Bacillus thuringiensis Cry2Ab2 protein and the Genetic Material Necessary for Its Production in Cotton (006487) Fact Sheet

I. Description of the Plant Pesticide

Bacillus thuringiensis Cry2Ab2 protein and the Genetic Material Necessary for Its Production in Cotton

- **OPP Chemical Code:** 006487
- **Trade Name:** Bollgard II®
- **Year of Initial Registration:** 2002
- **Pesticide Type:** Plant-incorporated Protectant
- **U.S. and Foreign Producers:**

  Monsanto Company
  700 Chesterfield Parkway North
  St. Louis, MO 63198

II. Use Sites

Full Commercial Use in Cotton.

III. Registration

Bollgard II®, the new biotech cotton plants were developed using particle acceleration plant transformation procedures to insert the Cry2Ab2 insect control gene into an existing Bollgard cotton variety expressing the Cry1Ac protein.

On 6/14/02, BPPD granted Monsanto Company a seed increase registration for Bacillus thuringiensis Cry2Ab2 protein and the genetic material necessary for its production in cotton, EPA Reg. No. 524-422. This was a plant propagation registration.

On 12/23/02, BPPD amended this registration to allow for a full commercial section 3 registration which granted unlimited acreage for planting. This registration is set to expire May 1, 2004.

IV. Science Assessment

Monsanto's Bollgard II Cotton

Several methods were used to confirm that the identity of the Cry2Ab2 protein produced by fermentation and used in the toxicity testing was the same as that produced in the cotton plant. The EC50 and LC50 with a pest insect (*Helicoverpa zea*), protein purity, and protein stability were determined. The protein as tested appeared to have the expected molecular weight (a 63 kDa protein band) by gel electrophoresis, was relatively pure, immunoreactive with appropriate antibodies, and stable through the 87 day time point. The tested substance was lyophilized Cry2Ab2 protein powder (Lot# 6312829) isolated from *Bacillus thuringiensis* strain EG7699.

A solution of Cry2Ab2 protein (approximately 1, 2, and 3 µg total protein) was applied to a polyacrylamide gel (4->20%) run under reducing conditions. Molecular weight markers were used to determine the weight of the Cry2Ab2 and contaminant proteins. Densitometric analysis was used to determine the relative percent of Cry2Ab2 protein and contaminant proteins. Protein molecular weight was estimated by comparison to marker proteins. The purity of the ~63 kDa protein (Cry2Ab2) was estimated to be 65.5% of total protein.

A. Human Health

1. Mammalian Toxicity

Monsanto submitted information which adequately described the Cry2Ab2 delta-endotoxin from Bt, as expressed in cotton, along with the genetic material necessary for its production. Because it would be difficult, or impossible, to extract sufficient biologically-active toxin from the plants to perform toxicology tests, Monsanto used delta-endotoxin produced in bacteria. Product analysis data was submitted to show that the microbially expressed and purified Cry2Ab2 delta-endotoxin is sufficiently similar to that expressed in the plant to be used for mammalian toxicological purposes. Plant and microbially produced Cry2Ab2 delta-endotoxin were shown by these studies to have similar molecular weights and immunoreactivity (SDS-PAGE and Western blots), to lack detectable post-translational modication (glycosylation tests), to have identical amino acid sequences in the N-terminal region and to have similar results in bioassays against Heliothis virescens and Helicoverpa zea. While it is difficult to prove that two proteins are identical, the combined results of the above studies indicate a high probability that these two sources produce proteins that are essentially identical by available protein analytical assays.

2. Toxicology Assessment

The data submitted and cited regarding potential health effects for the Cry2Ab2 protein include information on the characterization of the expressed Cry2Ab2 delta-endotoxin in cotton, the acute oral toxicity, and the in vitro digestibility and heat stability of the delta-endotoxin. The results of these studies were determined to be adequate to evaluate human risk and the validity, completeness, and reliability of the available data from the studies were considered.

There is a reasonable certainty that no harm will result from aggregate exposure to the United States population, including infants and children, to the Cry2Ab2 protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information.

The data submitted regarding potential health effects of Cry2Ab2 include information on the characterization of the expressed protein in cotton. The acute oral toxicity data submitted support the determination that the Cry2Ab2 protein is non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, et al., 1992). Since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry2Ab2 delta-endotoxin protein is not considered toxic. Because these proteins break down into their constituent amino acids almost immediately upon ingestion, there would be no chronic exposure to the protein and therefore no need for chronic toxicity testing. Because there is no chronic exposure, the mutagencity, developmental toxicity, subchronic toxicity, chronic exposure and oncogenicity assessment studies are not required.

The Agency has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity to these plant-incorporated protectants, there are no cumulative effects.

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectants residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to

negligible. Oral exposure, at very low levels, may occur from ingestion of processed products and drinking water. However, a lack of mammalian toxicity and the digestibility of the plant-incorporated protectants has been demonstrated.

3. Tolerance Exemption Conclusions

All active and inert ingredients resulting from the use of Bollgard II are currently covered by the following tolerance exemptions:

Bacillus thuringiensis Cry2Ab2 protein and the genetic material necessary for its production in corn or cotton are exempt from the requirement of a tolerance when used as a plant-pesticide in the food and feed commodities of corn, sweet corn, popcorn, cotton seed, cotton oil, cotton meal, cotton hay, cotton hulls, cotton forage and cotton gin byproducts.[40 CFR 180.1215; 66 FR 24066, May 1, 2001]

Bacillus thuringiensis subspecies *kurstaki* CryIA(c) delta-endotoxin and the genetic material necessary for its production in are exempt from the requirement of a tolerance when used as a plant-pesticide in all plant raw agricultural commodities.[40 CFR 180.1155; 62 FR 17722, April 11, 1997]

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry 2Ab2 protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because no toxicity to mammals has been observed for the plant-incorporated protectants and anticipated exposures are negligible.

B. Gene Flow Potential

EPA has reviewed the potential for gene capture and expression of the B.t. d-endotoxin in cotton by wild or weedy relatives of cotton in the United States, its possessions or territories. There is a possibility for gene transfer in locations where wild or feral cotton relatives exist. Therefore, EPA requires stringent sales and distribution restrictions on B.t. crops within these areas to preclude hybridization of the crop with sexually compatible relatives. Additionally, research plots and breeding nurseries have isolation or mitigation requirements to reduce the likelihood of cross-pollination with feral or indigenous populations of sexually compatible species.

C. Environmental Fate

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.

A Cry protein DT50 (time to 50% degradation) study was submitted for registration of Bollgard II cotton containing Cry2Ab2 and Cry1Ac (MRID 453371-01). According to this study, Cry2Ab2 + Cry1Ac proteins degrade rapidly in this sandy loam soil (typical soil type for cotton production). The DT50 was 2.3 days, DT90 was 15 days, and 75% of the protein degrades in the first week of incubation. However, this study uses the cotton bollworm (Helicoverpa zea) as the indicator species in the insect bioassay. The cotton bollworm is not as sensitive to Cry2Ab2 as other lepidopterans and it is less sensitive to Cry2Ab2 than Cry1Ac. However, the presence of Cry1Ac was not considered in the data analysis. An accurate degradation time (DT50) cannot be determined from this study since there is not a high dose of Cry2Ab2 or Cry1Ac expressed to control the cotton bollworm.

D. Ecological Effects

The Agency has determined that the non-target organisms most likely to be exposed to the protein in transgenic cotton fields were beneficial insects feeding on cotton pollen and nectar, upland birds feeding on cotton seed and soil invertebrates. Thus, toxicity tests were required utilizing representatives of those organisms. The toxicity of the Cry2Ab2 protein has been evaluated following challenge of several species of vertebrates and invertebrates, including: northern bobwhite quail, catfish, adult and larval honeybees, a parasitic hymenopteran (Nasonia), green lacewings, ladybird beetles, Daphnia, earthworms, and collembola. Waterfowl, freshwater and estuarine/marine fish, and aquatic invertebrate tests were waived due to lack of substantive exposure. Aquatic invertebrate testing was performed with cotton pollen containing Cry2Ab2 protein since cotton pollen may drift into the aquatic environment. Since Cry2Ab2 is an insect toxin that has never shown any toxicity and/or pathogenicity to plant species, terrestrial and aquatic plant studies have also been waived.

Wild mammal hazard assessment is being performed on the basis of rodent toxicity data prepared for human health risk assessment purposes. The data submitted to the Agency indicate no toxicity to rodents during the acute oral testing at the maximum hazard dose. These data show a lack of toxicity to mammals from exposure to high levels of Cry2Ab protein. Therefore no further wild mammal testing is required.

Avian Testing

This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on Harmonized Test Guideline Series 885.4050 Nontarget Avian Testing, Tier I. The study is scientifically sound and no treatment mortality or behavior change was observed between the dosed and control replicates.

The dietary LC50 for Cry2Ab2 protein in cottonseed meal when fed to juvenile northern bobwhite for 5 days was determined to be greater than 100,000 ppm diet. The no observed effect concentration was 100,000 ppm. These data show that there will be no adverse effects on avian wildlife from incidental field exposure to Cry2Ab2 protein. These data, however, are not sufficient to make a hazard assessment from repeated avian exposure to higher doses of Cry2Ab2 in their diet. A 10% cottonseed meal in the diet is not representative of all poultry diets. Prior to full commercial section 3 registration, a six week study with appropriate proportions of cottonseed meal in the diet is requisite to assess hazards to domesticated fowl from continuous exposure to higher levels of Cry2Ab2 protein. Therefore this study is classified as supplemental.

Freshwater Fish Testing

This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This is a non-guideline study based on Nontarget Freshwater Fish Testing (Harmonized Test Guideline Series 885.4200), Tier I.

The dietary LC50 and the NOEC for Cry2Ab2 protein in cottonseed meal when fed to channel catfish for 8 weeks was determined to be greater than 20% of diet. The data indicate that cottonseed meal derived from genetically modified cotton lines,15813 and 15985 (Cry2Ab2) can be used as a feed ingredient in channel catfish diets up to levels of about 20% without adverse effects on fish growth, feed conversion efficiency, survival, behavior, or body composition. These adverse effects may be due in part to the significant reduction in the concentration of the Cry2Ab2 protein in the modified

cottonseed as compared to raw cottonseed prior to commercial processing of cottonseed (toasting). A similar study performed with corn meal which contained Cry2Ab2 protein that was not denatured (MRID 450863-19) showed no adverse effects on catfish.

Aquatic Invertebrate Acute Toxicity Testing

The study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Parts 160 with certain exceptions that did not affect the integrity of the test. The testing was conducted based on Static-Renewal-Acute Toxicity Test with the Cladoceran (Daphnia magna) Harmonized Test Guidelines, Series 850.1010.

During the 48-hr exposure period to Cry2Ab2 containing cotton pollen, there were no observations of mortality, immobility or other behavioral effects in any of the treatments. Therefore, the EC50 is estimated to be > 120 mg cotton pollen/L and the NOEC was also > 120 mg pollen/L indicating that corn pollen containing Cry2Ab2 protein at these levels are either not available or non-toxic to Daphnia magna, a representative of aquatic invertebrate species.

Nontarget Invertebrate - Earthworm Testing

The study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Parts 160 and 792; Organization for Economic Development (OECD) Principles of Good Laboratory Practice; and Japan Ministries of Agricultural Forestry and Fisheries (MAFF), with certain exceptions that did not affect the integrity of the test. The testing was conducted based on Harmonized Test Guideline Series 850.6200 Earthworm Subchronic Toxicity Test and OECD Guideline 207

The 14-day LC 50 for earthworms exposed to Cry2Ab2 protein in an artificial soil substrate was determined to be greater than 330 mg Cry2Ab2 mg protein/kg dry soil; the no observed effect concentration was determined > 330 mg Cry2Ab2 mg protein/kg dry soil, the highest concentration tested. The study was procedurally sound and the data show that no adverse effects to earthworms are expected at Cry2Ab2 levels 12 and 83 times higher than the maximum expected environmental concentration for corn and for cotton respectively. Thus, an observable deleterious effect on earthworms is not expected to result from the growing of Cry2Ab protein containing cotton plants. This study meets current testing requirements for assessing subchronic risks to earthworms from plant-incorporated protectants derived from Bacillus thuringiensis.

Non-Target Arthropod Invertebrate Testing:

Honey Bee Larvae

This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. An acceptable study was conducted based on Harmonized Test Guideline Series 885-4380, Honey bee testing Tier I.

It can be determined from this study that the no-observed-effect concentration (NOEC) for Cry2Ab2 protein fed to honey bee larvae (Apis mellifera) is >100µg/mL (ppm) (MRID 453371-02). The test was scored for survival to capping, adult emergence, and adult survival. The larvae developed into adult honey bees normal in behavior and appearance. A NOEC could not be determined from the results of an additional study submitted for review (MRID 450863-07). However, results from this study supplement results from MRID 453371-02 in demonstrating a lack of risk from larval honey bees feeding on Cry2Ab2 protein.

Adult Honey Bee Testing

This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on Harmonized Test Guideline Series 885-4380, Honey bee testing Tier I. This study showed the no-observed-effect concentration (NOEC) for Cry2Ab2 protein fed to adult honey bees (Apis mellifera) is >68 µg/mL Cry2Ab2 protein.

Cry2Ab2 protein showed no measurable deleterious effects on honey bee larvae and adults up to the level tested.

Parasitic Hymenoptera Larva Testing

This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on Harmonized Test Guideline Series 885-4340 Nontarget Insect Testing, Tier I.

The guidelines recommend terminating the test when 20% mortality is reached in the control group or after 30 days. Since this study was terminated prematurely, an additional study should be conducted that continues for 30 days or until 20% mortality is reached in the assay control group. However, there was a high rate of morality in the assay control group; equal to the mortality in the 100 ppm potassium arsenate reference group which suggests that there was a non-treatment related effect occurring. This test should have been conducted until 20% mortality was achieved in the vehicle control group or for 30 days as described in Harmonized Test Guideline 885.4340. Due to the high rate of mortality in the assay control and 220 ppm Cry2Ab2 protein treatment group, and premature termination of the study, an LC50 could not be determined.

On April 18, 2002, Monsanto submitted a letter to the Agency requesting a waiver from parasitic Hymenoptera toxicity testing. This waiver request was based on a lack of exposure of parasitic Hymenoptera to the Cry2Ab2 protein. In addition, parasitic Hymenoptera are not expected to be susceptible to Cry2Ab2 since it is highly specific against lepidopterans and dipterans. Due to the lack of exposure and susceptibility of parasitic Hymenoptera to the Cry2Ab2 protein expressed in cotton or corn, The Agency has accepted Monsanto's request to waive this data requirement.

Green Lacewing Larva Testing

This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test.

This study was conducted based on Harmonized Test Guideline Series 885-4340 Nontarget Insect Testing, Tier I except the test was terminated when 50% pupation was reached in the assay control group. The guidelines recommend terminating the test when 20% mortality is reached in the control group or after 30 days. However, it is known that younger larvae are more susceptible to Bt proteins than older larvae. It can be assumed that adverse effects related to green lacewing larvae feeding on Cry2Ab2 protein would be observed once 50% pupation occurred. Based on this study, the no-observed-effect concentration (NOEC) for Cry2Ab2 protein fed to green lacewing larvae is >1,100 ppm Cry2Ab2 protein and the LD50 is >4,500 ppm. The NOEC represents 5.5x

the maximum concentration in corn plant material and 21.6x the maximum concentration in cotton plant material. Based on these results it can be concluded that green lacewing will not be adversely effected when exposed to Cry2Ab2 in the field.

Ladybird Beetle Testing

This study was conducted in accordance with Good Laboratory Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on Harmonized Test Guideline Series 885-4340 Nontarget Insect Testing, Tier I.

The primary route of exposure to Cry2Ab2 protein by ladybird beetle adults and larvae would be from cotton pollen ingestion. Since some of beetles in the treatment and control groups were observed to be immobile/and or lethargic, a NOEC cannot be determined from this study. However, it can be concluded that the LC50 for adult ladybird beetles feeding on Cry2Ab2 protein is >4,500 ppm which is a significantly greater level than would be encountered in the field.

This study does not adequately show that there will not be a hazard to ladybird beetle populations from Cry2Ab2 because lethargic/immobile effects were observed. In addition, ladybird beetle larvae would potentially have a higher risk of exposure to Cry2Ab2 than adults. Therefore, a dietary toxicity study should be conducted to determine the NOEC for ladybird beetle larvae.

Collembola feeding on Cotton Tissue

Although this study was not conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160, The Agency has determined that the study is scientifically valid. This study was conducted based on Harmonized Test Guideline Series 885-4340 Nontarget Insect Testing, Tier I.

This study determined that the presence of Cry2Ab2 protein was not toxic to Collembola. Cry2Ab2 protein also did not adversely affect the rate of Collembola reproduction. Mortality demonstrated in the positive control

group and observations of green digestive tracts in the other groups verified that Collembola are ingesting the test cotton tissue material. Results of this study showed the no-observed-effect concentration (NOEC) of Collembola exposed to Cry2Ab2 protein from cotton leaf tissue in the diet was >313 µg Cry2Ab2 protein/g diet. This study adequately addresses potential concerns for Cry2Ab protein expressed in transgenic cotton to Collembola (Folsomia candida) a representative of beneficial soil insect species. The results of this study demonstrate that Cry2Ab proteins found in transgenic cotton pose no hazard to soil inhabiting Collembola species, and by inference to other beneficial soil insects.

Combined effects of Cry1Ac and Cry2Ab proteins.

Bollgard II contains both Cry1Ac and Cry2Ab2 proteins. Nontarget testing with Cry1Ac (006445) and Cry2Ab2 proteins separately did not show any hazard to nontarget species. Any unexpected synergistic effects from Bollgard II which produces both Cry1Ac and Cry2Ab2 proteins are not anticipated because no adverse effects were seen in several nontarget tests (avian, earthworm and collembolla species) which were performed on tissue containing both Cry proteins.

Endangered Species Considerations

Based on the submitted Cry1Ac and Cry2Ab2 protein toxicity and exposure data there will not be a "may effect" situation for endangered mammals, birds, plants and aquatic species. The nontarget testing confirms the expectation that Cry1Ac and Cry2Ab2 protein toxicity is confined to Lepidoptera species larvae. Cotton is insect pollinated and pollen containing the Cry protein is not likely to drift out of fields. Nevertheless, relatively high Cry1Ac and Cry2Ab2 dosages were not toxic to the test species representative of organisms likely to be exposed to such pollen (e.g. ladybird beetles, green lacewings, honeybees). In addition, the larvae of endangered Lepidoptera species in cotton growing counties (Quino Checkerspot butterfly, Riverside County CA; Saint Francis' Satyr butterfly, Cumberland and Hoke Counties, NC and Kern Primrose Sphinx moth, Kern County CA) are not going to be exposed to the Cry proteins because their habitats do not overlap with cotton fields (e.g. the Quino Checkerspot butterfly is found only in the coastal sage scrub habitat in southern California, the Kern Primrose Sphinx moth is found only on a privately owned ranch in Walker Basin, Kern County, California, and the only known populations of Saint Francis' Satyr butterfly are found in wetlands dominated by sages and grasses on Government property in North Carolina) and their larvae do not feed on cotton and will not be exposed to Cry protein in pollen. The amount of pollen that would drift from these cotton plants onto plants fed upon by endangered/threatened species, would be very small compared to the levels fed to the test species. Therefore,

EPA does not expect a "may effect" scenario to any endangered/threatened species from cotton containing the Cry1Ac and Cry2Ab2 protein

E. Resistance Management

Insect resistance management strategies need to account for both Cry1Ac and Cry2Ab being pyramided in Bollgard™ II cotton lines.

Pest Biology

Knowledge of pest biology is critical for the development of effective IRM strategies. For example, refuges must be designed with a solid understanding of the target pest to maximize the production of susceptible insects and increase the likelihood of random mating between susceptible and potentially resistant pests.

TBW, CBW, and PBW differ in their impact on cotton on a regionally-specific basis. For example, in the Southeast, CBW is the predominant pest. In the Midsouth (Mississippi Delta), TBW is the most important pest; whereas, PBW is the only lepidopteran pest of importance in Arizona and California. However, there are many parts of the cotton belt in which TBW and CBW are both significant economic pests.

Key literature information (Caprio and Benedict 1996) regarding pest biology, adult movement, mating behavior, gene flow, and alternate hosts for TBW, CBW, and PBW has been reviewed previously by the Agency in its 1998 White Paper on Bt plant-pesticide resistance management (US EPA 1998) and most recently, in its 2001 Bt Plant-Incorporated Protectants Biopesticides Registration Action Document (USEPA 2001).

Based on the published research, TBW and CBW are highly mobile insects, with CBW being more mobile than TBW. Both TBW and CBW are polyphagous, but the utilization and effectiveness of alternate hosts has not been sufficient to prove that non-cotton hosts are effective refuges. PBW has limited mobility and dispersal (although it has extensive spring flights) and limited host range. Additional information is needed to further address larval and adult movement, mating behavior and dispersal, ovipositional preferences, population dynamics, gene flow, survival and fecundity, fitness costs, and the use of alternate cultivated or wild hosts as refuges. The varied cropping systems for cotton, including local and regional differences, should also be considered for evaluating the biology, ecology, and population dynamics and genetics of the target pests. This research will improve the strength and reliability of an IRM plan to effectively reduce the likelihood that TBW, CBW, or PBW will become resistant to the Cry1Ac delta-endotoxin. Therefore,

for Bollgard cotton, the Agency made the determination that some additional IRM data are needed to characterize better the impact of alternate hosts and supplemental insecticide treatments on refuge effectiveness, and north-south movement of CBW (USEPA 2001, see Section III. "Bt Cotton Confirmatory Data and Terms and Conditions of the Amendment" and Registration Decision Memorandum dated September 29, 2001). These same data requirements should also apply to Bollgard II cotton.

Insecticidal Activity and High Dose Determination

Insecticidal Activity Against Lepidopteran pests

Monsanto has provided the results of in vitro and in planta studies of the efficacy of the Cry1Ac and Cry2Ab proteins. Both proteins are highly active against the three primary target lepidopteran pests of cotton: TBW, CBW, and PBW. The level of insecticidal activity against certain pests for either Cry1Ac and Cry2Ab is summarized in Table 1 below. There are some differences in insecticidal activity of these proteins against the secondary lepidopteran pests such as fall armyworm (FAW), beet armyworm (BAW), and soybean looper (SL). Cry2Ab has some greater activity against FAW and BAW than Cry1Ac, but Cry1Ac is more efficacious against TBW and CBW (see Table 1 below).

Bollgard II cotton, which expresses both the Cry1Ac and the Cry2Ab proteins, exhibits substantially higher control of all target species than does Bollgard cotton, which expresses Cry1Ac alone. The data provided in Appendix 4 (MRID# 455457-01) indicate that the insecticidal activity of the combination of proteins is increased over either protein tested alone. These data also demonstrate that both the Cry1Ac and Cry2Ab proteins are present at consistently high levels across all plant parts for the duration of the growing season. This means that the insect pests feeding on Bollgard II cotton would be exposed to both of the insecticidal proteins simultaneously.

Monsanto (MRID# 450293-01, January 28, 2000 submission) has analyzed data involving the influence of Bollgard cotton on secondary lepidopteran pests: cabbage looper (Trichoplusia ni Hubner), soybean looper (Pseudoplusia includens Walker), saltmarsh caterpillar (Estigmene acrea Drury), cotton leafperforator (Buccalatrix thurberiella Busk), and European corn borer (Ostrinia nubilalis Hubner). Based on the analysis of Cotton Insect Loss Surveys from 1996 through 2000, no change in the secondary status of these pests was observed nationally or regionally. Further study of how Bollgard and Bollgard II cotton and insect resistance management plans have impacted or will impact secondary lepidopteran pests is recommended.

Bollgard II High Dose Determination for TBW, CBW, and PBW

Monsanto has provided laboratory studies to demonstrate that the Cry2Ab protein alone and the Cry2Ab + Cry1Ac proteins as expressed in Bollgard II produce a functional "high dose" in Bollgard II cotton for control of CBW, TBW, and PBW. These studies will be discussed below. EPA has previously concluded that a moderate, non-high dose of Cry1Ac is produced in current Bollgard lines to control CBW and a functional high dose of Cry1Ac is produced to control TBW and PBW (USEPA 1998, 2001).

The level of Cry2Ab expression measured in the ELISA is greater than 10 times the level of Cry1Ac expression seen in Bollgard II plants (mean levels were 3.5-fold greater) (see MRID# 455457-01, Appendix 4, Figure 6). This relationship is seen for all sites, sampling times, and tissue types. The expression of Cry2Ab in Bollgard II plants does not appear to compromise the expression of Cry1Ac levels. That is, the level of expression of Cry1Ac in Bollgard II cotton is essentially the same as in Bollgard cotton. Higher overall expression of Cry2Ab2 compensates for its lower unit activity against the target pests. Overall, the data suggest that the co-expression of the two insecticidal proteins, Cry2Ab and Cry1Ac, is likely to result in increased and prolonged lepidopteran activity in all tissue types, especially in the reproductive tissues.

TBW

Insecticidal activity against TBW was measured in Bollgard II cotton tissues in field trials conducted in 1998 and 1999 to assess the efficacy of Bollgard II cotton against the TBW as compared to the efficacy of Bollgard cotton using a quantitative bioassay (i.e., measured in Cry1Ac equivalents per protein-specific ELISA assays described in Greenplate 1999). The mean insecticidal activity was generally 3.5 times higher, but at least 2.5 times higher, than for Bollgard cotton in all plant tissues (see MRID# 455457-01, Appendix 4, Figures 1-5). These increased insecticidal activity levels can be seen at all sites, sampling times and in all tissue types. Lower insecticidal activity in Bollgard II tissues was observed in large leaves compared to terminal or square activity, but this activity was still higher than in any Bollgard tissue.

EPA (USEPA 1998, 2001) and two SAPs (1998, 2001) have previously concluded that the Cry1Ac in Bollgard cotton represents a high dose against TBW. Data presented by Monsanto show that the Cry2Ab protein in Bollgard II carries even more insecticidal activity than the Cry1Ac protein in Bollgard II cotton. Therefore, Cry2Ab in Bollgard II represents a high dose against TBW. Thus, Bollgard II cotton expresses a high dose of Both Cry1Ac and Cry2Ab proteins against TBW.

PBW

The relative PBW activity of Cry1Ac (LC50 = 0.006) is greater than Cry2Ab (LC50 = 0.1). PBW is more sensitive to the Cry1Ac and Cry2Ab proteins than TBW (see Table 1 above). EPA (USEPA 1998, 2001) and two SAPs (1998, 2001) have previously concluded that the Cry1Ac in Bollgard cotton represents a high dose against PBW. Data presented by Monsanto show that the Cry2Ab protein in Bollgard II carries even more insecticidal activity than the Cry1Ac protein in Bollgard II cotton. Since there is a high dose for both of these proteins for TBW, it logically follows that there is also a high dose of these same proteins for PBW. Thus, Bollgard II cotton expresses a high dose of both Cry1Ac and Cry2Ab proteins against PBW. Data by Marchosky et al. (2001) collected from field trials, conducted in 2000 to assess efficacy and yield, indicate that the Bollgard II cotton lines achieved a level of control about one order of magnitude higher than the Bollgard comparison lines (at least 99% control). In addition, data for cotton lines expressing just the Cry2Ab protein showed these lines to be as least as effective against PBW as Bollgard cotton lines containing only the Cry1Ac protein.

CBW

EPA (USEPA 1998, 2001) and two SAPs (1998, 2001) have previously concluded that the Cry1Ac in Bollgard cotton (expressing only Cry1Ac) represents only a moderate (non-high) dose against CBW. Monsanto presents three separate sets of laboratory studies to demonstrate that the Cry2Ab protein alone and the Cry2Ab + Cry1Ac proteins are expressed at a "high dose" in Bollgard II cotton for control of CBW. These three methods taken together provide a strong case that the Cry2Ab protein represents a high dose against CBW. (Sharlene Matten, Ph.D., October 24, 2002). For more information please refer to Dr. Matten's review entitled "EPA Review of Monsanto Company's Bollgard II Cotton Insect Resistance Management Plan For Section 3 Full Commercial Registration [Reg. No. 524-522; Submissions: S607615 and S620787; DP Barcode: D280082 and D285169; Case: 068818; MRID: 455457-01 and Monsanto Letter dated August 16, 2002]

Sequence Homology of Cry1A Versus Cry2A Proteins

Based on information presented by Monsanto, Cry1A and Cry2A proteins share less than 20% sequence homology. Crickmore et al. (1998) indicate that the Cry1A and Cry2A

classes are among the most divergent. Tabashnik et al. (1996) show that Cry2Aa2 clusters in a group distant from Cry1A toxins in a domain II loop on an amino sequence similarity dendogram examining cross-resistance potential of the diamondback moth. Previous work examining insect resistance to Bt indicate that when cross-resistance occurs, it occurs when the proteins are structurally similar and the insecticidal mechanisms are also similar (reviewed in Ferré and Van Rie, 2002). When proteins are dissimilar, as are Cry1A and Cry2A, it is likely that the insecticidal mechanisms would be different. Research by Jurat-Fuentes and Adang (2001) on domain II supports this conclusion. That is, toxins with low homology to Cry1A toxins in domain II loops are reasonable alternative toxins to Cry1A toxins in Bt crops or in Bt microbial formulations. Thus, lack of sequence homology supports the hypothesis that there will be a low likelihood of cross-resistance in the target insect pests for the Cry1Ac and Cry2Ab proteins.

Structural Comparison of Cry1Ac and Cry2Ab Proteins

Monsanto provides arguments that support the conclusion that the low likelihood of substantial sequence similarity between the Cry1Ac and Cry2Ab proteins suggests that there is a difference in their tertiary structure. There were two compelling pieces of information presented. Morse et al (2001) determined the three-dimensional crystal structure of the Cry2Aa toxin and defined the putative receptor binding epitope on the toxin. Their work indicates that the three-dimensional structure of Cry2A proteins are very different from Cry1A proteins. Cry2Ab (one of the toxins of interest in Bollgard II) shares 87% sequence identity with Cry2Aa (Widner and Whiteley, 1989). A second piece of evidence is provided by Kolwyck et al (2000). Their research showed that anti-Cry2Ab antibodies do not cross-react with the Cry1Ac proteins, nor do the anti-Cry1Ac antibodies cross-react with the Cry2Ab2 protein. Lack of cross-reactivity shows that the epitope binding sites for antibody recognition are different and therefore the tertiary structure is different. Lack of similar tertiary structure supports the conclusion that there will be a very low likelihood of high levels of cross-resistance in the target insect pests for the Cry1Ac and Cry2Ab proteins.

Mechanism of Action and Binding Characteristics

Cross-resistance is most likely when toxins share key structural features, which allows one resistance mechanism to confer resistance to more than one toxin. This is, if two separate Bt toxins bind to the same midgut receptor or share more than receptor , the likelihood of cross-resistance increases. In their submissions, Monsanto provides information from the literature that support the finding that Cry1Ac and Cry2A proteins do not have the same mechanism of action and binding characteristics. While some low level of cross-resistance is possible, it is unlikely that high levels of cross-resistance would be

conferred by resistance to Cry1A or Cry2A toxins because of the difference in their binding characteristics and mechanism of action.

English et al. (1994) concluded that binding characteristics of cotton bollworm to Cry1A and Cry2A toxins were different. These authors demonstrated that Cry2Aa did not bind to a specific, high affinity receptor that was capable of binding of Cry1Ac. Binding of Cry2Aa was non-saturable regardless of the amount of toxin added. Monsanto also included unpublished work by English (Monsanto letter, August 16, 2002) that examined the binding of Cry2Ab and Cry1Ac proteins to target insect gut brush border membrane vesicles (BBMV) in CBW, TBW, and PBW using the BIACORE 2000 instrument (Piscataway, NJ) and a hydrophobic sensor chip (L1). The BBMV were pretreated with 1% bovine serum albumin (BSA) prior to each assay to block non-specific protein binding. No specific binding was observed between the full-length Cry2Ab protein and any BBMV of CBW, TBW, and PBW. This research indicates that Cry2Ab, like Cry2Aa, does not exhibit specific binding kinetics in the presence of BBMV. This additional work supports the conclusion that the Cry2Ab protein, and Cry2 proteins in general, produce highly potent ion channels to compensate for binding either to themselves or to a large collection of non-specific binding sites. Proteolytic digestion experiments using BBMV isolated from CBW and TBW showed that the Cry2Ab protein does not have a trypsin- or chymotrypsin-resistance core as described for the Cry1Ac protein and other Cry1 proteins. Conversely, proteolytic treatment of the Cry1Ac protein resulted in removal of the insecticidally inactive carboxyl terminal half of the protein and a small amino terminal region to yield a stable core protein of approximately 60 kDa. Proteolysis (using trypsin) has a positive impact on the ability of the Cry2Ab protein to form ion channels. Collectively, these studies demonstrate that the Cry1Ac and Cry2A proteins differ significantly with respect to presence of a protoxin, saturable binding kinetics and pore formation.

Activity of Cry2Ab Against Cry1A-resistant Colonies

Monsanto provided a series of studies examining the activity of Cry2Ab against Cry1A-resistant colonies. This evidence indicates that when Cry1A-resistant colonies are challenged with Cry2Ab that the potential for cross-resistance is low in TBW (Appendix 1), in CBW (Appendix 2), and in PBW (Appendix 3). Based on the information presented below, there is a low likelihood of cross-resistance (especially for high levels) in the target insect pests for the Cry2Ab and Cry1Ac proteins.

Gould (Appendix 1 of MRID# 455457-01) examined the adaptation of highly-resistant or broadly-resistant TBW colonies to the Cry1Ac toxin to Cry2Ab alone or to Cry2Ab + Cry1Ac. These studies showed no survivorship of the YHD2 (>20,000-fold resistant to the Cry1Ac toxin) on cotton tissue expressing Cry2Ab or both the Cry2Ab and Cry1Ac proteins. A second colony (KCB) had lower resistance to Cry1Ac and resistance was

relatively broad-based. When these insects were place on plant tissue expressing both the Cry1Ac and Cry2Ab proteins, few or no insects survived. The few survivors did not develop beyond the first instar.

Bradley et al. (Appendix 2 of MRID# 455457-01) used one laboratory-selected CBW colony selected on Cry1Ac (13 generations) to examine potential cross-resistance. Their data indicate that for the lab-selected resistant strain, 47% survived on conventional cotton compared to 19% on Bollgard cotton. However, when the lab-selected resistant strain was tested against the Bollgard II cotton lines, less than 5% of the larvae survived. No fruit penetration was observed in Bollgard II cotton by the lab-selected resistant strain.

Work with TBW and CBW resistant (to Cry1Ac) colonies indicates that there is some low potential for cross-resistance and that there are likely to be a range of Bt resistance mechanisms. Previously, published research indicates that there is evidence for broad cross-resistance (low levels of resistance) to Cry1A and Cry2A in laboratory-selected strains of beet armyworm (Moar et al. 1995) and TBW (Gould et al. 1992). Preliminary bioassays conducted on PBW by Dennehy et al. (Appendix 3 of MRID# 455457-01) showed that resistance to Cry1Ac in a resistant PBW strain (AZP-R) does not appear to confer cross-resistance to Cry2Ab. There were no survivors of the AZP-R strain on Bollgard II cotton tissue (Event 15985, the leading event to be commercialized

Resistance Management Models for Pyramided Traits

Resistance simulation models predict that the greatest benefits of combining toxins in single plants by "pyramiding" or "stacking" are achieved when no cross-resistance occurs, when there are no fitness costs, when resistance to each toxin is rare and recessive, and when a refuge of plants without toxins are present. Modeling simulations of two-gene products predict that the resistance risk associated with a two-gene product will be significantly less than for a single-gene product (for example, Caprio 1998; Roush 1998; Hurley 2000). Monsanto concludes that modeling simulations predict that the two-gene product will have a life expectancy greater than six-fold compared to a single-gene product. This, they indicate, will add a degree of conservatism to the currently required IRM program for Bollgard.

Pyramiding relies on the idea that each protein is used individually in a way that would kill all insects susceptible to that protein, and in so doing, kills insects that are resistant to the companion protein (Roush, 1998). This has been described as "redundant killing" in the sense that most of the population is susceptible to both proteins and thus is killed twice. The extent to which the individuals that are resistant to one protein are killed by the other is central to the effectiveness of the pyramiding strategy.

Given that there are two insecticidal proteins, Cry1Ac and Cry2Ab, which have different modes of action, there is a very low likelihood of cross-resistance to Cry1Ac and Cry2Ab. Most likely, there would have to be multiple mechanisms of Bt resistance that occur in the field for Bollgard II to fail. If there is no cross-resistance, then the use of proteins jointly in a pyramided variety (assuming 70% mortality of RS heterozygotes for each protein) is considerably better in delaying resistance than the use of each protein sequentially (i.e., introduction of one protein after another) (see Roush 1998, Figure 2). These simulations indicate that a two-protein pyramid with a 5% structured (unsprayed) refuge can delay resistance for as long as if the two proteins are deployed sequentially with a 30% structured (unsprayed) refuge. That is, there is a six-fold advantage observed for the two-protein pyramid versus the single-protein sequential introductions. Thus, this conservative model illustrates the advantage of two-gene products over single-gene products as long as the control of susceptible insects is high. Based on the high dose determinations above, Bollgard II produces a high dose of Cry2Ab for control of TBW, CBW, and PBW, a high dose of Cry1Ac for control of TBW and PBW, and a moderate dose of Cry1Ac for control of CBW. This means that the control of susceptible TBW and PBW by Cry2Ab and Cry1Ac is very high; while, the control of susceptible CBW by Cry2Ab is very high and by Cry1Ac is more moderate. Even without a high dose for CBW in the case of Cry1Ac, when both the Cry2Ab and the Cry1Ac are pyramided together, Bollgard II should still have the predicted advantages of the pyramid for delaying resistance because it is expected that at least 50% of the heterozygotes will be killed (see discussion in Roush 1998). Thus, pyramiding two or more proteins into a cultivar increases the chance that at least one of the proteins will be especially favorable to resistance management. Modeling simulations predict that pyramids (with high mortality) can reduce the need for larger refuges (Roush 1998, Hurley 2000, Livingston et al. 2002). A reduction in refuge size, under the ideal conditions of the pyramid (no other single-gene products) offers growers an easier opportunity for grower compliance (Hurley 2000 and Livingston et al. 2002). A pyramid may also reduce the reliance by cotton growers on maize and other hosts as refuge for Helicoverpa species (Roush 1998).

The durability of the pyramid is dependent on when the pyramided varieties are released (see Roush 1998, Figure 4). If the initial resistance allele frequencies are still low, a greater advantage can be gained for early introduction of the pyramided varieties. For Bollgard II cotton, this means that the initial resistance allele frequencies for Cry1Ac and Cry2Ab would have to be low to maximize the greatest advantage. Bollgard cotton varieties expressing the Cry1Ac protein have been commercialized since the 1996 growing season (seven years). Research by Burd et al. (2000) in North Carolina indicated that CBW resistance to the Cry1Ac protein may be inherited as a single dominant or partially dominant trait and that the resistance allele frequency has been estimated to be 4.3 X 10-4 (Burd et al. 2001). Burd et al. (2001) also estimated the resistance allele frequency for Cry2Ab to be 3.9 X 10-4. Modeling simulations using these resistance allele

frequencies indicate greater than a 3-fold advantage for the pyramid (e.g., Cry2Ab + Cry1Ac) over the single-protein products (Cry1Ac alone (Bollgard) or Cry2Ab alone (Bollgard II segregant)), i.e., 65 generations v. 20 generations (see Roush 1998, Figure 4,).

How quickly the resistance management benefits of a two-gene product are realized will depend upon the speed of introduction. It is expected that some overlap among Bollgard cotton (one gene = Cry1Ac), Bollgard II cotton (two genes = Cry2Ab and Cry1Ac) and potentially, other transgenic Bt cotton varieties will occur in the next five or more years. Livingston et al. (2002, unpublished) used a stochastic, spatial model of population and genetic dynamics to simulate resistance evolution in CBW to both Bt corn and Bt cotton varieties that express one or two proteins in eastern North Carolina, a mixed cropping season under different scenarios over the course of 15 years. Their simulations predict that Cry2A resistance evolution is maximized when single-protein varieties expressing Cry1A and two-protein varieties expressing Cry1A and Cry2A were both available. Cry2A resistance evolution is best managed when the introduction of two-protein varieties were early rather than late because initial Cry1A resistance allele frequencies increased with the delivery date. Cry1A resistance evolution is delayed when two-protein varieties expressing Cry1A and Cry2A and single-protein varieties expressing Cry2A were available. That is, the introduction of the second protein, Cry2A, reduces the risk of resistance to Cry1A, but increases the risk of resistance to Cry2A. Cry2A and Cry1A resistance evolution was managed most effectively when single-protein varieties expressing these proteins were not commercially available. Their results suggest that two-protein minimum refuge requirements for Cry1A and Cry2Ab pyramided products may be lower than for each single-protein.

Hurley (2000) performed a bioeconomic evaluation of the gradual introduction of different Bt corn products containing single or multiple Bt proteins over 30 years. The results demonstrate that adding a second high-dose protein to an existing high-dose or moderate-dose protein decreases the risk of resistance relative to a single high-dose protein or a single moderate-dose protein when the amount of refuge is identical. Adding a second high-dose protein to an existing high-dose protein provides the greatest protection. Evaluation of Bollgard II indicates that Cry2Ab is more effective in controlling TBW, CBW, and PBW than Cry1Ac. Hurley (2000) indicates that if the second protein is more effective, the decrease in resistance to the initial protein and the increase in resistance to the second protein are larger. Thus, extending this argument to Bollgard II, because Cry2Ab is more effective than Cry1Ac, the predicted durability of this stacked product will be somewhat less than if Cry2Ab and Cry1Ac were equally effective and both were expressed at a high dose to control TBW, CBW, and PBW. Still, the overall durability of Bollgard II will be greater than if Bollgard (Cry1Ac alone) or Bollgard II segregant (Cry2Ab segregant) were introduced sequentially or in a mosaic.

Both Livingston et al. (2002) and Hurley (2000) provide simulations that predict that adding a second protein to an existing single protein variety decreases the risk of resistance to the initial protein, while increasing the risk of resistance to the new protein. Their simulations also demonstrate that less refuge is necessary to preserve the same durability for a pyramided variety than for a single-protein variety. The results of both of these analyses indicate that rapid introduction of the stacked variety will not increase the risk of resistance and will likely delay resistance than would the sequential introduction of single proteins. They also demonstrate that the benefits of introducing a stacked variety of Bt cotton declines when the two proteins are not equally effective (both are not high dose), but are still higher than either single protein introduced sequentially.

Structured Refuge

Monsanto has proposed to incorporate the use of Bollgard II cotton into the currently required refuge options: 1) 5% external, unsprayed structured refuge (must be within ½ mile of Bollgard fields and at least for Bollgard cotton. These are: 150 feet wide, but preferably 300 feet wide), 2) 5% embedded refuge (must be at least 150 feet wide, but preferably 300 feet wide), 3) 20% external, sprayed structured refuge (must be within 1 mile of the Bollgard fields), and 4) community refuge (either 5% external, unsprayed or 20% external, sprayed refuge options allowed). The current refuge options for Bollgard cotton are discussed in detail in Section III, "Bt Cotton Confirmatory Data and Terms and Conditions of the Amendment", of EPA's recent Bt Crops Plant-Incorporated Protectant Biopesticides Registration Action Document (USEPA 2001).

Based on the modeling results discussed above, the currently required IRM program for Bollgard cotton is more than sufficient for Bollgard II. That is, all three refuge options are more protective against insect resistance for the three target pests, TBW, CBW, and PBW, using Bollgard II which expresses two insecticidal proteins, Cry2Ab2 and Cry1Ac, than for either Bollgard cotton expressing just the Cry1Ac protein or for a Bollgard II segregant expressing just the Cry2Ab2 protein. While a structured refuge is still necessary for pyramiding to be effective in delaying resistance, the size of the refuge may be smaller for the two proteins deployed in a pyramid (e.g., Bollgard II expressing both Cry1Ac and Cry2Ab2) to produce a similar delay when the two proteins are deployed sequentially (e.g., Bollgard cotton expressing only Cry1Ac and Bollgard II segregant expressing only Cry2Ab) (see discussion in Roush 1998). However, because both Bollgard II and Bollgard (and other Bt cotton varieties not yet commercialized) will both be deployed commercially for some overlapping period of time, potentially more than five years, it would be prudent, conservative, practical and provides growers a uniform message regarding IRM, for Bollgard II cotton and Bollgard cotton to have the same structured refuge requirements. In addition, until there is further evidence that other hosts are proven to be suitable, only non-Bt cotton should be relied upon as refuge.

Resistance Monitoring

Monsanto states that a Bollgard II monitoring plan will be developed as an extension of the current Bollgard monitoring plan for the TBW/CBW and PBW programs. Monsanto indicates that baseline susceptibility data to the Cry2Ab (specifically the Cry2Ab2) toxin for the key pests, TBW, CBW, and PBW, were being collected during the 2002 growing season at various locations across the Cotton Belt. Monsanto will submit an interim report on the 2002 Cry2Ab2 protein baseline data to EPA for review in 2003 (Arthur, 2002). Monsanto will continue to collect baseline data during the 2003 season and submit a final report to EPA in 2004. It is recommended that Monsanto provide the baseline susceptibility data for the Cry2Ab2 toxin for the 2002 and 2003 growing seasons, establish diagnostic concentrations for testing for resistance to Cry2Ab2, and provide a detailed resistance monitoring plan for both the Cry1Ac and Cry2Ab2 toxins. It is also recommended that the current resistance monitoring requirements mandated for Bollgard be mandated for Bollgard II (see USEPA 2001, see Section III. "Bt Cotton Confirmatory Data and Terms and Conditions of the Amendment" and Registration Decision Memorandum dated September 29, 2001 for the monitoring requirements).

The need for proactive resistance detection and monitoring is critical to the survival of Bt technology. For Bollgard, Monsanto is required to monitor for insect resistance (shifts in the frequency of resistance-conferring alleles) to the Bt toxins as an important early warning sign to resistance development in the field and to determine whether IRM strategies are working. An additional value of resistance monitoring is it may provide validation of parameters used in IRM models. Effective monitoring programs should have well-established baseline susceptibility data, sensitive detection methods, and a reliable collection network. Chances of finding resistant larvae in Bt cotton depend on level of pest pressure, frequency of resistant individuals, number of samples, and sensitivity of the detection technique. Therefore, as the frequency of resistant individuals or the number of collected samples increases, the likelihood of sampling a resistant individual increases (Roush and Miller 1986). The goal is to detect resistance in an insect population before the occurrence of widespread crop failures, and if possible, in time so that mitigation practices can delay the development of resistance.

EPA has imposed specific monitoring requirements on Monsanto for its Cry1Ac plant-incorporated protectant as expressed in cotton (Bollgard™ cotton) (USEPA 2001, Section III). EPA has mandated that Monsanto will monitor for resistance and/or trends in increased tolerance for TBW, CBW, and PBW. There were approximately 5.7 million acres of Bollgard™ Bt cotton planted in the 2001 growing season and 4.5 million acres planted in the 2000 growing season (Monsanto 2002; USEPA 2001). It would be logistically and practically impossible to sample every farm that planted Bollgard™ (or in the future Bollgard II) cotton. Therefore, current resistance monitoring programs have focused

sampling in areas of highest adoption of the Bt crops as the areas in which resistance risk is greatest.

For TBW and CBW, at least 20 specific collection sites will be established in time for the 2003 growing season. Sites must be focused in areas with high risk of resistance (e.g. where adoption is at least 75% of the cotton planted in that county or parish) while overall being distributed throughout the areas where TBW and CBW are important pests with a goal of having sites in AL, LA, AR, MS, FL, VA, GA, NC, SC, TN, and TX. For PBW, collection sites must be focused in areas of high adoption, with the goal of including all states where PBW is an economic pest (i.e., AZ, CA, NM, TX). There is a sampling goal stipulated to collect at least 250 individuals from any one location with a target of least 20 locations for TBW, CBW, and PBW. The greater the number of samples and number of locations, the greater the probability that resistant individuals will be collected.

The currently required, basic detection method has been a discriminating dose/diagnostic dose bioassay system that would distinguish between resistant and susceptible phenotypes, but such tests have been criticized as being too insensitive to be able to provide early detection before resistance develops or can spread very far, especially if the alleles for resistance are rare in the insect population. Discriminating dose bioassays are most useful when resistance is common (homozygous recessive alleles, i.e., field failure levels) or conferred by a dominant allele when the resistance allele frequency is greater than 0.01 (Andow and Alstad, 1998; Andow et al., 1998). It is currently considered as one of the central components of any monitoring plan, but other monitoring methods, such as the F2 screen and DNA markers, may have value in conjunction with the discriminating concentration assay. Diagnostic concentration assays are already in use for the Cry1Ac toxin for testing for resistance development in TBW, CBW, and PBW. Monsanto recommends the use of diagnostic concentration assays to test for resistance development to the Cry2Ab toxin.

Grower Education and Compliance

Grower education and compliance are central to the success of any IRM program. Monsanto has committed to implement comprehensive education programs that would be appropriate to convey the importance of complying with the IRM program to growers of both Bollgard and Bollgard II. A detailed discussion of Monsanto's education programs and the results of grower surveys (regarding compliance, data indicate greater than 91% with size requirements) for Bollgard (since 1996) are found in the Agency's Bt Plant-Incorporated Protectants Reassessment Document (USEPA 2001, Section IID.). The grower education requirements are described in this same document (USEPA 2001, see Section III. "Bt Cotton Confirmatory Data and Terms and Conditions of the Amendment" and the Registration Decision Memorandum dated September 29, 2001). Because of the

importance of grower education, these same requirements are required for Bollgard II cotton.

Grower compliance with refuge and IRM requirements is a critical element for resistance management. Significant non-compliance with IRM among growers may increase the risk of resistance for Bt cotton. However, it is not known what level of grower non-compliance will compromise the risk protection of current refuge requirements. Therefore, in addition to carrying out an effective IRM education for growers, Monsanto must also establish a broad compliance program for Bollgard II just as it is required to do for Bollgard cotton. The current compliance program requirements are described in the Agency's Bt Plant-Incorporated Protectants Reassessment Document (USEPA 2001, see Section III. "Bt Cotton Confirmatory Data and Terms and Conditions of the Amendment" and the Registration Decision Memorandum dated September 29, 2001). Ideally, this compliance program would 1) establish an enforcement structure that will maximize compliance, 2) monitor level of compliance, and 3) investigate effects of noncompliance on IRM. Grower compliance with IRM strategies for Bollgard cotton (or any pesticide technology) is tied into the belief that new technologies, such as Bollgard II cotton (cotton expressing multiple Bt toxins (Cry2Ab and Cry1Ac), other new synthetic insecticides or other biological controls, will reduce the risk of resistance.

V. Benefits and Public Intrest Findings

Monsanto Company indicates that the target market for Bollgard II cotton is 45% of the U.S. cotton acreage that experiences consistent lepidopteran pest pressure. They intend to replace Bollgard cotton with Bollgard II cotton. They project that after five years following commercial introduction of Bollgard II cotton, approximately 80 percent of the Bollgard cotton acres will be replaced. Bollgard cotton acreage planted in 2001 was approximately 5.8 million acres (37% of the total Upland cotton acreage).

Efficacy Benefits

Bollgard II cotton has significant efficacy benefits including improved performance (relative to Bollgard cotton) against cotton bollworm (CBW) and certain secondary pests including: soybean looper (SL), cabbage looper (CL), saltmarsh caterpillar (SMC), beet armyworm (BAW), and fall armyworm (FAW). Little additional efficacy benefits from use of Bollgard II cotton are expected for tobacco budworm (TBW) and pink bollworm (PBW). This is due to the fact that Bollgard cotton provides almost complete control of these pests and little or no insecticide is used on Bollgard cotton acreage specifically for TBW or PBW.

An assessment of annual grower benefits is based on the construction of demand curves for Bollgard cotton and Bollgard II cotton. Grower benefits are defined as the difference between the willingness to pay and the actual technology fee. The analysis of the two demand curves, and in particular the marginal revenue per acre of additional Bollgard II cotton, suggests that the technology fee would likely increase by approximately $5 per acre. For all growers, the gross benefit is $11.20 per acre and the net benefit is $5.24 per acre for Bollgard II cotton if the increased in technology fee is included. U.S. total annual net incremental benefits are predicted to be $43.8 million for Bollgard II cotton as compared to Bollgard cotton.

Economic benefits

The major economic benefits of Bollgard II cotton are that it will expand both the pest spectrum and life of the Bollgard technology. Based on Monsanto Company's projections (Monsanto, 2002), Bollgard II cotton is projected to displace eighty percent of Bollgard cotton within five years following initial commercialization. The present value of total U.S. benefits of Bollgard II cotton are estimated to exceed $12 million at a minimum to approximately $900 million, depending upon the discount rate used. This analysis is based on extending the life of the Bollgard technology from 10 to 25 years.

An assessment of annual grower benefits is based on the construction of demand curves for Bollgard cotton and Bollgard II cotton. Grower benefits are defined as the difference between the willingness to pay and the actual technology fee. The analysis of the two demand curves, and in particular the marginal revenue per acre of additional Bollgard II cotton, suggests that the technology fee would likely increase by approximately $5 per acre. For all growers, the gross benefit is $11.20 per acre and the net benefit is $5.24 per acre for Bollgard II cotton if the increased in technology fee is included. U.S. total annual net incremental benefits are predicted to be $43.8 million for Bollgard II cotton as compared to Bollgard cotton.

Insecticide use reduction benefits

Use of Bollgard II cotton will result in some additional chemical insecticide use reduction and potential yield improvement. The gross benefits of $11.20 per acre will likely result from some combination of chemical savings ($16/acre is cost of average application) and yield improvement of $6 per acre (see Williams, 2002). Using the $43.8 million total annual net incremental benefits for Bollgard II cotton as compared to Bollgard cotton, this translates into a chemical saving of $50 million or 3.1 million acre treatments, which is approximately 14% of the 22.9 million acre treatments in 2001.

Although the exact amount cannot be quantified at this time, the Agency has previously documented the benefits and reduction in insecticide use for Bollgard cotton (see U.S. EPA 2001, Section E. "Benefits Assessment"). A qualitative analysis indicates that supplemental insecticidal

applications for control of CBW will be further reduced and may be zero in many areas. However, the grower will still need to control for other insect pests such as plant bugs and stink bugs. Bollgard II cotton appears to produce a functional high dose for control of TBW, CBW, and PBW (see U.S. EPA, 2002). For the secondary pests, the greatest insecticide use reduction will be for soybean looper, beet armyworm, and fall armyworm. The exact amount of pesticide reduction will vary from year-to-year depending on the sporadic nature of these pests and other local conditions.

VI. Additional Contact Information

Ombudsman, Biopesticides and Pollution Prevention Division (7511P)
Office of Pesticide Programs
Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, D.C. 20460