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EPA REFUGE REQUIREMENTS FOR BOLLGARD™ COTTON AND THE ROLE OF MODELING AND RESISTANCE MONITORING

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Abstract

The United States Environmental Protection Agency (EPA) has required an unprecedented insect resistance management (IRM) program as part of the Bollgard™ cotton (Cry1Ac plant-incorporated protectant (PIP) expressed in cotton) registration since it was first registered in 1995. New IRM requirements were mandated as terms and conditions of the September 29, 2001 registration decision following an extensive reevaluation of the risks and benefits of Bollgard™ cotton. IRM models are an important tool in comparing resistance management options and helping the Agency set IRM requirements. Resistance monitoring is needed to determine whether insect susceptibility has changed and whether the IRM plan should be altered. The current registration for Bollgard™ cotton expires September 30, 2006 except for the external, unsprayed structured refuge option which will expire September 30, 2004.

Introduction

Insects, fungi, and weeds developing resistance to pesticides are well documented in agriculture. As resistance begins to develop, more pesticide is needed to achieve control until total failure of that pesticide occurs. Integrated Pest Management or IPM grew out of insect resistance to insecticides and pesticide resistance management remains a common component of IPM programs today. Monitoring for the increased pesticide tolerance of the pest is a valuable asset in an IPM program, but it is frankly rarely done in a proactive fashion.

Insect resistance management (IRM) is the term used to describe practices aimed at reducing the potential for insect pests to become resistant to a pesticide. *Bt* IRM is important because insect resistance poses a threat to future use of microbial *Bt* pesticides and *Bt* technology as a whole. Academic scientists, public interest groups, organic and other farmers have expressed concern that the widespread planting of these genetically transformed plants will hasten the development of resistance to pesticidal *Bt* endotoxins. Effective insect resistance management can reduce the risk of resistance development.

Having an IRM plan is not specifically required under the United States (US) pesticide law or regulations. Rather under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), the United States Environmental Protection Agency (EPA) is mandated to ensure that will be no unreasonable adverse effects from the use of a pesticide when economic factors are taken into account. In this specific case, EPA has stated that we are working to prevent adverse effects that could happen if *Bt* could not be used and more toxic compounds would be used to control the insect pests.

The Agency has also addressed resistance management for synthetic pesticides and microbial pesticides. In June 2001, the Agency published a final policy notice regarding labeling statements for most pesticides as part of a project under the North American Free Trade Agreement (NAFTA) Technical Working Group on Pesticides (USEPA, 2001a). This notice provides a numerical system to identify the mode of action of the pesticide and label statements to encourage users to rotate between pesticides with different modes of action.

The *Bacillus thuringiensis* subsp. *kurstaki* Cry1Ac delta-endotoxin insect control protein (and the necessary genetic material for its production) was registered for full commercial use in Bollgard™ cotton in October 1995 (USEPA, 2000). The Cry1Ac delta-endotoxin is toxic to certain lepidopteran insect pests, in particular, *Helicoverpa zea* (Boddie) (cotton bollworm, CBW), *Heliothis virescens* (Fabricius) (tobacco budworm, TBW), and *Gossypiella pectinophora* (Saunders) (pink bollworm, PBW). In 1995, the Agency determined that the unrestricted use of Cry1Ac as expressed in cotton (Bollgard™ cotton) is likely to lead to the emergence of resistance in one or more of the target insect pests unless measures are used to delay or halt the development of resistant insects. Because some cotton pests also attack other crops, not only would the emergence of resistance affect the benefits of Cry1Ac cotton, such insect resistance could also affect the efficacy of *Bt* corn products and microbial formulations of *Bt*. The loss of *Bt* as an effective pest management tool – in cotton or other crops – could potentially have serious adverse consequences for the environment to the extent that growers would shift to the use of more toxic pesticides and a valuable tool for organic farmers would be lost. The emergence of resistance in cotton pests could also have significant economic consequences for cotton growers. Therefore, EPA required Monsanto (the registrant) to implement an IRM program to mitigate

the possibility that pest resistance will occur. This IRM program for Bollgard™ cotton is unprecedented, detailed, and proactive as compared to any other registered synthetic pesticide used on cotton or any other crop.

At a Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) meeting in March of 1995, EPA laid out a multi-faceted program we considered appropriate for *Bt* crop products (SAP, 1995). The elements are: knowledge of pest biology and ecology, dose (level of toxin expressed in the *Bt* crop), refuge design and deployment (non-*Bt* plants producing *Bt*-susceptible insects), cross-resistance between different *Bt* proteins, effective field monitoring for insect resistance, remedial action if resistance occurs, integrated pest management, development of alternate modes of action, and grower education. Repeatedly, the Science Advisory Panel has agreed with EPA that an appropriate resistance management strategy is necessary to mitigate the development of insect resistance to *Bt* proteins expressed in transgenic crop plants.

Resistance management programs for *Bt* plant-incorporated protectants (PIPs) are based on the use of both a high *Bt* toxin concentration coupled to the use of structured refuges planted nearby to provide sufficient numbers of susceptible adult insects. The "high dose/ structured refuge strategy" assumes that resistance to *Bt* is recessive and is conferred by a single locus with two alleles resulting in three genotypes: susceptible homozygotes (SS), heterozygotes (RS), and resistant homozygotes (RR). It also assumes that there will be a low initial resistance allele frequency and that there will be extensive random mating between resistant and susceptible adults. Under ideal circumstances, only rare RR individuals will survive a high dose produced by the *Bt* crop. Both SS and RS individuals will be susceptible to the *Bt* toxin. A structured refuge sets aside some percentage of the crop land for non-*Bt* varieties of that crop. The refuge provides for the production of susceptible (SS) insects that may randomly mate with rare resistant (RR) insects surviving the *Bt* crop to produce susceptible RS heterozygotes that will be killed by the *Bt* crop. This will remove resistant (R) alleles from the insect populations and delay the evolution of resistance. The 1998 SAP defined a high dose as 25 times the toxin concentration needed to kill susceptible larvae (SAP, 1998). This standard has been used by EPA since 1998. The Scientific Advisory Panels held in 1998 and 2000 noted that insect resistance management strategies should also be sustainable and to the extent possible, strongly consider grower acceptance and logistical feasibility (SAP, 1998, 2001).

EPA has just completed an extensive reevaluation of the *Bt* crop products including Bollgard™ cotton including IRM (USEPA, 2001b). The EPA IRM requirements including the refuge requirements and the role of modeling and resistance monitoring are discussed below.

Discussion

Scientific Basis for IRM Program

As an IRM program is developed and implemented, each pest's unique biology must be factored into the plan. For example, how far do the larvae move within the field and how far do the adults move affects the distance between the refuge and the *Bt* crop. The susceptible insects from the non-*Bt* refuge need to be in close enough proximity to randomly mate with the resistant insects that emerge from the *Bt* fields to produce heterozygous offspring that are fully susceptible to the *Bt* protein. Additional important questions that need to be addressed are how many generations of insects are produced each year, what is the mating behavior and the oviposition behavior, what is the host range of the insect, population dynamics, pest ecology, and if possible, the genetics and mechanism of resistance and the frequency of resistance alleles in the insect population. In addition, how the crop is grown including other pest management practices, when it matures, the extent of the acreage, and the overlap in distribution with other *Bt* crops are all important in the development of an appropriate program.

IRM Program Elements

The Agency has mandated seven basic IRM requirements for *Bt* (Bollgard™) cotton in its recent *Bt* PIPs reassessment document (USEPA, 2001b). These are as follows:

1. "Requirements relating to creation of a non-*Bt* cotton refuge in conjunction with the planting of any acreage of *Bt* cotton;
2. Requirements for the registrant to prepare and require *B*t cotton users to sign "grower agreements" which impose binding contractual obligations on the grower to comply with the refuge requirements;
3. Requirements for the registrant to develop, implement, and report to EPA on programs to educate growers about IRM requirements;
4. Requirements for the registrant to develop, implement, and report to EPA on programs to evaluate and promote growers' compliance with IRM requirements;

5. Requirements for the registrant to develop, implement, and report to EPA on programs to evaluate whether there are statistically significant and biologically relevant changes in susceptibility to Cry1Ac protein in the target insects;
6. Requirements for the registrant to develop, and if triggered, to implement a "remedial action plan" which would contain measures the registrant would take in the event that any insect resistance was detected as well as to report on activity under the plan to EPA;
7. Submit annual reports on or before January 31st each year."

Specific Refuge Requirements

There are three structured refuge requirements: 5% external, unsprayed structured refuge, 5% embedded refuge, and 20% external, sprayed structured refuge. There is an additional requirement for a 2002 community refuge pilot that allows multiple growers to work together to meet the refuge requirements. A 5% unsprayed structured refuge and/or a 20% external, sprayed structured refuge may be used in the community refuge program. In 2001, there were 144 community refuges (consisting of 166,000 Acres of Bollgard™ cotton). The primary refuge option used was the 20% external, sprayed structured refuge. Arkansas and Mississippi had the most community refuges. The refuge requirements specified as part of the terms and conditions are stated below (USEPA, 2001b).

"All growers of *Bt* cotton must employ one of the following structured refuge options:

1. External, Unsprayed Refuge. Ensure that at least 5 acres of non-*Bt* cotton (refuge cotton) is planted for every 95 acres of *Bt* cotton. The size of the refuge must be at least 150 feet wide, but preferably 300 feet wide. This refuge may not be treated with sterile insects, pheromones, or any insecticide (except listed below) labeled for the control of tobacco budworm, cotton bollworm, or pink bollworm. The refuge may be treated with acephate or methyl parathion at rates which will not control tobacco budworm or the cotton bollworm (equal to or less than 0.5 lbs active ingredient per acre). The variety of cotton planted in the refuge must be comparable to *Bt* cotton, especially in the maturity date, and the refuge must be managed (e.g., planting time, use of fertilizer, weed control, irrigation, termination, and management of other pests) similarly to *Bt* cotton. Ensure that a non-*Bt* cotton refuge is maintained within at least ½ linear mile (preferably adjacent to or within 1/4 mile or closer) from the *Bt* cotton fields. This option expires after the 2004 growing season unless extended by amendment as described below. EPA intends to review the data specified in the data requirements concerning alternate hosts and chemical insecticide sprays applied to *Bt* cotton, and decide in 2004 whether the new data support continuation of an external, unsprayed refuge as part of a larger requirement that would also likely involve alternative host plants. If these data support the continued availability of the external, unsprayed refuge option, EPA may approve an amendment to this registration to maintain the availability of this option.
2. External Sprayed Refuge. Ensure that at least 20 acres of non-*Bt* cotton are planted as a refuge for every 80 acres of *Bt* cotton (total of 100A) . The variety of cotton planted in the refuge must be comparable to *Bt* cotton, especially in the maturity date, and the refuge must be managed (e.g., planting time, use of fertilizer, weed control, irrigation, termination, and management of other pests) similarly to *Bt* cotton. The non-*Bt* cotton may be treated with sterile insects, insecticides (excluding foliar *Btk* products), or pheromones labeled for control of the tobacco budworm, cotton bollworm, or pink bollworm. Ensure that a non-*Bt* refuge is maintained within at least 1 linear mile (preferably within ½ mile or closer) from the *Bt* cotton fields.
3. Embedded Refuge. Plant at least 5 acres of non-*Bt* cotton (refuge cotton) for every 95 acres of *Bt* cotton. The refuge cotton must be embedded as a contiguous block within the Bt cotton field, but not at one edge of the field (i.e., refuge block(s) surrounded by *Bt* cotton). For very large fields, multiple blocks across the field may be used. For small or irregularly shaped fields, neighboring fields farmed by the same grower can be grouped into blocks to represent a larger field unit, provided the block exists within one mile squared of the Bt cotton and the block is at least 150 feet wide, but preferably 300 feet wide. Within the larger field unit, one of the smaller fields planted to non-Bt cotton may be utilized as the embedded refuge. The variety of cotton planted in the refuge must be comparable to *Bt* cotton, especially in the maturity date, and the refuge must be managed (e.g., planting time, use of fertilizer, weed control, irrigation, and management of other pests) similarly to *Bt* cotton. This refuge may be treated with sterile insects, any insecticide (excluding foliar *Btk* products), or pheromones labeled for the control of tobacco budworm, cotton bollworm, or pink bollworm whenever the entire field is treated. The refuge may not

be treated independently of the surrounding *Bt* cotton field in which it is embedded (or fields within a field unit).

4. Embedded Refuge for Pink Bollworm Only. Plant the refuge cotton as at least one single non-*Bt* cotton row for every six to ten rows of *Bt* cotton. The refuge may be treated with sterile insects, any insecticide (excluding foliar *Btk* products), or pheromones labeled for the control of pink bollworm whenever the entire field is treated. The in-field refuge rows may not be treated independently of the surrounding *Bt* cotton field in which it is embedded. The refuge must be managed (fertilizer, weed control, etc.) identically to the *Bt* cotton. There is no field unit option.
5. Optional Community Refuge Pilot. This option allows multiple growers to manage refuge for external, unsprayed and external, sprayed refuge options or both. This option is not allowed for the embedded/in-field options. A community refuge program will be allowed as a continuing pilot for the 2002 growing season. EPA will evaluate the community refuge program following the 2002 growing season. The community refuge for insect resistance management must meet the requirements of either the 5% external unsprayed refuge and/or the 20% sprayed option, or an appropriate combination of the two options. The registrant must implement the 2002 community refuge pilot program as described in the Bollgard® Cotton 2002 Refuge Guide and perform the following actions. The community refuge pilot must consist of the following:

    - There will be a community refuge coordinator for each pilot site. Each community refuge coordinator must submit a signed community refuge form listing all of the participants at the pilot site to the registrant by May 31, 2002. The registrant must provide EPA with a copy of the signed form and the community refuge coordinator will maintain a copy of the field map (to scale) or suitable scalar representation of the community refuge for review by the registrant or EPA as part of the compliance program.
    - The registrant must conduct two phone audits of a statistically representative sample of community refuge coordinators from communities in all states participating in the community refuge. The first phone audit shall occur no later than June 30, 2002 and the second phone audit shall occur no later than November 30, 2002. EPA shall review the questions prior to each phone audit.
    - The community refuge program users must be included in telephone compliance survey and the on-farm visits to be conducted by the registrant under section 3.c. below.
    - The registrant must provide a written report to EPA at the end of the 2002 growing season on community refuge use and compliance (due by January 31, 2003).
    - The registrant must conduct a review of the community refuge program and submit that review to the Agency as to any proposed changes by January 31, 2003. An appropriate amendment for any proposed changes must be submitted to the Agency.

    At the request of the registrant and based on EPA's review of the results of the 2001 community refuge pilot program, the requirements for the 2002 pilot program may be modified."

The Role of Models in Making Refuge Requirements

Mathematical models to predict the potential for the development of resistance have helped EPA make decisions regarding the requirements for IRM for *Bt* crops. In general, the predictive models allow the EPA to compare the relative efficacy of different IRM strategies to mitigate the development of insect resistance. For example, these models allow for a qualitative comparison of different refuge sizes, the impact on efficacy of the refuge if chemical insecticides are used, and differences in having the refuge be within the field or external to the field.

EPA has used models developed to predict the estimated time that resistance would develop to compare IRM strategies for *Bt* crops. Because these predictive models cannot be validated without actual field resistance, they have limitations and the information gained from the use of such models can only be used as a part of the weight of evidence determination conducted EPA to assess the risks of resistance developing in target pest populations. Models are an important tool in determining appropriate *Bt* crop IRM strategies. The 2000 SAP recommended that the model design should be peer reviewed and parameters validated. The SAP also stated that in the absence of field resistance, models are the only scientifically rigorous way to integrate all of the biological information available. EPA agrees with the 2000 SAP. While the absolute number of years to resistance is not precisely determined from the models, the relative difference in effectiveness between refuge options can be determined.

Thus, the utility of the models is not that they make accurate quantitative predictions, rather, it is that they enable the Agency to make informed judgments of the potential effects of using various refuge options.

EPA has used at least five models in its comparative evaluation of refuge options for Bollgard™ (*Bt*) cotton (see Section IID in USEPA, 2001b). Each of these models has limitations based on the assumptions in the models. For example, the predictions generated by the models are very sensitive to assumptions about the genetics of resistance (gene frequency and functional dominance) about which little, if anything, is known. Each of these models has provided the Agency useful comparisons of refuge options. The Agency recognizes that the predicted years are not absolute, but provide a measure of the relative likely success of various refuge options (in terms of predicted years to resistance). EPA recognizes, however, there is uncertainty in the predicted outcomes of these models. The predictive reliability of the models increases as other factors such as level of *Bt* crop adoption, level of grower IRM compliance, fitness costs of resistance to the insect, presence and availability of alternate insect host plants, spatial components, stochasticity, and pest population dynamics are included. Such parameters, however, serve to increase the reliability of the predicted model results only to the extent that the inputs are verifiably validated.

Given the uncertainty of predictive models, EPA has asked for additional data to evaluate whether other factors, such as, alternate hosts, level of compliance, and level of adoption alter the predictions of the models. Until such time as these additional data become available, some of the models predictions may be overly conservative. However, given that EPA considers the development of resistance to be a significant adverse effect, the Agency believes it is prudent to err on the side of conservative regulatory practice.

Therefore, EPA believes that it is imprudent to allow the 5% external, unsprayed refuge option for more than a limited period of time because current data indicates that this option has a significantly greater likelihood of insect resistance than either of the other refuge options. The 2000 SAP stated that the external, unsprayed option poses the highest risk to resistance evolution especially for cotton bollworm. Therefore, the external, unsprayed option expires after three growing seasons (September 30, 2004) unless extended by amendment. During the next two years, Monsanto is required to develop considerable new data on alternative host plants as possible effective refuges. In addition, additional data are required to determine the effectiveness of supplemental insecticidal sprays on *Bt* cotton to control CBW. EPA intends to review the alternate host and supplemental effects data and decide in 2004 whether to the new data support continuation of the 5% external, unsprayed structured refuge option. EPA may approve an amendment to the Bollgard™ cotton registration to maintain the availability of this option.

The Role of Resistance Monitoring in IRM

Resistance monitoring has been part of the requirements of the *Bt* crop products registration from the beginning. The ambitious goal is to detect insect resistance before it occurs in the field or before it spreads and if possible, prevent the development of resistance by detecting increased pest susceptibility. EPA's required IRM program includes annual resistance monitoring for the important target pests. The effort has been evolving over the last six years. To be effective, the plan requires sensitive tools to be in place that can detect changes in resistance allele frequency to the particular *Bt* protein and to be able to differentiate between natural variation in the population and a trend indicates resistance is likely to happen soon or may have already happened. As one of our early steps in developing this program, EPA established a working definition for what is resistance versus what is natural tolerance variation and our analysis was reviewed by our Scientific Advisory Panel for confirmation. In addition, EPA, working with the pesticide companies, has established definitions for suspected verus confirmed resistance. An additional consideration is the time required to "confirm" resistance. EPA has also mandated that Monsanto must also follow up on grower, extension specialist or consultant reports of less than expected results or control failures (such as increases in damaged squares or bolls) for the target lepidopteran pests (TBW, CBW, PBW) as well as for cabbage looper, soybean looper, saltmarsh caterpillar, cotton leafperforator and European corn borer.

The basic resistance monitoring program is to gather the target insects in an adequate sample size from an appropriate number of locations and to test for susceptibility to the *Bt* protein. Samples can be collected from various live stages. Adults might be collected from light or pheromone traps and/or eggs masses might be collected either from *Bt* fields or other crop or non-crop areas. Depending on the life stage collected, the insects might have to be reared to a stage when they can be fed on the appropriate *Bt* protein to determine their level of susceptibility to the insect toxin (e.g., Cry1Ac).

Resistance monitoring is a difficult and imprecise task. The chances of finding a resistant larvae in a *Bt* crop depend on the level of pest pressure, the frequency of resistant individuals, the location and number of samples that are collected, and the sensitivity of the detection technique (Roush and Miller, 1986). Therefore, as the frequency of resistant individuals in the insect population increases or the number of collected samples increases, the likelihood of locating a resistant individual increases. The likelihood of resistance is dependent of the genetics and mechanism of resistance for a particular pest.

A resistance monitoring program is more important when the predicted time to resistance is small rather than when the models predict that resistance is expected to be delayed for a very long time. Based on predictive models, level of adoption, and compliance for European corn borer (*Ostrinia nubilalis* Hübner) resistance to *Bt* proteins expressed in field corn predict 75 years or more until resistance would develop, but for cotton bollworm, tobacco budworm, and pink bollworm, the predicted years to resistance to *Bt* proteins expressed in cotton is much shorter.

The resistance monitoring program needs to consider the pest biology and ecology, population dynamics, genetics of resistance, mechanism of resistance, sampling methodology, bioassay methodology, standardization procedures, detection technique and sensitivity, and the statistical analysis of the probability of detecting resistance. To determine if refuges or any other resistance management tactics are working, one must track the frequency of resistance in field populations. With typical bioassays used for resistance monitoring, resistance cannot be detected readily, especially when resistance is recessive (as often is the case) and resistance alleles are rare. For example, if the frequency of a recessive resistance allele is 0.001, only one in a million individuals is expected to be a resistant homozygote (carrying two resistance alleles) capable of surviving exposure to a high concentration of the *Bt* protein.

There are a number of issues associated with this program. The first issue is sample size. The number of samples and number of locations that need to be sampled is dependent on the pest biology and ecology and population dynamics. If the genetic variation in an insect is known, then sampling strategies can be constructed with a greater probability of detection and a low probability of non-detection. Both factors must be considered to reduce the likelihood of both Type 1 (false positive) and Type 2 (false negative) errors. Sampling should also be done in a uniform fashion. Uniformity and standardization in the bioassays is also critical to the interpretation of monitoring information. Related to sample size is finding enough insects to test. Sampling insects exposed to the *Bt* crop is preferred, but if sampling is predominately in the *Bt* crop, then there are few, if any, larvae of the target insect to be found in most *Bt* fields. This means that sampling methods need to be adapted to either collect adults or egg masses to generate the volume of individuals that are needed to increase the probability of detecting resistance or samples are taken from non-*Bt* fields.

Current resistance monitoring plans in the United States have a goal to collect at least 250 individuals from any one location with a target of least 20 locations for tobacco budworm, cotton bollworm, and pink bollworm. The greater the number of samples and number of locations, the greater the probability that resistant individuals will be collected.

Another issue is the sensitivity of the detection methods. If resistance is recessive (rather than dominant or co-dominant), resistance is less likely to develop, but it's more difficult to detect. It is useful to know the frequency of the resistance allele in the natural population. Estimates of the frequency of resistance alleles have been determined based on laboratory-selection experiments (surrogates for what might happen, but not necessarily what will happen in the field). Field verification of resistance allele frequency require reliable and sensitive detection methods. However, if extremely sensitive detection methods, especially if resistance is recessive, are available and economically feasible to use, changes in resistance allele frequency (and verification of estimates) prior to any signs of field failure and create opportunities for proactive, adaptive IRM.

EPA has evaluated the advantages and disadvantages of various detection methodologies and continues will continue to watch for and require a switch to a test that is highly effective and economically viable as the detection methodology improves and is accepted. The currently required, basic test method has been a discriminating dose/diagnostic dose bioassay system that would distinguish between resistant and susceptible phenotypes, but such tests have been criticized as being too insensitive to be able to provide early detection before resistance develops or can spread very far, especially if the alleles for resistance are rare in the insect population. Discriminating dose bioassays are most useful when resistance is common or conferred by a dominant allele (resistance allele frequency > 0.01) (Andow and Alstad, 1998; Andow et al., 1998). It is currently considered as one of the central components of any monitoring plan, but other monitoring methods may have value in conjunction with the discriminating concentration assay.

A second detection technique is the F_2 screen (Andow and Alstad, 1998; Andow et al., 1998). The F_2 screen may be the best method for detection of rare recessive resistant alleles. The F_2 screen is conducted by taking mated females and sib-mating the F_1 progeny producing the F_2 progeny that are tested using an appropriate screening procedure such as a discriminating concentration assay or *Bt* crop, and performing statistical analysis. The technique also allows fewer samples to be collected to detect potential susceptibility shifts than the discriminating dose assay. The F_2 screen may be most useful to analyze populations that are expected to be at high risk to the development of resistance. Each isofemale line allows for characterization of four genomes, thus improving the sensitivity over the discriminating dose assay. effective method for detecting changes in the allele frequency of a recessive or partially recessive allele and can be used to verify some of the assumptions underlying high dose/refuge resistance management. If resistance alleles are found, they can be characterized to estimate the fitness of the

genotypes, determine whether there is a cost of resistance, and enable predictions of the evolution of resistance. A potential obstacle to the F_2 screen is that it may be might be too expensive because it is highly labor intensive and may not be suitable for routine screening purposes, especially if there is replication at each site. In general, the F_2 screen is more expensive than other methods for detecting dominant resistant alleles when the resistance allele frequency is >0.01. However, for recessive alleles, the F_2 screen is the least expensive method and can estimate resistance allele frequencies to a high level of precision (<0.005) for under $5,000 per location.

Additional tests include grower reports of unexpected damage, sentinel plots or use of in-field screening procedures, screening against resistant test stocks (allelic recovery method), and use of in-field detection (using DNA markers) kits.

In a first step toward more efficient DNA-based monitoring, Gahan et al. (2001) describe using a DNA-based screening system for detecting Cry1Ac-resistant tobacco budworm that have developed resistance through a specific mutation in the cadherin gene (characterized the mechanism of *Bt* resistance found in the YHD2 strain, see Gould et al., 1997). This mutation results in a truncated cadherin that lacks the toxin-binding region and thus cannot bind Cry1Ac. The power of DNA-based screening depends on the diversity of resistance-conferred mutations. Field populations of tobacco budworm might harbor this same mutation, other mutations of the same gene, or other genes and mechanisms of resistance. The Gahan et al. findings are the first to identify a DNA-based screening for *Bt*-resistant tobacco budworm heterozygotes by directly detecting the recessive allele. The Gahan et al. DNA marker is being evaluated in the field and other DNA markers are being screened.

Gould et al. (1997) used a series of genetic crosses with test stocks of highly resistant tobacco budworm (YHD2) selected on Cry1Ac in the laboratory to estimate the resistance allele frequency in a natural population of tobacco budworm. This method can identify recessive or incompletely dominant resistance alleles from field-collected males. By using an assay that discriminates between heterozygotes, they could establish which wild males carried a resistance allele. Using this allelic recovery method, Gould et al. (1997) estimated the resistance allele frequency to be 1.5×10^{-3}. This method is only useful when there are previously identified resistance alleles. As noted above, Gahan et al. (2001) were able to identify the mechanism of resistance in this YHD2 line and be the first develop a DNA-marker that might be used in the field to screen for resistance.

Venette et al. (2000) proposed the use of an in-field screen to examine resistance allele frequency. This method uses *Bt* sweet corn to screen for European corn borer and corn earworm (*Helicoverpa zea* Boddie) that are resistant to the *Bt* protein. That is, the *Bt* crop is the discriminatory screen for resistant individuals. By sampling large numbers of *Bt*-expressing plants for live corn borer larvae, the frequency of resistance can be estimated and resistant individuals can be collected for documentation of resistance. A high number of false positives can reduce the efficiency and accuracy of resistance allele measurement. One source of false positives is the occurrence of weakly or non-expressing "off-type" plants among the sampled plants. Another source might be surviving susceptible larvae that are incorrectly scored as resistant larvae because of larval movement between *Bt* and non-*Bt* off-types or weeds. Another problem is that there might not be sweet corn varieties that contain the same *Bt* genes as the field corn varieties. This would reduce the efficiency of sampling.

In addition to sampling and detection sensitivity, other equally complex issues are related to cost and feasibility. It would be virtually impossible and economically prohibitive to sample every farm in which *Bt* crops are used. For example, there are approximately 14,000 *Bt* cotton producers (out of approximately 25,000 cotton producers). These producers planted approximately 4.5 million acres of Bollgard™ cotton in the 2000 growing season and 5.7 million acres of Bollgard™ cotton in the 2001 growing season. Current resistance monitoring programs have focused sampling in areas of highest adoption of the *Bt* crops as the areas in which resistance risk is greatest. For tobacco budworm and cotton bollworm, at least 20 specific collection sites will be established in time for the 2003 growing season. Sites must be focused in areas with high risk of resistance (e.g. where adoption is at least 75% of the cotton planted in that county or parish) while overall being distributed throughout the areas where tobacco budworm and cotton bollworm are important pests with a goal of having sites in AL, LA, AR, MS, FL, VA GA, NC, SC, TN, and TX. For pink bollworm, collection sites must be focused in areas of high adoption, with the goal of including all states where pink bollworm is an economic pest (i.e., AZ, CA, NM, TX).

The cost of the US monitoring program is borne chiefly by the companies although academic institutions and the U.S. Department of Agriculture researchers who carry out the bioassays probably bear some costs (i.e., University of Arizona for pink bollworm and USDA/ARS at Stoneville, Mississippi for tobacco budworm and cotton bollworm).

Related to who will pay for resistance monitoring programs, is the issue of cost-effectiveness. An important issue to consider is if money is not limiting, will the resistance monitoring programs be more proactive, more expansive, and more sensitive. What is the best test to be used considering how much information is found for the money involved? Cost-effectiveness is related

to the perceived and real value of the technology and the likelihood of resistance. Those who believe there is little likelihood of resistance development are less enthusiastic for a rigorous monitoring program.

Remedial Action Plans

EPA required a remedial action plan be available in the unfortunate situation that resistance is suspected or actually does develop. Again, as for resistance monitoring plans, remedial action plans are specific for the crop and pest. For example, because the pink bollworm is predominately a pest of cotton in the western US and has such a different biology than the other two target pests of *Bt* cotton, the remedial action plan for pink bollworm is quite different than that for either cotton bollworm and/or tobacco budworm in the southeastern US. These plans define not only suspected and confirmed resistance, but the key steps and actions needed if and when resistance develops. Generally, if resistance is confirmed, the farmers involved will treat their *Bt* crop with alternative pest control measures. This might be a chemical pesticide known to be highly effective against the insect or it might mean measures such as crop destruction. In addition, the sales and distribution of the *Bt* crop would be suspended in that area and the surrounding area until it can be determined that insects in that area have regained their susceptibility to the *Bt* protein. There would also need to be increased monitoring to define the remedial action area(s). Other remedial action strategies include increasing refuge size, changing dispersal properties, use of sterile of insects, or use of other modes of actions. Geospatial surveys would help define the scale of remedial action and where to intensify monitoring.

Because no field resistance has yet been found to any of the *Bt* crops, all of these tactics are untested. However, EPA believes that a key attribute of these plans are having involvement in the plan's development by the local farmers who would be affected most by the loss of this technology. So far there is only a regional remedial action plan for the Arizona area where pink bollworm is the chief pest controlled by *Bt* cotton. An interim remedial action plan is required and is being revised to address tobacco budworm and cotton bollworm resistance to *Bt* cotton, key economic pests of cotton in the mid-South and Southeastern United States.

Summary

EPA has required an unprecedented insect resistance management (IRM) program for Bollgard™ cotton in order to delay or prevent the target insects from becoming resistant to the Cry1Ac delta-endotoxin. EPA has mandated seven basic IRM elements as part of the terms and conditions of the amended Bollgard™ cotton registration. There are three specific structured refuge options: 5% external, unsprayed; 5% embedded, and 20% external, sprayed. The current registration expires September 30, 2006, except for the 5% external, unsprayed structured refuge option which expires September 30, 2004 pending further review of certain data. A community refuge pilot was renewed for 2002. This program will allow multiple growers to work together to comply with the refuge requirements. The Agency used multiple IRM predictive models as tools to compare refuge options. Models are imperfect, but in the absence of field resistance, are the only tools available that integrate all of the available biological and genetic information to predict the likelihood of resistance. Further data are needed to validate input parameters, e.g., alternate host contribution to effective refuge size, level of adoption, level of compliance, and supplemental insecticidal spray effects. Uncertainty in models, also indicates that more focus should be on resistance monitoring, grower actions, and potential remedial action strategies.

Annual resistance monitoring should be proactive and focus on areas of highest risk of resistance. Sampling and detection methodologies need improvement. Insect resistance monitoring is expensive and the extremely high costs can be offset by more reliance on farmer actions to carry out robust IRM plans, on compliance monitoring, and thorough remedial action plans.

The science of insect resistance management, including models, is complex and is continuing to develop. Maintaining the Bollgard™ cotton IRM program, or any, IRM program requires the effective actions of farmers, pesticide companies, researchers, and government regulators. EPA will continue to monitor all of these activities closely for the *Bt* cotton products and make further IRM requirements if necessary.

Disclaimer

The opinions discussed in this article are those of the author and do not necessarily represent those of the United States government.

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