant colony is available to cross with the field collections, the F1 screen approach.
Plant-based bioassays provide relevant exposure to a plant-produced insecticidal agent and therefore can be useful in understanding field-relevant performance of insect collections. As with diet bioassays, plant-based bioassays can measure the mean fitness of an insect collection on the plant material. In situations where insects normally show a very uniform response, such as mortality within a fixed time, these bioassays can be used to estimate the frequency of individuals that do not show the normal response. When a reliable artificial diet is not available, or if there is a focus on behavioral rather than physiological mechanisms of resistance, a plant-based bioassay may be used as the primary resistance monitoring bioassay. As with diet based bioassays, plant-based bioassays can measure sublethal effects of a less-than-high-dose trait.

Plant-based bioassays can also characterize the potential effects of any putative resistance using measures of the amount of plant material (e.g. leaf area) consumed. Plant-based bioassays can use excised plant material (replaced with fresh material at regular intervals) or whole plants. Because these assays are often conducted with greenhouse- or growth chamber-grown plants, it is important that the concentration of the insecticidal protein in the plant material is measured as part of the bioassay protocol to ensure that it is representative of field-grown plants.

Plant-based bioassays, particularly whole-plant bioassays, provide the opportunity to investigate whether putative resistant insects are able to complete their development on the plant, which will determine whether a putative resistance allele can persist and spread through a population. Inability to complete development is an indication that the putative resistance is not field-relevant.

Moving from diet to plant tissue to whole plant increases field-relevance. However, this progression also may increase complexity, variability and labor costs. With the need for resistance monitoring programs to be reliable, scalable, and cost-effective, it is common for these programs to operate in a tiered manner, whereby unusual findings in diet bioassays are investigated for potential field relevance using plant tissue and/or whole plants.

In addition to bioassays, molecular screening tools can also be used if field-relevant resistance alleles have already been characterized. Molecular methods permit field-collected insects to be preserved and tested, obviate complexities of rearing pests, and greatly increase the efficiency of detecting specific resistance-conferring genetic mutations. This latter benefit is also a major limitation; sole reliance on molecular monitoring may result in not detecting resistance conferred by other genes. Thus, molecular monitoring methods typically must be conducted in parallel with bioassay-based monitoring efforts.

Interpretation of Bioassay Data for Resistance Determination

Resistance that is relevant to continued viability of a transgenic insecticidal trait ("field-relevant resistance") can be defined as a genetically heritable change in a target pest population that arises from exposure of the population to the trait in the field and reduces the sensitivity of the population to the trait. Field-relevant resistance reduces or has the potential to reduce the ability of the trait to provide protection of the crop.

Because a central component of the resistance definition is a change in sensitivity, it is important that the sensitivity of a field collection is compared with the sensitivity of previous collections. Previous collections can be from baseline studies conducted prior to or in the early years of commercialization of the transgenic crop, or from susceptible colonies maintained in the laboratory. Laboratory colonies that are both uniform and consistent over years can be powerful as a negative control for resistance monitoring bioassays to demonstrate equivalency of methodology when bioassays results are being contrasted through time. However, careful consideration needs to be applied to laboratory colonies when used as comparators. Colonies that have been maintained in a laboratory for many generations adapt to the benign conditions of laboratory rearing and may lose their ability to combat natural stressors, with a frequent outcome being that they are more susceptible to insecticidal proteins than field populations. Therefore, to be useful as comparators, the relative sensitivity of laboratory colonies and susceptible field populations needs to be

established. Laboratory colonies that are most similar to field populations are most appropriate as comparators for resistance monitoring bioassays. For laboratory colonies that are significantly more sensitive than field populations to be useful, a conversion factor, established using their relative sensitivity, may be employed when comparing fitness parameters.

Further information can be derived from comparing the sensitivity of a collection from contemporaneous field collections; the sensitivity of a putatively resistant population can be compared with collections from areas where field performance problems were not reported.

When insect collections are made during a field investigation of unexpected damage, interpretation of the bioassay data must acknowledge that the collected insects are those that survived on the transgenic crop and therefore do not represent the full range of sensitivity of the general population from which they originated. Products that routinely allow target pest survival are expected to disproportionately remove insects that are most sensitive to the insecticidal trait, raising both the mean fitness and the frequency of resistant individuals in the remaining insects that are subsequently sampled in the damaged field. The bioassay results apply to the collected insects and the insects that survived in the transgenic crop field, but are not applicable to the general population. Additional field collections of insects that are not under active selection are needed to characterize the general population. These collections can be made, for example, in fields without the insect protection trait or using traps placed at some distance from insect protected fields that attract adults from a large area. As noted above, the bioassay results can also be compared with results for collections from fields that contain the transgenic crop but that do not show unexpected damage under similar pest pressure and environmental conditions. It is unsound to extend to the general pest population conclusions based solely on insects collected from unexpectedly damaged fields.

The field-relevance of an unusual response in laboratory bioassays needs to be established before resistance can be confirmed. It is important that the finding be repeatable and that the response is heritable. Particularly when the insect collection was part of a population screening program, it is important to investigate whether the reduced sensitivity is sufficient to allow the insect to feed and survive on the transgenic crop at a higher rate than is normal for susceptible insects.

Commitment to Sustainability and Transparency

The impact that any given resistance will have on field performance of a transgenic insect-protected crop cannot be predicted reliably in advance of emergence of field-relevant resistance. The dynamics of resistance increase in the field level is impacted by many variables; for example, refuges, natural enemies, and fitness costs can delay resistance. Thus, we seek a fine balance in interpreting laboratory-based results prior to emergence of field problems and contextualization with greenhouse- and field-based observations. To achieve this balance requires communication between the technology providers and relevant pest management experts in the research, extension and regulatory sectors. Central to this is timely, transparent exchange of resistance monitoring results from all these sectors.

Resistance is a natural expectation stemming from the societal need to control key crop pests. The operational paradigm for successfully delaying resistance is multi-dimensional. It strives for deployment of effective agronomic and integrated pest management practices,

development of products with multiple insecticidal traits (pyramids) against target pests, and the presence of adequate refuges. IRM programs deploy these components and resistance monitoring informs us of when they are achieving their desired outcomes and when they have not. In the latter case, resistance should be confirmed with additional field collections. In some cases, however, resistance confirmation can take additional cropping seasons and it may be appropriate to begin remediation programs while the confirmation steps are continuing.

Appendix: Glossary of Terms Used in this Paper

There is confusion and inconsistency in the public literature in how many key terms are used. For clarity, we define several key terms as used in the present paper.

Resistance: A genetically heritable change in a target pest ***population*** that arises from exposure of the population to the transgenic insect protection trait in the field and reduces the sensitivity of the population to the trait.

Field-relevant resistance: ***Resistance*** that increases the fitness (survival and reproduction) of the insect population when developing on the transgenic insect protected crop. Field-relevant resistance reduces or has the potential to reduce the ability of the trait to provide protection of the crop.

Population (a.k.a. **general population, larger population**): A group of actually or potentially interbreeding organisms that are present in the same geographic area at the same time (this is the ecological definition of a population). A population therefore extends across multiple fields or counties depending on the biology, particularly dispersal behavior, of the pest species.

Collection: The insects that are sampled from a population as part of a resistance monitoring program. A collection is representative of the group of insects present at the location of the collection. If the insects are not under active selection, their susceptibility is representative of the general population.