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Introduction to Biotechnology Regulation for Pesticides

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I. OVERVIEW OF BIOPESTICIDES AND BIOTECHNOLOGY PESTICIDE PRODUCTS

A) Biotechnology and Traditional Breeding

For centuries, humans have improved crop plants through selective breeding and hybridization — the controlled pollination of plants. In more recent times, plant breeders created new varieties using chemicals or irradiation to provide unique traits in plants. Plant biotechnology is an form of plant breeding with one very important difference — plant biotechnology allows for the transfer of genetic information from species unrelated to the plant.

Traditional plant breeding involves the crossing of hundreds or thousands of genes, whereas plant biotechnology allows for the transfer of only one or a few desirable genes. This more precise science allows plant breeders to develop crops with specific traits and without relying on imprecise chemical mutation or random cross pollination that may include undesirable traits that have to be bred out of the new plant before it can be a commercially viable new variety.

Some of the beneficial traits in new plant varieties fight plant pests — insects, weeds and diseases — that can be devastating to crops. The pest fighting traits are pesticides as defined by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). The EPA regulates the use of pesticides under the authority of three federal statutes – FIFRA, the Federal Food, Drug, and Cosmetic Act (FFDCA), and the Food Quality Protection Act (FQPA). Federal oversight of pesticides is required to ensure that their use will not cause unreasonable adverse effects to human health or the environment. Other laws that may be taken into consideration when conducting risk assessments include the Endangered Species Act, the Migratory Bird Treaty Act and other environmental laws.

B) Early Regulatory Development for Biotechnology Products

Responding to the rapid increase in the production of biotechnology products, there was a realization for the need for some sort of guidelines to ensure that public health and the environment are adequately protected from the potential risks of this technology. As products began moving from the laboratory toward the market, regulatory agencies realized that there should be regulatory mechanisms to ensure that these new products did not adversely affect public health or the environment.

To clarify regulatory jurisdiction over biotechnology products, the Administration established an interagency working group under the White House Cabinet Council on Natural Resources and the Environment in 1984. The working group's principle goal was to ensure the regulatory process adequately considered health and environmental safety consequences of the products of biotechnology as they move from the laboratory to the marketplace. Safety was not their only concern; however, the Council also emphasized the

importance of not stifling innovation or enervating the competitiveness of the U.S. biotech industry. Thus, the interagency working group sought to establish a sensible framework that effectively protected human health and the environment while providing breathing room for a burgeoning industry.

Scientists also wanted the freedom and flexibility to engage in research and did not want Congress to pass unduly restrictive laws. In 1986, the Federal Government, through the Office of Science and Technology Policy issued the Coordinated Framework for the Regulation of Biotechnology. The Coordinated Framework concluded that the products of biotechnology do not differ fundamentally from unmodified organisms or from conventional products; that the product, rather than the process, should be regulated; that the regulations should be based on the end use of the product; and that review should be conducted on a case-by-case basis. The Coordinated Framework states that no new statutes (laws) are needed to regulate biotechnology products, but that new regulations would be required. All regulations would go through a process of public notice, a public comment period, and consideration of those comments before final regulations were established. EPA is also required under FIFRA to take such proposed regulations through our outside scientific peer review committee called the Scientific Advisory Panel.

As part of a coordinated federal framework for biotechnology, biotech crops undergo a food safety and environmental approval process by the EPA, Food and Drug Administration (FDA) and the Department of Agriculture (USDA). The FDA's role (FFDCA), is to insure the safety of the food supply which is typically done by verifying that any new biotech foods are as safe as the foods already being consumed. The USDA (Federal Plant Pest Act - FPPA, Plant Quarantine Act - PQA and the Virus, Serum, Toxin Act - VSTA) is responsible for protecting US agriculture by assessing biotech plants for their potential to become a plant pest in the environment, while EPA's mandate is to protect human health and the environment by ensuring that a plant derived from biotechnology produces no unreasonable adverse effects.

C) EPA's Regulation of Biotechnology Pesticide Products

EPA regulates three major classes of biopesticides: biochemical pesticides, microbial pesticides, and plant-incorporated protectants (PIPs). Within the office Pesticide Programs (OPP), the Biopesticides and Pollution Prevention Division (BPPD) regulates natural and engineered microbial pesticides, PIPs and biochemical pesticides. Microorganisms can be genetically engineered to produce biochemicals used as pesticides. Microbial pesticides can be naturally occurring or genetically engineered. Plant-Incorporated Protectants are pesticidal substances that are produced in a living plant along with the genetic material necessary to produce the substance, where the substance is intended for use in the living plant. EPA includes the genetic material necessary to produce the substance in the definition of a plant-incorporated protectant because the genetic material introduced into the plant will ultimately result in a pesticidal effect. In addition, the inclusion of the genetic material may be responsible for the spread of the pesticidal trait in the environment to related plant relatives.

Under the Coordinated Framework, EPA proposed regulations for genetically engineered microbial pesticides and for plant-incorporated protectants. Regulations for notification prior to small scale field testing of engineered microbial pesticides were final in 1994. At the end of 1994, EPA proposed a set of regulations aimed at establishing the Agency's scope for PIPs and proposing to exempt several classes of PIPs which had very low risk. Most of these proposed regulations were final in 2002, although some of the exemptions are being further evaluated before the Agency makes a final decision.

i. Genetically Engineered Microbial Pesticides

Genetically engineered microorganisms are regulated using essentially the same data requirements used for naturally occurring microbial pesticides. (See 40 CFR part 158.740.) Some additional data may be required concerning the genetic engineering process used

and the results from that process. EPA requires notification prior to small scale field testing of genetically engineered microorganisms to allow EPA to determine if an Experimental Use Permit is needed. (See 40 CFR part 172 subpart C.) When testing10 acres or more, EPA requires an Experimental Use Permit before field testing naturally occurring or genetically engineered microorganisms. Under FIFRA, microbial biotech products, as with all other pesticides, must be evaluated for their risks and benefits. Before any registration is granted, OPP considers such issues as potential adverse effects to non-target organisms, environmental fate of the microorganism, and the potential pathogenicity and infectivity of the microorganism to humans.

ii. Plant-Incorporated Protectants

All plants produce some substances which act to repel certain insects or kill various pathogens such as bacteria and fungi; plants can also have other mechanisms such as hairy leaves or other structure to help ward off pests. Plants which have more of these natural protections are less susceptible to pests than plants which have fewer of them.

In the 1994 policy statement and proposed rules for what are now called PIPs (plant-incorporated protectants), EPA explained that substances plants produce for protection against pests are pesticides under FIFRA, if humans intend to use these substances for pesticidal purposes. In 1994, EPA called these substances, produced and used in living plants, along with the genetic material necessary to produce them, “plant-pesticides.” With issuance of the policy statement and proposed rules, EPA began registering plant-pesticides under FIFRA and regulating residues of plant-pesticides under FFDCA. The name plant-incorporated protectants or PIPs was selected after EPA took comments on a substitute name for plant-pesticides as requested in the public comments in response to the 1994 proposed rules.

In 2001, EPA published final rules exempting from FIFRA requirements (except for an adverse effects reporting requirement pesticidal substances produced through conventional breeding of sexually compatible plants. EPA also exempted residues of these pesticidal substances and of all nucleic acids that are part of a PIP from FFDCA pesticide residue requirements. These residues of pesticidal substances were exempted based on a long history of human dietary exposure to these naturally occurring plant compounds, and epidemiological studies showing the health benefits of consuming foods which can contain low levels of these substances. Residues of nucleic acids that are part of PIPs were exempted as nucleic acids are common to all life forms, have always been present in human and domestic animal food, and are not known to cause adverse health effects when consumed as part of food. PIPs moved into plants from other organisms, including from plants not sexually compatible with the recipient plant, continue to be regulated. EPA believes this oversight is appropriate because of the new exposure to the PIP now expressed in the new host plant. An example of this type of PIP is the insecticidal protein from the bacterium *Bacillus thuringiensis* introduced into a plant for protection against lepidopteran (caterpillar) pests. EPA also proposed in 1994 to exempt three other categories of PIPs. However, in 2001, EPA decided to solicit further guidance from the public on the three proposed exemptions because of the wide range of varied comments received on the proposals. In addition, EPA placed in the public record for the rules and solicited comment on the information, analyses and conclusions of the 2000 National Academy of Sciences study entitled “Genetically Modified Pest-Protected Plants: Science and Regulation” that recommended EPA reconsider its proposed categorical exemption of viral coat proteins, PIPs which act by primarily affecting the plant, and PIPs produced by using biotechnology to move genes between sexually compatible plants.

In general, the data requirements for PIPs are based on those for microbial pesticides as, up to now, the PIP products have come from microorganisms. The exact data requirements for each product have been developed on a case by case basis. All of the products EPA has seen to date have been proteins, either related to plant viruses or based on proteins from the common soil bacteria *Bacillus thuringiensis* also known at Bt. The general data requirements include product characterization, mammalian toxicity, allergenicity potential,

effects on non-target organisms, environmental fate, and for the Bt products, insect resistance management to product from losing use of both the microbial sprays and the Bt PIPs. In December 1999 and June 2000 EPA asked the Scientific Advisory Panel (SAP) to reevaluate the data requirements for protein PIPs. The Agency presentations and the SAP reports are available on the website listed below. EPA has made adjustments to our data requirements based upon the SAP's recommendations.

EPA's recent reevaluation of the Bt PIPs shows that generally, these products reduce the use of more toxic insecticides. This is most obvious for Bt cotton. Traditionally, cotton is one of the most frequently treated crops, often with organophosphate compounds. The use of Bt cotton greatly reduces the use of these toxic compounds. New products currently being tested under Experimental Use Permits to control corn rootworm have the potential to reduce reliance on the millions of pounds of organophosphates and carbamates currently used to control this insect.

One should not, however, confuse support with advocacy. As a regulatory agency, the EPA maintains a neutral position regarding biotechnology. We require appropriate studies to determine if there is a risk and the studies must conform to established standards including being conducted by labs that are subject to inspection. The studies enable the agency to conduct risk assessments that are made available to the public. Additionally, the Agency strives to have a transparent review process that enables all stakeholders views to be heard. EPA maintains a public docket of all studies and the Agency's review of the studies; we subject our analyses to the Scientific Advisory Panel (SAP); and the Agency holds public hearings, workshops, and seminars to provide the public with information and to obtain input on our regulatory decisions.

All these efforts were designed to assess and regulate the human health and environmental impacts of biotechnology products, and to assure that the decisions taken by the Agency were based on the most current health and ecological data that provide consistency, effectiveness, and flexibility. As the science of biotechnology progresses, we anticipate that the types of products will also change. These changes will determine how we review these ingredients and the types of data that will be required. In the future, it is possible that compounds similar to protective compounds already produced by plants, such as phenols, ketones or aldehydes, will be used as PIPs . These types of compounds will require a very different set of data than that required to determine the safety of proteins. EPA will rely on the SAP and similar scientific bodies to guide the Agency on the appropriate data requirements. EPA remains fully committed to assuring that the review, assessment and registration of biotechnology products meet the stringent standards required by FIFRA and the FFDCA, and are fully protective of public health and the environment. It is EPA's goal to make our regulatory process and decisions within a sound and transparent process framework tuned into the most recent and scientifically sound information.

D) Herbicide Tolerant Plants

The EPA regulates the herbicides used on herbicide tolerant plants, but the regulation of the plants and the food crops is done by USDA and FDA, respectively. EPA does establish tolerances (legal limits of pesticide residue) for the herbicide used to control weeds in the crop fields. In addition, EPA and USDA have a Memorandum of Understanding to work together on resistance management for herbicide tolerant crops.

E) Useful Web Sites

The Environmental Protection Agency
http://www.epa.gov

The Biopesticides and Pollution Prevention Division
http://www.epa.gov/pesticides/biopesticides

Food and Drug Administration, Center for Food Safety and Applied Nutrition (CFSAN)
http://vm.cfsan.fda.gov/~lrd/biotechm.html EXIT disclaimer

United States Department of Agriculture, Animal and Plant Health Inspection Service
http://www.aphis.usda.gov/brs/index.html EXIT disclaimer

II. Data Requirements for the Registration of Plant-Incorporated Protectants

A) Product Characterization

Identification of the new Plant-Incorporated Protectants (PIP) character added to the plant generally follows guidance developed by the EPA, Canadian Food Inspection Agency (CFIA) and the USDA's Animal and Plant Health Inspection Service (APHIS). This includes a description of the source of the pesticidal gene (promoters, enhancers, open reading frame and closing and release signals for the pesticidal trait) and identifies the source of any other traits introduced such as selection markers (herbicide resistance, antibiotic resistance or a phenotypic trait such as cells turning blue when introduced $-glucuronidase is present). This information is given as a plasmid map of the vector DNA and includes the exact DNA sequence for the introduced traits. The discussion also includes the biology of the source organism(s) and describes any hazards associated with source organism(s) such as pathogenicity or toxin production. Rationale is also given as to why this trait was selected and any changes to the actual DNA sequence of the introduced gene(s) are described.

A description of the recipient plant is provided including the general biology and use as a crop. The details expected to be covered would include the possible production of toxins or anti-nutrients by the plant, major insect pests, weeds and diseases of the crop, reproductive biology, and presence of wild or weedy relatives of the crop in the United States.

Trait introduction is discussed to provide information about the plant cell culture and selection/regeneration technique used to produce the genetically engineered plant. Confirmatory data show what part of the vector DNA is actually incorporated into the plant genome. This consists of southern blot analysis for the trait's gene and also gives information about the possibility of multiple copies of the gene(s) being incorporated. A southern blot analysis is a DNA detection assay based on the high binding affinity of the two complimentary strands of DNA for each other. Using a radioactive or other labeled form of one of the complimentary strands and restriction endonuclease enzymes which cut the DNA at specific locations, information about the status and insertion of the introduced traits in the transformed plant can be surmised.

The stability and inheritance of trait is examined to determine any linkage of the introduced trait(s) and if there is more than one site of incorporation for the trait(s). The presence and performance of the trait over several plant generations is examined for determining stability of trait expression.

Protein characterization and expression data provide biochemical information about the actual expressed protein in the plant and its concentration in various tissues. This includes the amino acid sequence, the activity of the protein (usually information about the range of susceptible species) and identification of the expressed protein. Protein identification usually includes SDS-PAGE analysis, immunological recognition in an ELISA or western blot assay and N-terminal amino acid sequencing. For the proteins reviewed to date by the EPA, there has also been information to indicate if the protein was modified when produced in the plant versus its form in the original donor organism. For traits derived from bacteria, there is a possibility that the protein was modified after translation in the plant, for example by adding extra sugar residues to the protein (glycosylation), which does not occur in the

source bacterium. Protein glycosylation is a concern if it changes the stability or activity of the expressed protein and data from the microbial source is being used to determine safety. Expression data are provided to determine maximum exposure levels for the PIP in several plant tissue (stem, leaf, root, flower, pollen, etc). Expression levels are cogent for human health and environmental hazard assessment as well as insect resistance management.

B) Mammalian Toxicity

The mammalian toxicity data examined is guided by the fact that most PIPs seen to date are proteins. Given this fact a special set of data is generally considered to affirm the assumption that the introduced protein behaves like other dietary proteins. In vitro digestibility data is often considered part of the biochemical characterization of the expressed protein but may also address toxicity issues. The rationale behind its use is to determine if the expressed protein degrades in the presence of acid, heat or simulated gastric and intestinal digestive fluids as would be expected of a normal dietary protein. The stability to digestive fluids, acid or heat also is one of the complex of characteristics that is examined to determine if a protein with no dietary history has potential to be a food allergen. If a protein is not rapidly broken down by digestive fluids, it may have a longer time period to interact with the gastrointestinal mucosa and possibly induce an allergic reaction in susceptible individuals.

Acute oral toxicity is examined since the primary route of exposure is dietary for PIPs. The study is done with a gavage (forced oral) administration of a maximum hazard dose (2-5gm/kg body weight) and the animals are followed for 14 days of observation for clinical signs. At the end of 14 days the animals are sacrificed and the internal organs are examined by gross necropsy. The test substance used is the protein expressed from the gene introduced into the plant or the same protein produced from an alternate source. If the protein has no unusual persistence in digestive fluids and shows no toxicity in the acute oral toxicity tests, there is no reason to believe that the introduced protein would behave any differently than any other dietary protein and longer term toxicity testing would not be justified.

Test substance equivalence may be required if an alternate source for the producing test material has been employed. In all the PIPs examined to date, there has never been sufficient expression of the protein PIP in the plant itself to provide adequate purified material to supply an acute oral toxicity test. Therefore, to provide adequate protein test material for the study, an alternate production system for the protein is employed. This has been either the original source bacterium *Bacillus thuringiensis* or an industrial fermentation strain like a laboratory strain of *Escherichia coli*. In order to be able to utilize this alternate source, however, a series of tests similar to those done for product characterization need to be performed using both the plant expressed protein and the bacterial protein to insure these two are equivalent. These tests include the bioactivity of the protein (e.g., LD50 determinations against a range of insects), SDS-PAGE analysis, immunological recognition in an ELISA or western blot assay and possible post-translational modifications for the plant expressed material.

Amino acid sequence homology is examined for the introduced protein to determine if there are any similarities between the introduced protein and known protein toxins or allergens. This analysis is done by both a whole sequence homology comparison and also a step wise eight contiguous amino acid sequence comparison. The eight amino acid fragment analysis is done for comparison to known allergens since this is believed to be the smallest stretch of amino acids that can be recognized by an antibody. Since antibodies are intimately involved in the aberrant response causing food allergies, this analysis is critical for examining proteins with no previous dietary exposure for their potential to be related to a food allergen.

III. Gene Flow Assessment for Plant-Incorporated Protectants

The movement of transgenes from the host plant into weeds and other crops has been a significant concern for EPA due to the possibility of novel exposures to the pesticidal substance. This concern has been considered for each of the Bt plant-incorporated protectants (PIPs) currently registered.

The Federal Insecticide, Fungicide and Rodenticide Act directs the U.S. EPA to examine all potentially adverse environmental impacts, including those which may arise from gene flow of PIPs to wild or feral populations of sexually compatible plants. In addition to this mandate, the Federal Food, Drug, and Cosmetic Act requires the issuance of a food tolerance or exemption from the requirement of a tolerance for all pesticidal substances that may enter the food supply whether through seed mixing or cross pollination. To date, PIPs in three crop species have been registered by the Agency and all have received exemptions from the requirement of a tolerance. *Bt* Corn, cotton and potato were reviewed for their potential to hybridize with wild and feral relatives of sexually compatible plants in the U.S., its territories and possessions.

EPA has determined that with the conditions of registration in place there is no significant risk of gene capture and expression of any Bt endotoxin by wild or weedy relatives of corn, cotton, or potato in the U.S., its possessions or territories. In addition, the USDA/APHIS has made this same determination under its statutory authority under the Plant Pest Act.

The *Bt* corn and potato PIPs that have been registered to date have been expressed in agronomic plant species that, for the most part, do not have a reasonable possibility of passing their traits to wild native plants. Most of the wild species in the United States cannot be pollinated by these crops (corn and potato) due to differences in chromosome number, phenology and habitat. There is a possibility, however, of gene transfer from Bt cotton to wild or feral cotton relatives in Hawaii, Florida, Puerto Rico and the U.S. Virgin Islands. Where feral populations of cotton species similar to cultivated cotton exist, EPA has prohibited the sale or distribution of *Bt* cotton in these areas. These containment measures prevent the movement of the registered Bt endotoxin from Bt cotton to wild or feral cotton relatives.

EPA's evaluation of the possibility of movement of Bt transgenes into weedy relatives of potato, corn, and cotton are presented below:

A) Potato (*Solanum tuberosum*)

EPA has reviewed the potential for gene capture and expression of Bt plant-incorporated protectants (only Cry3A has been introduced into potato) by wild or weedy relatives of cultivated potato in the United States, its possessions or territories. Based on data submitted by the registrant and a review of the scientific literature, EPA concluded that there is no foreseeable risk of unplanned pesticide production through gene capture and expression of the Colorado potato beetle control protein (Cry3A) in wild potato relatives in the U.S. Tuber-bearing *Solanum* species, including *S. tuberosum*. *S. tuberosum* cannot hybridize naturally with the non-tuber bearing *Solanum* species in the U.S. Three species of tuber-bearing wild species of *Solanum* occur in the United States: *Solanum fendleri, Solanum jamesii*, and *Solanum pinnatisectum*. But, successful gene introgression into these tuber-bearing *Solanum* species is virtually excluded due to constraints of geographical isolation and other biological barriers to natural hybridization. These barriers include incompatible (unequal) endosperm balance numbers that lead to endosperm failure and embryo abortion, multiple ploidy levels, and incompatibility mechanisms that do not express reciprocal genes to allow fertilization to proceed. No natural hybrids have been observed between these species and cultivated potatoes in the U.S.

B Corn/Maize. (*Zea mays*)

EPA has reviewed the potential for gene capture and expression of the BtCry endotoxin protein in corn plants, by wild or weedy relatives of maize in the United States. EPA believes there is no significant risk of gene capture and expression of any of the Cry

endotoxins by wild or weedy relatives of maize in the United States because extan populations of sexually compatible species related to Zea mays are not present in the continental United States or its territories and possessions. *Zea mays* is a wind-pollinatec species, and the presence of spatially separate tassels (male flowers) and silks (female flowers) encourages outcrossing among nearby plants. Maize cultivars and landraces are known to be interfertile to a large degree. Recent studies have indicated that cross-pollination at 100 ft. from the source of genetically modified maize was 1 % and this proportion declined exponentially to 0.1 % at 130 ft and further declined to 0.03 % at the farthest distance measured (160 ft). For production of Foundation Seed, a distance of 660 f has been required to ensure separation of pollen types. Additionally, the relatively large size of corn pollen as compared to other grass species and the short time span that corn poller remains viable (i.e., typically less than 60 minutes) under natural conditions both preclude long distance transfer for purposes of outcrossing. Under conditions of high temperature and desiccation, corn pollen longevity is measured in minutes. These conditions may ever destroy the anthers before any viable pollen is shed. More moderate conditions can extenc the field life to hours.

For transformed plants to become weedy escapes as a result of the genetic modificatior (i.e., expression of *Bt* endotoxins that protect plants from insect damage), they would neec to inherit and express many other unrelated traits that provide selective advantage to a weedy growth habit (e.g., large numbers of easily dispersed seeds, propensity to grow or disturbed ground, vegetative propagation, seed dormancy, etc.). These traits do not exis within the corn complement of genetic characters, a species that has been selected fo domestication and cultivation under conditions not normally found in natural settings. The presence of a large cob or ear that does not shatter as the bearer of seeds severely limits the dispersing abilities of corn and it has been theorized that in the species as we know i would die out in a few generations due to competition amongst seedlings germinating from the cob.

Transformation of corn to express *Bt* endotoxin does not alter the ability of corn to outcross with teosintes (*Zea mays* ssp. *mexicana*, *Z. mays* ssp. *parviglumis*, *Z. luxurians*, *Z perennis*, *Z. diploperennis*) or *Tripsacum* species. Teosintes exist as special plantings (e.g. in research plots, botanical gardens, and greenhouses) and some are used to a smal extent as forage crops in the western United States. Many native teosintes in Mexico, E Salvador, Guatemala, Nicaragua and Honduras are interfertile with corn to varying degrees and have been known to produce viable seedlings. Despite having coexisted and co-evolved in close proximity to corn in the Americas over thousands of years, however, corn and teosintes maintain distinct genetic constitutions even with this sporadic introgression Given the cultural and biological relationships of various teosinte species and cultivatec corn over the previous millennia, it appears that gene exchange has occurred (basec largely upon morphological characters) between these two groups of plants and that no weedy types have successfully evolved as a result. More recent cytogenetic, biochemica and molecular analysis has indicated that the degree of gene exchange is far less thar previously thought and evidence for gene introgression into teosinte from corn may be considered as circumstantial at present. The teosintes retain a reduced cob-like fruit/inflorescence that shatters more than cultivated corn, but still restricts the movement o seeds as compared to more widely dispersed weedy species. Hence, the dispersal of large numbers of seeds, as is typical of weeds, is not characteristic of teosintes or corn. In thei native habitat, some teosintes have been observed to be spread by animals feeding on the plants. Teosintes and teosinte-corn hybrids do not survive even mild winters and would no propagate in the U.S. Corn Belt. Additionally, some types have strict day length requirements that preclude flowering within a normal season (i.e., they would be induced to flower in November or December) and, hence, seed production under our temperate climate. Based on the ability of corn to hybridize with teosintes, the results of previous genetic exchange amongst these species over millennia, and their general growth habits any introgression of genes into wild teosinte from Zea mays is not considered to be a significant agricultural or environmental risk. The growth habits of teosintes are such tha the potential for serious weedy propagation and development is not biologically plausible ir the United States.

Sixteen species of *Tripsacum* are known worldwide. Most of the 16 different *Tripsacum* species recognized are native to Mexico, Central and South America, but three occur within the U.S. The Manual of Grasses of the United States reports the presence of three species of *Tripsacum* in the continental United States: *T. dactyloides*, *T. floridanum* and *T. lanceolatum*. Of these, *T. dactyloides*, Eastern Gama Grass, is the only species of widespread occurrence and of any agricultural importance. It is commonly grown as a forage grass and has been the subject of some agronomic improvement (i.e., selection and classical breeding). *T. floridanum* is known from southern Florida and *T. lanceolatum* is present in the Mule Mountains of Arizona and possibly southern New Mexico. For the species occurring in the United States, *T. floridanum* has a diploid chromosome number of 2n = 36 and is native to Southern Florida. *T. dactyloides* includes 2n = 36 forms which are established in the central and western U.S., and 2n = 72 forms which extend along the Eastern seaboard and along the Gulf Coast from Florida to Texas, but which have also been found in IL and KS; these latter forms may represent tetraploids (x = 9 or 18). *T. lanceolatum* (2n = 72) occurs in the Southwestern U.S. Eastern Gama Grass (*T. dactyloides*) differs from corn in many respects, including chromosome number (*T. dactyloides* commonly n = 18; *Zea mays* n = 10). Many species of *Tripsacum* can cross with *Zea*, or at least some accessions of each species can cross, but only with difficulty and the resulting hybrids are primarily male and female sterile. *T. dactyloides*, is considered by some to be an ancestor of *Zea mays* or cultivated corn, while others dispute this, based largely on the disparity in chromosome number between the two species, as well as radically different phenotypic appearance. Albeit with some difficulty, hybrids between the two species have been made. In most cases these progeny have been sterile or viable only by culturing with in vitro 'rescue' techniques. Relatively few accessions of *T. dactyloides* will cross with corn and the majority of progeny aren't fertile or viable even in those that do. In controlled crosses, if the female parent is corn, there is a greater likelihood of obtaining viable seed. When these hybrids have been backcrossed to corn in attempts to introgress *Tripsacum* genes for quality enhancement or disease resistance, the *Tripsacum* chromosomes are typically lost in successive generations.

Even though some *Tripsacum* species occur in areas where corn is cultivated, gene introgression from corn under natural conditions is highly unlikely, if not impossible. Hybrids of *Tripsacum* species with *Zea mays* are difficult to obtain outside of the controlled conditions of laboratory and greenhouse. Seed obtained from such crosses are often sterile or progeny have greatly reduced fertility. Approximately 20% of corn-*Tripsacum* hybrids will set seed when backcrossed to corn, and none are able to withstand even the mildest winters. The only known case of a naturally occurring *Zea* - *Tripsacum* hybrid is a species native to Guatemala known as *Tripsacum andersonii*. It is 100 % male and nearly 99% female sterile and is thought to have arisen from an outcrossing to a teosinte, but the lineage is uncertain. *Zea mays* is not known to harbor properties that indicate it has weedy potential and other than occasional volunteer plants in the previous season's corn field corn is not considered as a weed in the U.S. The risk of *Tripsacum* /corn hybrids forming in the field is considered minimal. *Tripsacum* species are perennials and seem more closely related to the genus *Manisurus* than either to corn or teosinte.

Since both teosinte and *Tripsacum* are included in botanical gardens in the U.S., the possibility exists (although unlikely) that exchange of genes could occur between corn and its wild relatives. EPA is not aware, however, of any such case being reported in the United States. Gene exchange between cultivated corn and transformed corn would be similar to what naturally occurs at the present time within cultivated corn hybrids and landraces. Plant architecture and reproductive capacity of the intercrossed plants will be similar to normal corn, and the chance that a weedy type of corn will result from outcrossing with cultivated corn is extremely remote. Like corn, *Zea mays* ssp. *mexicana* (annual teosinte) and *Zea diploperennis* (diploid perennial teosinte) have 10 pairs of chromosomes, are wind pollinated, and tend to outcross, but are highly variable species that are often genetically compatible and interfertile with corn. *Zea perennis* (perennial teosinte) has 20 pairs of chromosomes and forms less stable hybrids with corn. Corn and compatible species of teosinte are capable of hybridization when in proximity to each other. In Mexico and Guatemala, teosintes exist as weeds around the margins of corn fields. The F1 hybrids have been found to vary in their fertility and vigor. Those that are fertile are capable of

backcrossing to corn. Except for special plantings as noted above, however, teosinte is no present in the U.S. or its territories. Its natural distribution is limited to Mexico, Honduras Nicaragua and Guatemala. *Tripsacum*/corn hybrids have not been observed in the field, bu have been accomplished in the laboratory using special techniques under highly controlled conditions.

C Cotton. (*Gossypium* spp.)

EPA has reviewed the potential for gene capture and expression of the Cry1Ac endotoxin ir cotton by wild or weedy relatives of cotton in the United States, its possessions o territories. There is a possibility for gene transfer in locations in Hawaii, Florida, Puerto Ricc and the U.S. Virgin Islands where wild or feral cotton relatives exist. Therefore, EPA required stringent sales and distribution restrictions on Bt crops within these areas to preclude hybridization of the crop with sexually compatible relatives.

There are four species of cotton, *Gossypium*, in the United States. Two of them, *Gossypium hirsutum* (upland cotton) and *Gossypium barbadense* (sea island cotton, pulpulu haole Pima), are used commercially and escaped plants can be found growing in the wild ir climates where they can survive the winter. In addition, two native wild species o *Gossypium* occur in the United States: *G. thurberi* Todaro and *G. tomentosum* Nuttall e Seeman. *G. thurberi* Todaro (*Thurberia thespesiodes* Gray) occurs in the mountains o Southern Arizona and northern Mexico at 2,500 to 5,000 feet (rarely at 7000 feet), and is rather common on rocky slopes and sides of canyons in late summer and autumn. An gene exchange between plants of *Gossypium hirsutum* and *Gossypium thurberi*, if it dic occur, would result in triploid (3x=39 chromosomes), sterile plants because *G. hirsutum* is an allotetraploid (4x = 52 chromosomes), and *G. thurberi* is a diploid (2x = 26 chromosomes). Such sterile hybrids have been produced under controlled conditions, bu they would not persist in the wild; in addition, fertile allohexaploids (6x = 78 chromosomes) have not been reported in the wild.

The second wild native species, *Gossypium tomentosum*, occurs in Hawaii on the six islands of Kahoolawe, Lanai, Maui, Molokai, Nihau and Oahu. Upland, Hawaiian and sea island cotton are all tetraploids (4x = 52) that can crossbreed. Introgression has beer claimed for what one author considered hybrid populations of *G. barbadense* X *G tomentosum*. As *G. tomentosum* may bloom at the same time as domestic cotton, there is no guarantee of either geographic or temporal isolation. For these reasons, EPA imposed stringent sales and distribution restrictions on the registration for cotton expressing the Cry1Ac delta endotoxin grown in Hawaii. The Agency required the following labeling statement to mitigate the potential for the Cry1Ac gene to move from cultivated cotton to *G tomentosum*:

> "Not for commercial sale or use in Hawaii. Test plots or breeding nurseries established in Hawaii must be surrounded by either 12 border rows of non-cotton if the plot size is less than 10 acres or 24 border rows if the plot is over 10 acres and must not be planted within 1/4 mile of *Gossypium tomentosum*."

The inability of plants or seeds of either of *G. hirsutum* or *G. barbadense* to survive freezing temperatures restricts their persistence as perennials or recurrent annuals to tropical areas Feral *G. hirsutum* occurs in parts of southern Florida in the Everglades National Park and the Florida Keys. Cotton is not grown commercially in these areas at this time (cultivated cottons are found in the northernmost portions of the state), but the containment provisions of the initial registration must continue for areas in Florida where feral cotton occurs. Wild cotton is a potential concern as it may increase the spread of resistance in Florida (with intensive vegetable production). EPA imposed sale and distribution restrictions on *Bt* cottor in Florida, restricting its use to those sites North of Tampa (Route 60). The Agency is satisfied that the planting restrictions on *Bt* cotton (i.e., no *Bt* cotton south of Tampa) wil mitigate concerns for gene transfer to wild cotton:

> " Do not plant in Florida South of Tampa, Route 60."

A restriction on the planting of *Bt* cotton in Florida south of Route 60, near Tampa precludes any chance of outcrossing with feral *Gossypium* spp. due to the extremely large distance between any commercial plantings and wild populations in the extreme south of the state. Previously there were no restrictions on commercial planting of *Bt* cotton in the Virgin Islands or Puerto Rico, both of which are known to have extant populations of wild *G hirsutum* or *G. barbadense* or hybrids of the two species. In the former case, no commercial plantings of cotton or test plots of *Bt* cotton are planned for the U.S. Virgin Islands, hence gene flow is not a concern there. Planting of *Bt* cotton should be restricted, however, if prevention of gene flow is desired. Puerto Rico has seen use as a winter nursery for some commercial cotton breeding and the populations of wild *Gossypium* are apparently commonly distributed throughout the island. Similar restrictions, as imposed for Hawaii, are appropriate for this situation based upon the distribution of wild populations around the island and the propensity of insect pollinators to move substantial differences. In both cases, monitoring of native populations of established *Gossypium* spp. may be necessary to assess the efficacy of this isolation procedure for *Bt* cotton. This would entail monitoring of wild populations for evidence of gene introgression through PCR or similarly sensitive methods. Alternatively, the absolute restriction of planting *Bt* cotton in Puerto Rico and the U.S. Virgin Islands, would of course, alleviate any concerns over gene flow.

Given that the distribution of feral *Gossypium* spp. in Puerto Rico is not well documented the mapping of native populations with related distance to breeding nurseries of *Bt* cottor may be sufficient to indicate the probability of cross pollination. If any populations of fera *Gossypium* spp. are eliminated over time, this could potentially change the proximity to research areas and influence the isolation requirements with respect to gene flow issues.

EPA analyzed the possibility of gene flow from transgenic cultivars expressing *Bt* to wild native plants which acquire the *Bt* genes through cross pollination as part of its risk assessment of *Bt* potato, *Bt* corn, and *Bt* cotton as summarized above. EPA has concluded that there is no significant risk of gene capture and expression of the *Bt* toxin(s) by wild or weedy relatives of corn, cotton, or potato in the U.S., its possessions or territories. The creation of "*Bt*-enhanced weeds" from *Bt* gene movement from *Bt* corn or *Bt* potato is only a remote possibility in the U.S. For *Bt* cotton, where the possibility of gene movement may exist in certain geographically distinct areas, EPA has mitigated the potential for such movement by imposing strict geographic restrictions on the sale and distribution of *B* cotton.

IV. Ecological Non-Target Organism Risk Assessment process for Plant-Incorporated Protectants

A) Non-Target Organism Hazard Assessment Process:

Hazard to non-target organisms is a function of toxicity AND exposure. Non-target organisms that are not exposed to a toxic substance are not at risk. Organisms that may be exposed to limited amounts of a toxic substance may also not be at risk. Therefore, the risk assessment process depends on determination of the toxicity of a given substance and the degree of environmental exposure of non-target species.

The primary issue which defines the type of toxicity data needed for a risk assessment of PIPs is that the pesticidal substance is contained within the plant parts thus resulting in minimal exposure to non-target organisms. This exposure scenario is quite different from spray applications of pesticides. Exposure of non-targets to plant-incorporated protectants would occur primarily when wildlife feed on the pesticidal plants, on exposure to dispersed pollen or if sexual transfer of the new trait(s) to non-target wild/weedy relatives by cross-pollination takes place. Therefore, each risk assessment is made from an analysis of the nature of the gene being introduced, the plant receiving the gene, the environment where the plant will be grown and the species susceptible to the effects of the introduced gene This amounts to a case-by-case analysis.

The Basic Issues In Non-target Organism Risk Analysis are as follows:

1. The non-target organism endpoints of concern for PIPs are initially based on containment of the gene product to the modified plant (except for pollen dissemination).

2. This containment limits the exposure of the gene product to non-target organisms which are expected to interact with the plant.

3. Where exposure occurs, the possible toxicity to non-target organisms is evaluated through non-target wildlife testing.

4. If toxicity to the non-target wildlife in question is observed, then the amount of exposure is determined to ascertain if adverse effects to any non-target could occur under field conditions.

In addition, the concentration of the active ingredients in plant tissues, soil residues and their degradation rates, are also measured to more accurately determine exposure of non-target organisms.

The Agency believes that the single species laboratory toxicity testing at doses 10X to 100X the levels encountered in the environment (using a “safety factor”) represents a reasonable approach to evaluating risk related to the use of biopesticides, and is one in which negative results at high exposures would allow a high degree of confidence in the safety of the test agents. Where testing shows a hazard at doses approaching the expected environmental concentrations, a determination of the LD50, LOEC and NOEC is required. If necessary, higher tier testing (to assess population level effects) is performed.

B. Non-Target Organism Data Requirements

The following basic ecological effects testing requirements on representative non-target terrestrial and aquatic species listed in the OPPTS Harmonized Pesticide Test Guidelines are addressed by submission of data or waiver requests with credible justification:

The testing required by the OPPTS Harmonized Pesticide Test Guidelines is as follows:

Tier I:

- **Avian oral toxicity test** (on upland game bird and waterfowl species) (A 6 week broiler dietary study for chronic exposure effects has also been added)

- **Wild mammal oral toxicity test** (rodent species)

- **Freshwater fish oral toxicity test** (on cold water and warm water species) (Including farmed fish feeding studies for chronic exposure effects)

- **Freshwater invertebrate testing** (on Daphnia or aquatic insect species)

-**Estuarine and marine animal testing** (grass shrimp and fathead minnow species)

- **Non-target plant studies** (for terrestrial, aquatic and out-crossing issues)

- **Honey bee testing** (for larval and adult bee toxicity)

- **Non-target insect testing** (on predators and parasites, most commonly green lacewing larvae, the ladybird beetle, a parasitic wasp and soil

invertebrates).

- **Tritrophic testing of selected beneficial insects** is also being submitted by the registrants.

- **Terrestrial environmental expression testing** (which is a determination of degradation rates of the proteins in soil)
(and)
- **Plant tissue expression data and degradation rates**

If the results of Tier I testing show adverse non-target species effects at field use rates, then testing of additional species and/or testing at a higher Tier level is required:

Tier II Freshwater and marine or estuarine environmental expression testing (aquatic environmental fate).

Tier III: Chronic, reproduction, life cycle and population effects (and host range) testing.

[If the results from environmental fate studies show a plant protein that is toxic to non-target species persists in the environment at significant levels, Tier III studies are designed to show effects of chronic exposure to these levels on selected fish and wildlife species. Tier III studies are also used to determine non-target effects of plant proteins designed to inhibit insect molting, reproduction, disease resistance and similar properties]

Tier IV: Simulated or actual field testing (however, field scouting for non-target insect abundance is currently recommended as a Tier I test)

Tier IV studies are used to determine if there is a noticeable shift in wildlife populations under field use conditions.

OPP recognizes the potential value of Tier IV field tests as an additional check on the presence of ecosystem effects. Therefore, field data are being gathered concurrently with full-scale efficacy testing during the product development stage. This provides the opportunity to evaluate pesticidal effects (both direct and indirect) on a much broader spectrum of non-target species under more natural exposure conditions than is possible in single species no-choice laboratory feeding studies.

Non-target insect field studies also address the long term use effects at the ecosystem level.

C) Candidate Test Organisms

An additional factor in determining the extent of testing necessary for risk assessment is the degree of pest species specificity shown by the protein in question. This is of primary importance in assessing ecological risk. Most protein plant-incorporated protectants produce adverse effects against a specific class of target species. Careful scientific consideration on a case-by-case basis is given to the selection of non-target species to be tested in order to include species that are most likely to be susceptible. Selection of the test species for any specific plant construct is done jointly by the Agency and the registrant during pre-registration conferences. Rationale for selection is discussed and the final test species list may be smaller or greater than the guideline data requirements.

The selection of test species is limited by their availability, the ability to rear them in captivity-in sufficient quantities for testing, or their ability to survive captivity without unacceptable mortality levels.

Endangered and threatened species hazard is addressed by extrapolating toxicity from related species, and if a hazard is identified, after consultation with the Fish and Wildlife Service possible risk is mitigated by preventing field exposure.

D) Test Substances and Dosing

Wherever possible, the whole modified plant tissue or pollen is used as the dosing substance. The use of pure test substance alone may be inadequate since it does not test for inadvertent, possibly harmful changes in the plant tissue itself (i.e. unintended effects).

In addition to the general data requirements discussed above, data derived from additional tests may be required by the Agency in order to make judgments regarding safety to non-target organisms on a case-by-case basis. Such data may also be required where special problems with Tier I testing are encountered. Test methods will usually be derived from protocols already described or cited in OPPTS Harmonized Guidelines, or other sources, such as the OECD Guidelines, or new protocols may be developed on a case-by-case basis.

V. Environmental Fate

A. Fate and Environmental Effects of Transgenic *Bacillus thuringiensis* d-Endotoxins in the Soil

Soil organisms may be exposed to d-endotoxins from current transgenic crops by exposure to roots, incorporation of above ground plant tissues into soil after harvest, or by pollen deposited on the soil. Root exposure may occur by feeding on living or dead roots or, theoretically, by ingestion or absorption after secretion of d-endotoxin into the soil. In addition, evidence suggests that some soil components, e.g. clays and humic acids, bind d-endotoxins in a manner that makes them recalcitrant to degradation by soil microorganisms, but without eliminating their insect toxicity. Therefore, exposure to d-endotoxin bound to soil particles may also be a route of exposure for some soil organisms.

Experiments addressing the amounts and persistence of d-endotoxins in the soil have been submitted and reviewed for the current registrations. A number of publications in the scientific literature have also addressed the degradation of Cry proteins in the soil. These experiments consist of the incorporation of purified d-endotoxin or transgenic plant material in soil in a laboratory setting. Cry protein half-life studies were submitted for registration for corn containing Cry1Ab, and published studies were available for Cry1Ac cotton. Cry1Ab produced estimated degradation rates of 1.6 days for the Cry protein as expressed in transgenic corn tissue and 8.3 days for the purified protein (Sims and Holden 1996). Data produced by Monsanto for Cry1Ac protein and transgenic Cry1Ac in cotton give degradation rates of approximately 9-20 days for the purified protein, and 41 days for the protein in cotton tissue. Published data for Cry1Ab or Cry1Ac in cotton tissue or as purified protein produced degradation rates of 2.2 to 46 days, where measurable (in 4 of 11 experiments), with degradation rates in transgenic tissue shorter than for purified protein in two of three experiments (Palm et al. 1994). Degradation rates of purified Cry1Ac in two different non-sterile soils were 22 d and 40 d (Palm et al. 1994). None of the studies discussed above have been performed under field conditions, although most have used field soil in laboratory microcosms.

Several studies indicate that Cry proteins bind to clays and humic acids (Crecchio and Stotzky 1998, Koskella and Stotzky 1997, Tapp and Stotzky 1995, Tapp and Stotzky 1998, Stotzky 2000a). The results of these studies suggest that this binding slows the rate of microbial degradation of these toxins compared to when these soil components are not present (Stotzky 2000a). However, this protection is not absolute, since degradation does in fact occur under several experimental conditions. Several factors influence either the affinity of binding or the rate of degradation. In particular, pH near neutrality generally substantially increases degradation. At pH above 5.8 to near neutrality, degradation of Cry

protein bound to clay minerals in soil was much faster than degradation at pH 4.9-5.0 (Tapp and Stotzky 1998). For example, it was found (Tapp and Stotzky 1998) that Cry toxin added to nonsterile soil containing kaolinite or montmorillonite showed little degradation even after around six months at lower pH (pH~5), while substantial degradation occurred over this time period at higher pH.

Corn does not grow well below ~pH 5.6 (Aldrich et al. 1975), and therefore most corn growing soils are expected to be at a higher pH. Potato prefers acid soils (Smith 1977), and the optimum range is pH 5.0-6.5 (Ware and McCollum 1980). The optimal range for cotton is pH 6.0-6.5 (Donahue et al. 1990). Therefore, under most production conditions, cotton and corn would not be grown on soils that would inhibit the rate of degradation compared to what is seen at near neutral pH. On the other hand, potato may be grown at soil pH levels that approach those in which a substantial reduction in degradation rates has been shown to occur. However, effects of pH on degradation rates in the range of pH 5.0-5.8, which overlaps with potato growing conditions, has not been explored.

Studies have shown a substantial degradation (loss of biological activity) occurring rapidly in the first several weeks, with much slower subsequent breakdown (Tapp and Stotzky 1998, Palm et al. 1994, Palm et al. 1996). These experiments suggest that testing for persistence in the field should be determined over sufficiently long periods to assure an accurate assessment of degradation.

Many of the experiments examining persistence of Cry proteins reported in the published literature have apparently been conducted in bulk soils or soil components. Bulk soil generally does not support populations of microorganisms as high as in the rhizosphere or where plant residues are incorporated into the soil. Therefore, degradation rates under field conditions may be higher than those shown in bulk soil experiments.

It is important to consider that a number of factors are expected to influence persistence under actual field conditions, including: humic acid and clay content of the soil, clay type, pH, moisture, soluble ion content and type, and temperature, all of which affect microbial activity, composition, and population levels. These factors may also may affect binding affinity of Cry proteins for soil components. Since these factors may vary considerably in the field, persistence of Cry proteins could likewise vary considerably. However, the conditions examined by the registrants generally replicate common field soil conditions, although performed in a laboratory setting. Consideration should be given to performing field tests of degradation under a range of conditions that may be expected under actual *Bt* crop cultivation.

A previously unconsidered issue regarding exposure of soil organisms to Cry proteins concerns the possibility that one of the *Bt* crops, specifically *Bt* 11 corn, exudes Cry1Ab protein into the soil (Saxena et al. 1999). Exudation would likely cause continuous exposure of soil organisms to Cry1Ab protein. This situation differs from previous risk assessment considerations, which examined the effects of a single incorporation of *Bt* plant material or Cry protein, as would occur with incorporation at the end of the growing season. It is also possible that soil organisms could be exposed to higher levels of Cry1Ab than would occur with a single incorporation. Finally, since only *Bt*11 corn was examined, it is unknown whether the proposed exudation could occur in other *Bt* crops.

In the March 12, 2001 SAP Report No. 2000-07 on *Bt* Plant-Pesticides Risk and Benefit Assessment, the October 2000 Scientific Advisory Panel (SAP) concluded that published data at that time did not adequately address the persistence of Cry proteins from *Bt* crops in the soil. Since it is difficult to correlate the relevance of the published laboratory studies to field situations, the SAP recommended field studies be conducted in established *Bt* fields in a variety of soil types and climatic conditions. The SAP suggested that the amount, accumulation and persistence of biological activity of Cry proteins in the soil are areas that should be investigated. However, the SAP also concluded that this data was not necessary for an EPA preliminary risk assessment but may be needed for a final assessment. In general, the Panel believed that studies on the mechanism Cry proteins enter soil (e.g.,

secretion, shedding of root hairs, degradation of biomass pollen) were primarily of academic interest. Knowledge of the potential environmental impacts is the important issue.

B. Exposure of Non-Target Organisms to Cry Proteins in the Soil

Most available data do not suggest toxic effects on non-lepidopterans from Cry1Ab or Cry1Ac proteins, or of non-coleopterans by Cry3A, even at doses from about 102 to 10⁴ fold higher than the amounts estimated from the Saxena et al. paper. The *Bt*11 Cry1Ab truncated protein was tested by the registrant on two species of Collembola (*Folsomia candida* and *Xenylla grisea*) and one species of earthworm (14 days) at 200 µg Cry1Ab/g feed with no observable effects. A published longer term continuous feeding study using transgenic cotton containing either Cry1Ab, Cry1Ac, or potato containing Cry3A at an estimated concentration of 10-20 PPM were performed on the collembolan *Folsomia candida* and the orbatid soil mite *Oppia nitens* (Yu et al. 1997) for 7-8 weeks, and offspring examined for developmental effects. No adverse effects were detected in these studies for any of the three Cry proteins. Therefore, high dose and continuous feeding studies on several invertebrates, including several important soil species, do not indicate likely adverse effects on non-lepidopteran species in the field.

The route of exposure of non-target organisms must also be considered. In the case of significant root exudation of Cry protein, some differences in exposure compared to single incorporation of transgenic plant material may be expected. Organisms that pass soil through their digestive systems, such as earthworms, could be exposed to higher levels of Cry protein due to exudation compared to a single incorporation of plant material. Organisms at higher trophic levels that feed on soil feeders may, secondarily, be exposed to toxin. Testing on birds and rodents however, showed that there was no toxicity to these species.

It is very difficult to determine the importance of shifts in the structure of microbial soil populations unless these changes can be associated with measurable ecological parameters. In most cases, such research has not been performed for microbial populations. Limited data do not indicate that Cry proteins have any measurable effect on microbial populations in the soil. No observable effects on microbial populations were found due to Cry1Ab or Cry1Ac protein exposure for up to 28 days for the former and 56 days for the latter in a single incorporation of 50 ng/g soil (Donegan et al. 1995). Small transient changes in microbial populations have been associated with some *Bt* transgenic plant material (cotton) rather than the transgenic Cry protein itself in experiments where transgenic plant tissue was used (Donegan et al. 1995). The authors of the study suggested that the process of making the transgenic plant, e.g tissue culture which may introduce genotypic changes unrelated to the transgene, may have been responsible for these temporary population shifts. Other work comparing transgenic Cry3A potato and the non-transgenic isoline found no significant difference on the populations of several groups of soil microorganisms including fungal species diversity and three plant pathogenic fungi in this season long field study (Donegan et al. 1996).

C. Exposure of Soil Organisms to Cry Proteins from In Situ Roots or Incorporated Plant Tissue

Primary exposure to soil organisms has been considered to be from incorporation of crop residues at the end of the growing season, or to a lesser extent from deposition of pollen onto the soil. Therefore, degradation and possible accumulation of Cry proteins has been examined by determining degradation rates of Cry proteins, either in isolation or as expressed in the plant tissue, incorporated at a single point in time. In addition determination of degradation rates of Cry proteins in soil is more feasible using a discreet starting point. Estimates of total Cry protein incorporated into the soil have been based upon the biomass of total plant tissue, although it is not clear whether root biomass has been included in these calculations.

In contrast, the roots of a crop may be comparable in biomass to the above ground portions

of the crop at the end of the growing season. During the growing season, soil organisms are exposed to roots and their contents. In particular, organisms that feed on living roots will ingest expressed Cry protein directly from this source. Data for expression levels of Cry proteins in the roots are not available for all registered transgenic crops. Cry3A is expressed in potato tubers at 1.01 µg/g tissue (NAS/NRC 2000). An average of 20.2 µg/g total root protein (seasonal average for actively growing corn) for *Bt*11 corn containing Cry1Ab is expressed (Rusch and Kendall 1995). Because of the variability of expression levels between transformation events, the levels of root expression in the other transgenic *Bt* crops cannot be predicted from these numbers or from expression in other tissues in those plants.

Similar to the case with above ground plant tissue, organisms that feed on root feeding organisms may be exposed indirectly and over an extended period of time. In addition, a significant amount of root tissue has been estimated to be lost during plant growth. Estimates of loss of root tissue range from about 11-72 percent of total root tissue and about 4-20 percent for rhizodeposition of insoluble root material into soil (Newman 1985). This dead tissue may consist of exuded high molecular weight materials such as root cap mucilage, root cortical cells, or whole root tissue (Newman 1985). The composition of this material is generally believed to consist largely of higher molecular weight materials, such as the structural components of the roots. It is therefore difficult to estimate whether the proportion of Cry protein in this material differs from that of living roots. Deposition of some Cry protein in the soil will likely occur during degradation of root tissue, in addition to Cry protein incorporated into the soil from the above ground parts of the crop at the end of the growing season.

As with the above ground portions of the plant, the root biomass increases during the growing season. Therefore, assuming that other factors are comparable, exposure of soil and soil organisms will be minimal early in the growing season and will increase with root volume. Therefore, the assumptions underlying a discrete, relatively short duration of exposure of both soil and soil organisms is likely to be inaccurate.

As discussed above, several soil invertebrates have been used to examine the toxicity of Cry proteins. Submitted studies have examined high dose acute toxicity to earthworms and collembola, while published studies have examined the effects of longer term exposure to collembola, orbatid mites (Yu et al. 1997) and microorganisms (Donegan et al. 1995, Donegan et al. 1996). Also discussed above is new unpublished data (Stotzky 2000b) that indicates no apparent impact on a range of soil microorganisms, total biomass, etc. Results of all these studies have revealed little or no adverse effects. The adequacy of these studies should be considered in the context of exposure during the growing season to root material, as well as incorporation of above ground material and roots at the end of the growing season. In particular, studies with all Cry proteins currently found in transgenic crops, and examining adults and offspring of the collembolan *Folsomia candida* and the orbatid mite *Oppia nitens*, both detritus feeders, showed no adverse effects at expression levels at or above those found in the above ground parts of the plant (Yu et al. 1997). An additional study conducted by Saxena and Stotzky (2001) reported that *Bt* Cry protein released from root exudates and biomass of *Bt* corn has no apparent effect on earthworms, nematodes, protozoa, algae, bacteria, actinomyces and fungi in soil in spite of the fact that enough detectable Cry protein is bound to soil particles to show toxicity to the target pest. In addition, toxicity studies of above ground invertebrates, discussed elsewhere, also show few adverse effects to taxa that are not closely related to those known to be affected. While not soil organisms themselves, these data generally suggest the lack of substantial adverse effects to soil invertebrates.

Available studies on the impact of transgenic Cry producing plants indicates that adverse effects on soil microorganisms are unlikely. No effects have been seen due to the protein itself, and only a minimal, transient increase observed in soil microbes attributed to the transgenic cotton plant tissue rather than the Cry protein expressed in that tissue (Donegan et al. 1995). No adverse effects have been observed in a similar season long field study with Cry3A potato (Donegan et al. 1996).

D. Horizontal Transfer of Trangenes From *Bt* Crops to Soil Organisms

Concern has been expressed about the possible transfer of transgenes from crop plants to related or unrelated species of plants or other organisms. Also of concern is the possibility of horizontal gene transfer, that is transfer to unrelated species. Horizontal gene transfer regardless of the source of the gene, has been considered in the literature, and evidence presented for possible impact on the evolution of organisms over long periods of time (Smith et al. 1992). However, the likelihood of such transfer over a human time scale, and where the transgene has a positive impact on the fitness of the recipient, has not been demonstrated. Horizontal transfer of several traits might be of concern for the current *Bt* crops, for example the cry genes and antibiotic resistance genes.

There is no evidence that horizontal gene transfer occurs from plants to microbes or bacteria to bacteria. *Bt* genes which naturally occur in many soils have never demonstrated horizontal gene transfer. The October 2000 SAP concluded that the "horizontal gene transfer assessment appear to be adequate, and no additional data are probably necessary for the Agency to complete a risk assessment."

E. Determination of Cry Protein Levels in Soils Following Several Years of *Bt* PIPs

In the March 12, 2001 SAP Report No. 2000-07 on *Bt* Plant-Pesticides Risk and Benefit Assessment, the October 2000 Scientific Advisory Panel (SAP) concluded that published data at that time did not adequately address the persistence of Cry proteins from *Bt* crops in the soil. Since it is difficult to correlate the relevance of the published laboratory studies to field situations, the SAP recommended field studies be conducted in established *Bt* fields in a variety of soil types and climatic conditions. The SAP suggested the determination of the amount, concentration, and persistence of biological activity of Cry proteins in the soil are areas that should be investigated. The EPA agrees with the SAP that actual field data or Cry protein levels in soil will yield relevant data on persistence and natural variation of plant-produced *Bt* proteins in soil. If high levels of Cry proteins are found in field soils reevaluation of the risks to certain non-target organisms might be required. Therefore, EPA is requiring additional supplementary studies regarding Cry protein in soil.

The Agency is requiring testing of Cry1Ab and Cry1F protein under a range of conditions typical of *Bt* corn cultivation. EPA requires each registrant or the registrants in cooperation to submit test protocols before the studies are actually conducted. In general, the Agency anticipates that soils would be sampled from fields where *Bt* corn has been grown continuously for at least 3 years compared with fields where no *Bt* crop has been grown. These paired fields would include several locations throughout the corn growing area of the US representing different soil and climatic variations. The Agency anticipates that samples would need to be taken 2 or 3 times during the growing season.

VI. Insect Resistance Management in *Bt* Crops (*Bt* Crop IRM)

(*Chapter VI is based on a presentation given at the OECD Living Modified Organisms and the Environment conference held November 27-30, 2001 in Research Triangle Park, North Carolina*)

A. 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. 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. Academic and government scientists, public interest groups, and organic and other farmers have expressed concern that the widespread planting of genetically transformed plants will hasten the development of resistance to *Bacillus thuringiensis* (*Bt*) endotoxins. Sound IRM will prolong the life of *Bt* pesticides and

adherence to the plans is to the advantage of growers, producers, researchers, and the American public. EPA's strategy to address insect resistance to *Bt* is twofold: 1) mitigate any significant potential for pest resistance development in the field by instituting IRM plans, and 2) better understand the mechanisms behind pest resistance. IRM is important for transgenic crops expressing *Bacillus thuringiensis* (*Bt*) insecticidal proteins (*Bt* crops) because insect resistance poses a threat to future use of microbial *Bt* pesticides and *Bt* technology as a whole.

The goal of IRM is to have the target pest continue to be susceptible to the pesticide. Each IRM program consists of strategies to reduce the likelihood that insect resistance will develop and strategies to manage insect resistance once it occurs. *Bt* IRM is of great importance because of the threat insect resistance poses to the future use of *Bt* microbial pesticides and *Bt* plant-incorporated protectants. Specific IRM strategies, such as the high dose/structured refuge strategy, will mitigate insect resistance to specific *Bt* proteins produced in corn, cotton, and potatoes. Effective insect resistance management can reduce the risk of resistance development.

At a Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP)1 meeting in March of 1995, EPA laid out a multi-faceted program that the agency considered appropriate for *Bt* crop products. 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.

B. Scientific Basis for an 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.

Current 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. This standard

has been used by EPA since 1998.

To be effective, an IRM plan must be specific to the target pest, the crop, and to the class of pesticide being used. The use of a high dose strategy is often not the best approach for chemical pesticides sprayed on a crop and pesticide rotation is one of the preferred approaches. Annually rotating between *Bt* crops and non-*Bt* crops treated with conventional insecticides is likely to wipe away many of the environmental benefits of increased non-target organisms we are seeing in areas where *Bt* crops are frequently grown.

C. Specific Refuge Requirements

The Agency has determined that a 20% non-*Bt* field corn refuge requirements for *Bt* field corn grown in the Corn-Belt and a 50% non-*Bt* corn field refuge requirements for *Bt* field corn grown in cotton-growing areas is scientifically-sound, protective, feasible, sustainable, and practical to growers. EPA believes that the use of predictive models provides confidence that resistance will not evolve to any of the target pests (i.e., European corn borer, corn earworm, southwestern corn borer, fall armyworm, and other stalk-boring pests) under the time frame of the registrations.

For *Bt* sweet corn, no specific refuge requirements are necessary because sweet corn is typically harvested much earlier than field corn, 18-21 days after silking, and before most lepidopteran larvae complete development. However, to mitigate the development of resistance, EPA has determined that crop residue destruction is necessary within 30 days. This practice will likely destroy any live larvae left in *Bt* sweet corn stalks and prevent overwintering of any resistant insects.

At this time, the Agency believes that available empirical data substantiate the success of the 5% external unsprayed, 20% external sprayed, and 5% embedded structured refuge options to delay insect resistance to *Bt* cotton. However, 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. Because of the greater risk of resistance development, the external, unsprayed option expires after three growing seasons (September 30, 2004).

Presently, the Agency is mandating additional improvements to the current *Bt* corn (field and sweet) and *Bt* cotton IRM programs that will require: 1) anyone purchasing *Bt* corn and *Bt* cotton to sign a grower agreement which contractually binds the grower to comply with the IRM program and that there will be a mechanism by the year 2003 by which every grower affirms their contractual obligations to comply with the IRM program, 2) an ongoing IRM education program, 3) an ongoing IRM compliance monitoring program including a third party compliance survey and mechanisms to address non-compliance, 4) and ongoing insect resistance monitoring program for each target insect pest, 5) remedial action plans to be implemented if resistance does develop, and 6) annual reporting of the IRM (and other) activities. No other pesticide products than the *Bt* crop products have such extensive IRM requirements.

In considering the new IRM regulatory program for *Bt* plant-incorporated protectants (PIPs), EPA considered not only the science, but also factors such as grower costs and compliance, resistance monitoring, and remedial action if resistance should occur. EPA considered four important areas: Farmer Actions, Compliance Monitoring, Insect Resistance Monitoring, and Remedial Action Plans. Each of these areas was considered in making the decision to continue the registrations of the *Bt* PIPs.

D. Farmer Actions

Farmer adoption of IRM requirements is critical to the long-term, sustainability of IRM

strategies for *Bt* crops. The first essential farmer action is to become familiar with the IRM requirements for his (or her) *Bt* crop. Without farmer implementation of appropriate IRM strategies then pest resistance cannot be mitigated. Each farmer must sign a contract or grower agreement indicating that the farmer will abide by the IRM requirements and the farmer receives a technical bulletin describing the latest requirements. Educating the farmer also includes making sure that the farmer is aware of any changes that have occurred since he/she last grew the *Bt* crop, whether it was last year or two or more years ago. Educational materials are provided by each company and education sessions are held by the companies, sometimes by the seed dealer, the commodity group, and often by the United States Department of Agriculture's Cooperative Extension Service. Information is also provided through the internet and through newsletters and other media. Once educated to the requirements, it is the farmer's responsibility to plant and manage the refuge. It must be the correct size (such as 20 percent non-*Bt* corn to 80 percent *Bt* corn in the Corn Belt), it must be placed at the correct distance so that any *Bt*-resistant insects coming from the *Bt* crop will easily find mates from the *Bt*-susceptible insects coming from the refuge, and the farmer must plant a refuge using a crop variety compatible to the *Bt* crop in the time that adults would emerge from the refuge and the *Bt* crop. Farmers also play an important role in supplementing monitoring by reporting any failure of the *Bt* crop to control the target pest. Because farmers pay an extra fee when they buy the *Bt* seeds, they have an incentive to complain to the company who sold them the seed if it is not preforming correctly. An important farmer action in the overall IRM program, is that the farmer cooperate and accurately respond to questionnaires and surveys on what activities the farmer has actually done.

E. Compliance Monitoring Program

EPA recognizes that compliance is a complex issue for *Bt* crops and IRM; therefore, a balance must be achieved between refuge size and deployment with grower compliance. Currently, the financial burden of implementing refuge requirements is borne primarily by the growers. Increasing refuge size and/or limiting refuge deployment to better mitigate the risk of resistance will likely increase costs to growers and result in a higher rate of grower non-compliance.

The *Bt* crop reassessment has greatly strengthened the compliance monitoring to increase the likelihood of IRM adoption, to measure the level of compliance, and to institute penalties should non-compliance become a significant problem (see terms and conditions of registration).2 Until recently, monitoring for farmer compliance to the IRM program has been largely voluntary, but now it is mandatory. Key to this program is the grower agreement between the company and the farmer indicating the farmer will abide by the IRM requirements. Although it varies a bit by crop, the program is basically a tiered approach of actions depending on the results of a grower survey. The survey is conducted by a third party, independent organization using funds provided by the companies. The survey questions are developed in consultation with academic and government researchers knowledgeable on the subject and there is an EPA review of the survey also. The survey focuses on areas of highest risk, typically those areas of highest adoption. If the survey indicates that an area of the country is not fully complying with the requirements, increased education and more intense surveying will be implemented in that area. The degree of increased effort may be directly related to the type of problem. For example, if bad weather conditions cause farmers to plant their refuge late, that is quite different than if farmers in an area decide it is unimportant to plant the refuge at all. In addition, typical on-farm visits conducted by the companies and/or their representatives will report on farmers who are or are not complying with the requirements and what follow-up actions need to be taken. Any farmer determined to be out of compliance, will automatically receive an on-farm inspection the following year. If that farmer is still found to be significantly out of compliance, that grower would be denied the use of the *Bt* crop the following year. While that farmer may be able to buy the technology the third year, that farmer would again automatically receive an on-farm visit during that growing season and if that farmer again is out of compliance, the farmer would be denied the use of the technology permanently.

F. Insect Resistance Monitoring

Monitoring has been part of the requirements of the *Bt* crop products registration from the beginning. The 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 program includes monitoring for the important target pests. 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 the ability to differentiate between a natural variation in the population and a trend indicating that resistance is likely to happen soon or may have already happened. In one of the 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 versus confirmed resistance. An additional consideration is the time required to "confirm" resistance.

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 that attract the moths or larvae 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 susceptibility to the insect toxin.

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. 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 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, when the allele is recessive (as often is the case) and 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 and cotton bollworm, pink bollworm, and European corn borer. Additional sampling for southwestern corn borer is focused in those areas of the Corn Belt in which this pest is an economic problem. 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.

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.

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 X 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.

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 *Bt* cotton in the 2000 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. There were approximately 20 million acres of *Bt* corn planted in the 2000 growing season. 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 Nebraska for European corn borer, University of Arizona for pink bollworm, University of Missouri for southwestern corn borer, and USDA/ARS at Stoneville, Mississippi for tobacco budworm and cotton bollworm).

G. Remedial Action Plan

EPA requires 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 for cotton bollworm and 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, and key economic pests of cotton in the mid-South and Southeastern US. There is also a general remedial action plan to address resistance to European corn borer, southwestern corn borer, and corn earworm.

H. Conclusion: Balancing the Four IRM Activities

The four IRM activities described above (farmer actions, compliance monitoring, insect resistance monitoring, and remedial action plans) need to be balanced. To some extent, they are at least partial substitutes for each other. In other words, if the refuge is extremely large (95%), there is virtually no need to monitor for insect resistance because resistance is so unlikely to occur. However, having a 95 percent refuge would eliminate many of the benefits to growers and the environment for cotton growers. Monitoring every *Bt* field for insect resistance reduces the need for a compliance program, but such an intensive effort is infeasible and extremely costly. In our regulation of these *Bt* products, EPA has attempted to balance these activities. EPA believes that the increased quality and substance of the compliance monitoring and resistance monitoring programs required through our completed reassessment can compensate to some extent for the small refuge size for *Bt* cotton. In addition, EPA has required additional data on the effect of alternate plant hosts and alternative modes of actions on delaying cotton bollworm resistance to *Bt* cotton. EPA believes that technological improvements to detect resistance earlier in the field will result in scientifically valid methods that will be cost-effective for insect resistance management in the future. Our faith in improvements ahead comes from knowing that academic, company, and government research continues to be strong in the area of IRM.

1. See http://www.epa.gov/scipoly/sap for the reports of the Scientific Advisory Panel and http://www.epa.gov/pesticides for the meeting notes from the Office of Pesticide Programs Pesticide Program Dialog Committee. Scientific Advisory Panel meetings related to biotechnology can also be found through links from the Biopesticides web page at http://www.epa.gov/pesticides/biopesticides.

2. Sections III and V of the *Bt* Crops BRAD at http://www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm

VII. Benefits

Presently in development

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