UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC
SUBSTANCES

November 6, 2007

MEMORANDUM

SUBJECT: Technical Review of Monsanto Company's Submissions (dated September 22, 2006 and March 9, 2007) Regarding Insect Resistance Management for MON 89034 Corn (EPA registration 524-LTL). DP Barcode: 335208. Decision: 371189. MRID#: 469514-30; MRID 470794-02

TO: Susanne Cerrelli, Regulatory Action Leader
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

FROM: Sharlene R. Matten, Ph.D., Biologist
IRM Program Lead
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

11/6/07

And

Alan H. Reynolds, M.S., Entomologist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

11/6/07

Action Requested

BPPD[1] has been asked to review Monsanto Company's submission entitled, "Insect Resistance Management Plan for Second Generation Lepidopteran-Protected Corn, MON 89034" (MRID 469514-30) and supplemental information (MRID# 470794-02) to support the Section 3 Registration, 524-LTL.

[1] The use of BPPD in this review refers to BPPD IRM team consisting of Sharlene Matten, IRM Program Lead and Alan Reynolds, Entomologist.

Background

Monsanto Company (hereafter referred to as Monsanto) has developed through the use of genetic engineering, MON 89034, a corn product that produces the *Bacillus thuringiensis* (Bt)-derived insecticidal proteins Cry1A.105 and Cry2Ab2. The Cry1A.105 toxin is a "chimeric" protein containing domains I and II and the C-terminal from Cry1Ac and domain III from Cry1Fa (domain III). The Cry2Ab2 protein is exactly the same as that currently expressed in Monsanto's Bollgard II cotton. MON 89034 is protected from damage caused by larval feeding of *Ostrinia nubilalis* (European corn borer; ECB), *Diatraea grandiosella* (southwestern corn borer; SWCB) and *Diatraea saccharalis* (sugarcane borer; SCB), *Spodoptera frugiperda* (fall armyworm; FAW), and *Helicoverpa zea* (corn earworm; CEW). Monsanto presents data to support its proposed IRM plan for MON 89034. Monsanto wishes to demonstrate that: (1) resistance to Cry1A.105 and Cry2Ab2 proteins in MON 89034 is expected to be at least partially recessive; (2) the probability of cross-resistance between Cry1A.105 and Cry2Ab2 is low; and (3) the level of both Cry1A.105 and Cry2Ab2 produced in MON 89034 confer high level of control of susceptible target pests (*in vitro* and *in planta).* Monsanto proposes a 5% structured refuge in the U.S. Corn Belt (currently, a 20% structured refuge) and a 20% structured refuge in cotton growing regions (currently, a 50% structured refuge) to mitigate insect resistance to the Cry1A.105 and Cry2Ab2 proteins. Simulation modeling was provided to support this plan. BPPD's technical analysis of Monsanto's proposed IRM plan for MON 89034 is the subject of this review.

Conclusions and Recommendations

MON 89034 field corn uses in the Corn Belt

1. Pyramids can reduce the need for large refuges. Monsanto has proposed that a 5% structured refuge, rather than the current 20% structured refuge, be used with the field corn uses of MON 89034. However, Monsanto's data and modeling do not support a 5% structured refuge for MON 89034 for field corn uses in the Corn Belt. There are uncertainties in the dose determination for ECB, SWCB, CEW, FAW (SS and RS mortality), cross-resistance likelihood of Cry1A.105, Cry1Ac, and Cry1Fa and its impact on the durability of MON 89034, and limitations of the simulation modeling. It is recommended that the current 20% structured refuge requirement for field corn uses of MON 89034 in the Corn Belt be maintained until such time as Monsanto can address these uncertainties.

2. Monsanto relies on the Roush (1998) model to support the need for a 5% structured refuge rather than a 20% structured refuge in the Corn Belt. Roush's model (1998; Figure 2) indicates that a 5% structured refuge is equal to or greater than a 20% structured refuge for a highly effective, high dose single-gene product when a two-gene product (MON 89034 in this case) achieves at least 95% control of susceptible homozygotes **and** 70% control of heterozygotes assuming there is no cross-resistance. The dose information provided by Monsanto is not sufficient to demonstrate that each protein will kill 95% of the homozygous susceptible insects and 70% of the heterozygotes. BPPD recommends that Monsanto further investigate whether MON 89034 consistently has high mortality of susceptible homozygotes (>95%) and whether the heterozygote mortality is at least 70% for MON 89034 against the target pests (for the Corn Belt – ECB and SWCB). The 1998 SAP suggested several ways to estimate mortality for less susceptible larvae (i.e.

heterozygotes) (EPA 1998). These techniques included testing larger, later instar larvae that may be less susceptible

3. Monsanto has demonstrated that Cry1A.105 and Cry2Ab2 have different modes of action and, therefore, a low likelihood of cross-resistance. Cry1A.105 and Cry2Ab2 would be suitable partners in a pyramided product. Monsanto has also shown that there is a low likelihood of cross-resistance between Cry1A.105 and Cry1Ab. Monsanto has previously demonstrated that there is a low likelihood of cross-resistance between Cry2Ab2 and Cry1Ac.

4. Monsanto did not address the likelihood of cross-resistance of Cry1A.105, Cry1Ac, Cry1Fa, proteins already in existing Bt corn and Bt cotton products, and what impact such cross-resistance would have on the durability of MON 89034. It is recommended that Monsanto provide additional information on cross-resistance of Cry1A.105 and Cry1Fa and Cry1Ac (including binding site models and use of resistant colonies) for the target pests and determine how such cross-resistance may impact the durability of MON 89034. The Cry1A.105 protein is a chimeric protein consisting of Domains I and II and the C-terminus of Cry1Ac and Domain III of Cry1Fa. It is important to address not only the likelihood of cross-resistance potential of Cry1A.105 and Cry1Ab and, similarly, Cry1A.105 and Cry2Ab2 (which was done by Monsanto), but also that of Cry1A.105 and Cry1Ac and Cry1Fa.

5. Additional species-specific (e.g, ECB and SWCB for the Corn Belt), spatially-explicit, landscape modeling is recommended to explore the durability of MON 89034 versus single-protein Bt corn products. Modeling would need to consider the impact of other Bt proteins in the landscape that may confer some cross-resistance (to Cry1A.105, in particular) and how such cross-resistance would impact the durability of MON 89034 in the Corn Belt (use of simulation modeling). This is analogous to the species-specific simulation modeling that EPA required Monsanto do to support the use of natural refuge (instead of a structured refuge) for management of *H. virescens* and *H. zea* to the Cry1Ac and Cry2Ab2 proteins expressed in Bollgard II cotton.

6. *MON 89034 field corn use in cotton-growing areas.* A 20% non-Bt corn refuge for MON 89034 in the southern cotton-growing areas would be sufficient to manage the risk of resistance evolution to Bt corn and Bt cotton products assuming there is no cross-resistance. However, Monsanto did not sufficiently address the cross-resistance of Cry1A.105, Cry1Fa, and Cry1Ac in the cotton-growing landscape and how cross-resistance may impact the durability of MON 89034. Should cross-resistance be of concern then the durability of MON 89034 in the southern cotton-growing areas might be compromised. Monsanto needs to address this potential in subsequent simulation modeling.

7. *Sweet corn.* No structured refuge is needed in conjunction with the MON 89034 sweet corn use based on the destruction of potential resistant larvae through cultivation practices.

8. *Popcorn.* Monsanto provided no additional dose and/or efficacy data to what was provided for field corn to support the use of MON 89034 on popcorn. Without these data, the popcorn use cannot be supported.

9. *Other Important Elements of the IRM Plan.* Monsanto's proposed program for resistance monitoring, grower education and compliance monitoring as part of the MON 89034 IRM program is "acceptable." No Cry1A.105 baseline susceptibility studies have been conducted, but are planned by Monsanto. Monsanto has indicated that baseline susceptibility information for ECB to Cry2Ab2 has been collected over a two-year period (summarized in Monsanto's submission). For each protein, a discriminatory concentration (diagnostic dose) will have to be determined for use in the annual resistance monitoring program. Annual reporting to the Agency of the results of the resistance monitoring, grower education, and compliance monitoring is needed (as is required for all other Bt PIPs). If there is confirmed resistance to either protein then it must be reported to the Agency (see FIFRA 6(a) incident reporting requirements and the requirements as part of the Remedial Action plan).

BPPD Review

1. **Assessment of the Probability of Cross-Resistance to the Cry1A.105 and Cry2Ab2 Proteins**

The Cry1A.105 toxin is a "chimeric" protein containing domains I and II and the C-terminal from Cry1Ac and domain III from Cry1Fa (domain III). The Cry2Ab2 protein is exactly the same as that currently expressed in Monsanto's Bollgard II cotton. There are a number of Bt corn products on the market that produce the insecticidal proteins, Cry1Ab and Cry1Fa (potential cross resistance with Cry1A.105/Cry2Ab2 and Cry1Ab is discussed in section 3, **"Impact of Prior Use of Cry1Ab-Expressing Bt Corn Products on MON 89034"**). There are also Bt cotton products that produce the Cry1Ac, Cry1F, and Cry2Ab2 insecticidal proteins. Mathematical models indicate that the IRM values of a Bt corn product with two insecticidal proteins, like MON 89034, would be the greatest if there is a low probability of cross-resistance (e.g., Roush 1998). Cross-resistance is most likely when proteins share key structural features, which allows one resistance mechanism to confer resistance to more than one protein (Tabashnik, 1994; Gould et al., 1995).

There are three models that have been proposed to explain the mode of action of Cry1A toxin mode of action (see discussion in Piggott and Ellar, 2007). The most accepted Bravo model proposes that both the cadherin and aminopeptidase (APN) receptors are required for full Cry1A toxicity. This model suggests that receptor binding is sequential: 1) ingestion of the protein inclusions by a susceptible insect larva, 2) solubilization of the protein in the insect midgut, 3) cleavage of the protoxin by host proteases and release of the active toxin, 4) binding of the active toxin to specific receptors on the midgut epithelieum, 5) oligomerization of toxin subunits to form pore structures that inject into the membrane, 6) passage of ions and water through the pores, resulting in swelling, lysis, and the eventual death of the host. Differences in any of these steps will reduce the probability of cross-resistance between any two Cry proteins. The more controversial Zhang model suggests that receptor binding activates an Mg^{+}-dependent signaling cascade that promotes cell death. The Jurat-Fuentes model suggests that cytotoxicity is due to the combined effects of osmotic lysis and cell signaling. The later two models are, at present, more speculative.

Resistance associated with modification of the binding site receptor has been the primary Bt resistance mechanism reported to date (reviewed in Ferré & Van Rie 2002). Other Bt

resistance mechanisms have been reported that are based on alterations in the proteases that cleave the protoxin, processing it into a smaller active toxin (Candas et al. 2003) and most recently, the discovery that esterases can bind and detoxify Bt toxins (Gunning et al. 2005). Only the binding reduction mechanism has a demonstrated causal link between the biochemical modification and resistance (Ferré and Van Rie 2002). Ferré and Van Rie (2002) indicate that in all cases of binding site modification, resistance is due to a recessive or partially recessive mutation in a major autosomal gene, and cross-resistance extends only to Cry proteins sharing binding sites. Cry proteins that do not share high levels of sequence similarity tend to have different binding sites and different modes of action. Analyses of resistance to Bt Cry proteins indicate that cross-resistance occurs most often with proteins that are similar in structure (Tabashnik, 1994; Gould et al., 1995).

With this information in mind, Monsanto has assessed the probability of cross-resistance between Cry1A.105 and Cry2Ab2 on three levels: 1) structural similarity between the proteins, which is indicative of mode of action; 2) characterization of elements of the mode of action, such as the biophysical nature of binding of the Bt proteins to the target insect midgut; and 3) demonstration that the individual proteins are effective in controlling resistance to the other protein. Results of these efforts are discussed below.

The first piece of the analysis relates to whether the Cry1A.105 protein has high sequence similarity with the Cry2Ab2 protein. Monsanto provided BPPD with a summary of current information about the structural and functional similarities of the Cry1A.105 protein to other Bt Cry1 proteins. The Cry1A.105 protein is a chimeric protein with overall amino acid sequence identity to the Cry1Ac, Cry1Ab and Cry1Fa proteins of 93.6, 90.0 and 76.7%, respectively. The Cry1A.105 protein expressed in MON 89034 corn plants results in increased activity against FAW, SCB, and CEW compared to Cry1Ab expressed in MON 810 corn plants (see BPPD review of efficacy data, Matten, 2007; Monsanto study MRID# 46951415). A structural model of the Cry1A.105 protein was developed using the X-ray crystal structure of the Cry1Aa protein. This model demonstrated high overall main chain structural similarity with Cry1Aa. Models of Cry1Ab and Cry1Ac were also prepared using the Cry1A.105 model. Comparison of the aligned folds of all three proteins showed that Cry1Ab and Cry1A.105 have essentially the same main chain structure (i.e., similar three domain structures) and that Cry1Ac differs slightly in its main chain structure from the other two in domain III. Thus, comparison of the modeled crystal structures of the Cry1A.105, Cry1Ab, and Cry1Ac with the experimental Cry1Aa X-ray crystal structure demonstrated high three-dimensional structural similarity between the four proteins (i.e., Cry1A.105, Cry1Ab, Cry1Ac, and Cry1Fa).

In the case of Cry1A.105 and Cry2Ab2 proteins, however, there is only a 14% amino acid sequence identity. Based on the available data, Monsanto has sufficiently demonstrated that there is low sequence similarity between the Cry1A.105 protein and the Cry2Ab2 protein. Lack of sequence similarity would suggest that cross-resistance between the Cry1A.105 and Cry2Ab2 proteins would be unlikely. On the other hand, high sequence similarity between the Cry1A.105 protein and Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Fa proteins is one indicator that cross-resistance may be a concern for these proteins. This is important because Cry1.105 is composed of domains I and II and the C-terminus of Cry1Ac and domain III of Cry1Fa. This subject will be discussed further in the review.

Previous studies have shown that Cry1A proteins are activated by proteolytic cleavage of the C-terminal domain and the N-terminus of domain I in the insect gut. In contrast, Cry 2A proteins are activated by cleavage of the N-terminus of domain I and the C-terminal part of domain III. Different activation mechanisms would tend to decrease the likelihood of cross-resistance between the Cry1A and Cry2A proteins.

Assessment of binding characteristics is one way of determining the potential for cross-resistance between the two proteins. As noted above, changes in the nature of protein binding to the insect midgut is the mode of action step that has most often been associated with insect resistance to Bt Cry proteins (for reviews, see Tabashnik, 1994; Baxter et al., 2005). Biacore is used to quantify the interaction kinetics of Bt proteins with the insect brush border membranes (BBM). Competitive and non-competitive binding may not always be distinguished by Biacore and other analyses, such as ligand blotting, may be used. Ligand blotting is a qualitative tool used to identify protein bands that have the specific secondary modification to bind Bt proteins. Monsanto used both Biacore and ligand blotting to characterize Cry1A.105 and Cry2Ab2 binding to ECB brush border membranes (studies by Li and Guzov, 2006 were provided in Appendix 1 of Monsanto submission, MRID# 469514-30, Head, 2006 and are discussed below).

Binding constants for the interaction of Cry1A.105 and Cry2Ab2 with immobilized BBMV (ECB) differed by more than an order of magnitude with essentially no BBMV-specific binding being observable for Cry2Ab2. The Biacore system could not distinguish unique aspects of non-competitive binding for Cry1A.105 and Cry2Ab2 on BBMV. This result suggests that there are very different binding sites for Cry1A.105 and Cry2Ab2 in the ECB midgut. Additional Biacore analyses indicated that Cry1A.105 and Cry2Ab2 bound to different glycosyl moieties linked to bovine serum albumin (BSA). Cry1A.105 preferentially bound to galactosamine (K_D=1.5x10^{-8}M and R_{max}=2419 RU). Cry2Ab2 preferentially bound to N-acetyl glucosamine (K_D=7.0x10^{-11}M and R_{max}=32 RU), but also bound galactosamine with a K_D =2.0x8^{-8}M and R_{max}=625 RU. Furthermore, Cry1A.105 binding to galactosamine filled a two-binding-site model as evidenced by the reduction in the Chi^2 value from 2583 to 53, but the fit of Cry2Ab2 binding was similar for both models suggesting that the Cry2Ab2 and Cry1A.105 proteins not only bind to different sugars but also differ in their binding kinetics.

The ligand blotting analysis demonstrated that the Cry1A.105 and Cry2Ab2 proteins bound to different components on ECB brush border membrane filaments (BBMF) separated by SDS-Page and immobilized on a nitrocellulose membrane. Trypsin-treated Cry1A.105 protein was shown to bind to a ~150 kDa band while the trypsin-treated Cry2Ab2 protein was shown to a ~130 kDa band, but weakly to a ~150 kDa band. The trypsin-treated Cry2Ab2 protein had a greater rate of binding than the Cry1A.105 protein. Overall these results support the conclusion, as Monsanto has described, that the Cry2Ab2 and Cry1A.105 proteins displayed different binding components and different kinetics in binding to ECB BBMF. These results are consistent with the differences in binding affinity for Cry1A.105 and Cry2Ab2 proteins observed with Biacore. In addition, Monsanto notes that Cry2Aa did not bind to a specific, high affinity Cry1Ac receptor in work performed by English et al. (1994).

In conclusion, Biacore and ligand blotting analyses demonstrate that Cry1A.105 and Cry2Ab2 proteins bind to some unique components on ECB brush border membranes. They also share

many common binding sites. Screening a limited number of glycosylated BSAs, indicated that galactosamine is recognized by Cry1A.105 only, while Cry2Ab2 demonstrated a high affinity for both N-acetylglucosamine and galactosamine. These data support the conclusion that Bt protein binding to carbohydrate moieties is the principal basis of the specific interactions between the Cry1A.105 and Cry2Ab2 proteins and the ECB brush border membrane. Specific binding of Bt proteins to the target insect gut membrane is a key step in their mode of action. Differences in the Cry1A.105 and Cry2Ab2 protein interactions with the BBM suggest that these two proteins have differences in mode of action. BPPD agrees with Monsanto that these differences should minimize the development of cross-resistance by the target insect pests to these two proteins.

Monsanto also provided evidence to show that there is a lack of cross-reactivity between Cry1A.105 and Cry2Ab2 antibodies. The homologous primary-secondary antibody pairs recognized only their corresponding antigens (i.e., trypsin-treated Cry1A.105 or Cry2Ab2) with no cross-reactivity. Similarly, Monsanto previously demonstrated that anti-Cry2Ab antibodies do not cross-react with the Cry1Ac proteins, nor do the anti-Cry1Ac antibodies cross-react with the Cry2Ab2 protein (Head and Reding 2001, MRID# 455457-01). The 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 Cry1A.105 (and all Cry1A proteins) and Cry2Ab proteins.

Monsanto provided indirect information (i.e., there are no colonies of lepidopteran corn pests resistant to either Cry1A.105 or Cry2Ab2 proteins) to indicate that insects resistant to one of the two insecticidal proteins, Cry1A.105 or Cry2Ab2, will be controlled by the other insecticidal protein. First, Monsanto cited to studies provided in support of the Bollgard II cotton registration (i.e, Cry2Ab2 and Cry1Ac Bt plant-incorporated protectants as expressed in cotton) that indicated that Cry1Ac-resistance did not confer Cry2Ab2 resistance to tobacco budworm, cotton bollworm, and pink bollworm (Head and Reding 2001; EPA 2007). In addition, Monsanto shared information that a Cry2Ab2-resistant colony (called SP15) of *Helicoverpa armigera* (Dr. Rod Mahon, CSIRO, Australia) showed little or no cross-resistance to Cry1Ac and the microbial insecticide, DiPel®, that contains the Cry1Ab, Cry1Ac, and Cry2Aa proteins. Monsanto tested this Cry2Ab2-(SP15) resistant colony against purified Cry1A.105, Cry1Ac, and Cry2Ab2 protein relative to a susceptible laboratory colony of *H. armigera*. The SP15 colony was found to be highly resistant to the Cry2Ab2 protein, but showed little or no cross-resistance to the Cry1Ac and Cry1A.105 proteins. Other published research indicates that there is evidence for broad cross-resistance (low levels of resistance) to Cry1A and Cry2A proteins in laboratory-selected strains of beet armyworm (Moar et al. 1995) and tobacco budworm (Gould et al. 1992). Collectively, results of resistant colony studies indicate that there is some low potential for cross-resistance, but those high levels of cross-resistance to Cry1A.105 and Cry2Ab2 is unlikely. In the field, this would translate to the efficacy of MON 89034 being maintained even though resistance might occur to one of the proteins.

2. Dose

The determination of dose, or the amount of toxin expressed by the transgenic crop relative to the susceptibility of the target pests, is a critical component of IRM. Models have shown that

a high dose of toxin, coupled with a non-transgenic refuge to provide a supply of susceptible insects, is the most effective strategy for delaying resistance in Bt crops. The high dose/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. In practice, a high dose PIP should express sufficient quantities of toxin to kill all susceptible insects (SS) as well as heterozygous insects with one resistance allele (RS). Lower dose PIPs might allow for survival of insects with at least one susceptibility allele (SS or RS), although effective IRM may still be possible with a suitable refuge strategy.

The 1998 Science Advisory Panel (SAP) defined high dose as a level of toxin 25 times greater than is needed to kill all susceptible insects. The SAP also outlined five techniques to determine high dose: 1) Serial dilution bioassay with artificial diet containing lyophilized tissues of Bt plants using tissues from non-Bt plants as controls; 2) Bioassays using plant lines with expression levels approximately 25-fold lower than the commercial cultivar determined by quantitative ELISA or some more reliable technique; 3) Survey large numbers of commercial plants in the field to make sure that the cultivar is at the $LD_{99.9}$ or higher to assure that 95% of heterozygotes would be killed (see Andow & Hutchison 1998); 4) Similar to #3 above, but would use controlled infestation with a laboratory strain of the pest that had an LD_{50} value similar to field strains; and 5) Determine if a later larval instar of the targeted pest could be found with an LD_{50} that was about 25-fold higher than that of the neonate larvae. If so, the later stage could be tested on the Bt crop plants to determine if 95% or more of the later stage larvae were killed.

It must be noted that both the high dose definition and verification techniques were developed in 1998 when all of the registered Bt crops were single toxin products targeted against lepidopteran pests. In recent years, PIPs (in Bt cotton) have been approved that contain two genes targeted at the same insect pest. These "pyramided" products can be beneficial for IRM, since target pests must overcome two toxins to develop field resistance to the PIP. The benefits are greatest for two toxins with unrelated modes of action (i.e. binding to different Bt receptor sites in the midgut) that are expressed at high doses in the plant (Roush 1994).

For pyramided products, the dose of each toxin should be evaluated separately. This can be easily accomplished if the pyramided product is created through conventional breeding -- in this case, the dose of the single toxin products has already been established and the combined dose in the pyramided PIP can be determined with comparative efficacy studies. However, for pyramids created by non-conventional breeding (e.g. recombinant DNA techniques), defining the dose can be more complicated since single toxin lines may not be available (or commercialized) for comparisons. The dual toxins can also be evaluated collectively to determine an "effective" high dose. In some examples, each toxin by itself may not supply a high dose, but in combination a sufficient control (>95% of heterozygotes) is provided to be considered high dose.

MON 89034 was created with recombinant DNA technology (and not conventional breeding) to express the Cry1A.105 and Cry2Ab2 toxins. Both of the toxins are located on the same plasmid in the MON 89034 plant genome. Because of this, there are no originating single gene lines (i.e. expressing Cry1A.105 or Cry2Ab2 only) for dose comparisons, although single

gene events were separately engineered. The Cry1A.105 toxin is a "chimeric" protein containing domains I and II and the C-terminal from Cry1Ac and domain III from Cry1Fa (domain III). By creating this chimera, Monsanto hoped to improve efficacy against several target pests including fall armyworm and corn earworm. The Cry2Ab2 protein is exactly the same as that currently expressed in Monsanto's Bollgard II cotton.

To evaluate dose, Monsanto conducted a number of laboratory and field studies with diet bioassays and MON 89034 plant material. Three sets of experiments were conducted: 1) bioassays with purified toxin incorporated into artificial diet to determine pest susceptibility, 2) leaf disk or kernel testing conducted in the laboratory, and 3) field tests with whole plants (artificial infestation of small corn plots) compiled over a several year period. Four target pests were evaluated including European corn borer (ECB), southwestern corn borer (SWCB), fall armyworm (FAW), and corn earworm (CEW). A description of the test procedures is included in Monsanto's submission (Head 2006; MRID# 469514-30). Toxin expression data was also obtained from MON 89034 leaf tissue and other tested lines.

Laboratory bioassays (Monsanto's submission, Head (2006), section 2.2.1) were conducted using purified protein in diet to determine susceptibility (molting inhibitory concentration, MIC_{90}) to the MON 89034 toxins. Molting inhibition is often used instead of straight mortality (i.e. an LC_{50} or LC_{90}) because it can be assumed that insects that fail to develop as larvae will be functionally dead in the field. A MIC_{90} bioassay can also reduce the amount of purified toxin needed for the testing relative to an LC_{90} determination, though it is unclear whether Monsanto had insufficient purified protein to determine LC_{90} values. The MIC_{90} tests showed that all four target species were more susceptible to Cry1A.105 than Cry2Ab2 (as measured in ppm). ECB was more sensitive to both Cry1A.105 and Cry2Ab2 than the other tested lepidoptera by at least an order of magnitude. For Cry1A.105, BPPD agrees with Monsanto that the amount expressed in plant leaf tissue is high relative to the susceptibility of the target insects. Toxin levels in leaf tissue measured throughout the growing season (V2 - Pre-VT) exceeded the MIC_{90} for all four species (both measured in ppm). On the other hand, the amount of Cry2A2b expressed in MON 89034 exceeded the MIC_{90} value only for ECB. For the other three pests, the level of Cry2Ab2 was at (for SWCB) or below (CEW and FAW) the MIC_{90} level. These data suggest that the Cry1A.105 component of MON 89034 may be expressed at a sufficient level for all four pests to be considered "high dose" while the Cry2Ab2 expression is less certain. However, BPPD concurs with Monsanto's contention that the results of laboratory bioassays are difficult to correlate with natural field systems and larval survival on plant tissue is more challenging than on artificial diet.

Unlike the artificial diet bioassays, the tests with plant material (leaf disks and whole plant) directly assessed the performance of MON 89034 against the target pests. Since MON 89034 expresses both Cry1A.105 and Cry2Ab2 simultaneously, the tests with MON 89034 plant material evaluate the "effective dose" of both toxins together. However, Monsanto was also able to include single gene lines producing either Cry1A.105 or Cry2Ab2, though none of these were ultimately commercialized or used to create MON 89034. To relate the single gene isolines to MON 89034, Monsanto supplied some plant expression data for the isolines which could be compared to the known toxin expression of the stacked product. For the leaf disk/kernel tests, two Cry1A.105 isolines were used; one (LAJ138) with toxin expression equivalent to that of MON 89034 and another (LAJ129) with less than half the expression. Both of the Cry2Ab2 lines that were used (70774 and 67620) had less toxin expression than in

MON 89034. Other single gene lines were used for the field tests, although no expression data were reported for those hybrids.

The results of the leaf disk tests generally supported the conclusions derived from the susceptibility diet bioassays (i.e. high efficacy against the target pests). Two-toxin MON 89034 was highly effective against all four target pests with at least 90% mortality among exposed larvae and significant growth inhibition in the survivors. On the other hand, mortality was more variable for the single gene isolines that were also tested. For ECB, both MON 89034 and the single gene (Cry1A.105 and Cry2Ab2) isolines killed nearly all exposed larvae. Low survival (4%) was noted only on a Cry2Ab2 isoline (67620) and on MON 89034, though the surviving larvae were stunted (< 41% the mass) relative to larvae on control leaf disks. The highest level of survival was noted for SWCB with some survival (up to 41% of the control group) observed on both the isolines and MON 89034, although the surviving larvae showed growth inhibition in all cases. For FAW and CEW, no survival was noted on MON 89034 or the isolines (though CEW survival on the control was only 26%, presumably due to CEW preference for feeding on corn ears instead of leaf tissue).

A second trial using kernels instead of leaf disks was performed for CEW. This test revealed relatively high survival (up to 35%) on the lower expressing isolines (LAJ129 and 67620) and 9% survival on MON 89034 (growth inhibition was not recorded).

Several sets of field tests (conducted in 2000 and 2002) showed high efficacy, though they provided less information on dose. The field tests were targeted primarily at ECB (one study was designed for SWCB) and assessed plant damage (as opposed to directly evaluating mortality). Single gene isolines were used, but no expression data were given (they were claimed to be lower than MON 89034) and MON 89034 was not included in the trials. The trials showed that Cry1A.105 and Cry2Ab2 isolines significantly reduced ECB and SWCB leaf and tunneling damage relative to the non-Bt control groups. Feeding damage was comparable to the commercial product MON 810, which is known to express a high dose for ECB and SWCB. While these studies demonstrated field efficacy of the Cry1A.105 and Cry2Ab2 isolines, they provide limited information for the assessment of MON 89034 dose. This is because 1) MON 89034 was not evaluated in any of the trials, 2) mortality was not assessed, and 3) CEW and FAW were not included in the trials. Monsanto recognized the limitations of the field work, but indicated that they should be considered *in toto* with the laboratory bioassays and leaf disk tests.

Overall, the dose studies present a mixed picture of the dose profile for MON 89034. Dose and efficacy data indicate that: (1) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provide essentially 100% control of ECB; (2) the Cry1A.105 protein in MON 89034 provides approximately 95% control of SWCB, while the Cry2Ab2 protein provides 80-90% control; (3) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provide >95% control of FAW; and (4) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provide 90-95% control of CEW. Clearly, the hybrid offers a high level of control against the four major target pests including greater than 95% control of ECB and FAW and greater than 90% control of CEW and SWCB. The actual level of control may be even higher due to growth inhibition among survivors that would likely preclude developmental completion. As demonstrated in the diet bioassays, the target pests appear to be somewhat more sensitive to Cry1A.105 than to Cry2Ab2. However, much of the dose information is circumstantial; the leaf disk assays were

the only trial phase the directly evaluated MON 89034. The other data were obtained from susceptibility assays with purified protein (that were compared to MON 89034 expression data) and tests with (non-commercialized) single gene isolines.

BPPD agrees with Monsanto that MON 89034 provides strong control; these tests demonstrate that MON 89034 will likely kill >90% of susceptible insects. On the other hand, the data do not support a high dose under the definition put forth by the 1998 SAP (a level of toxin 25 times greater than needed to kill susceptible larvae; i.e. a dose greater than the LC_{99} of the pest). Some survival of MON 89034 plant tissue was noted for ECB, SWCB, and CEW. Monsanto assumes that the survivors would not reach adulthood due to growth inhibition (and therefore are functionally dead), but that assumption was not tested due to the short time frame of the experiment.

Monsanto's dose studies did not directly evaluate the effect of MON 89034 on potentially heterozygous larvae (i.e. with one copy of a resistance allele). Since heterozygotes may be more tolerant of Bt toxins than susceptible homozygous larvae, the 1998 SAP indicated that a high dose product should kill at least 95% of homozygous susceptibles. Roush's modeling (1998) specifies that 70% of heterozygotes should be killed by the toxins expressed in the dual gene PIP. Monsanto assumes that MON 89034 meets the criteria for the Roush model (i.e. 95% susceptible and 70% heterozygote mortality), but no empirical evidence was presented regarding potential heterozygote mortality. Given that the major support for Monsanto's proposal to reduce corn refuge from 20% to 5% is the Roush model, BPPD recommends that Monsanto further investigate whether MON 89034 consistently has high mortality of susceptible homozygotes (>95%) and further investigate heterozygote mortality for MON 89034. BPPD recognizes that direct evaluations of heterozygote effects can be difficult, particularly if resistant colonies for the target pests are unavailable and given that there is no field resistance to either protein. Monsanto has not provided enough information to determine the "killing power" of each individual protein – it would be useful to assess whether Cry1A.105 and Cry2Ab2, individually, will kill greater than 95% of the susceptible homozygotes. The 1998 SAP suggested several ways to estimate mortality for less susceptible larvae (i.e. heterozygotes) (EPA 1998). These techniques included testing larger, later instar larvae that may be less susceptible to the toxins or with PIPs expressing lower levels of toxin than the commercial event (see the discussion of the SAP recommendations at the beginning of this section).

3. Impact of Prior Use of Cry1Ab-Expressing Bt Corn Products on MON 89034

Monsanto examined the impact of prior use of Cry1Ab-expressing Bt corn products on MON 89034 Bt corn. Cry1Ab-expressing Bt corn products have been on the U.S. market since 1997 and planted on millions of acres. This selection pressure could result in increased Cry1Ab-resistant allele frequencies in lepidopteran corn pests, particularly those that are more dependent on corn as a primary host such as ECB and SWCB. Should there be Cry1Ab-resistant insects that are cross-resistant to either the Cry1A.105 protein and/or Cry2Ab2 protein then IRM value of MON 89034 would be significantly reduced. Given that there is very high amino acid similarity between the Cry1Ab and Cry1A.105 proteins then the potential for cross-resistance between Cry1Ab and Cry1A.105 is an important consideration. In an earlier section of this review, BPPD concluded that there is a low likelihood of cross-resistance between Cry1A.105 (Cry1A proteins) and Cry2Ab2 proteins.

European corn borer (ECB) populations have been monitored for susceptibility to Cry1Ab since the 1995 growing season (diagnostic concentration information has been collected since 1999). Since 1998, monitoring has also been required for corn earworm (CEW), southwestern corn borer (SWCB), and fall armyworm (FAW, sweet corn only) susceptibility to Cry1Ab. All of the Cry1Ab monitoring data through the 2000 growing season were reviewed by the Agency during the 2001 Bt crops reassessment (EPA 2001). Data for the 2001 through 2005 growing seasons were independently reviewed by EPA (see Reynolds 2004a, 2004b, 2006; Milofsky 2007). Estimates of the frequency of Cry1Ab resistance in ECB indicate that Cry1Ab-resistant alleles capable of conferring ECB survival on a Bt corn plant are very rare (Andow et al. 2000; Bourguet et al. 2003, Stodola et al. 2006). The highest estimate of Cry1Ab resistance allele frequency in U.S. ECB populations was $<4 \times 10^{-4}$ at the 95% confidence level. The Cry1Ab resistance allele frequency has not increased significantly in frequency even with the ten years of widespread use of Cry1Ab-expressing corn products. There have been no instances of Cry1Ab resistance capable of conferring survival on Cry1Ab-expressing Bt corn plants in annual monitoring of ECB, SWCB, and CEW (see ABSTC 2006; Milofsky 2007 (EPA's technical review)). BPPD agrees with Monsanto that the frequency of Cry1Ab-resistance is very low and that it has not increased significantly in over ten years of widespread use of Cry1Ab-expressing corn products.

What is known about Bt toxin receptors is summarized in a recent review by Piggott and Ellar (2007). By far the most studied receptors have been lepidopteran receptors associated with Cry1A toxins. These authors summarized Cry toxins for which a putative receptor has been identified (see Table 2 in Pillar and Ellar 2007). The Cry1A proteins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca, Cry1Fa) all have aminopeptidase N receptors (APNs) that can serve as Cry-binding proteins that mediate pore formation, but their relevance to toxin susceptibility has not been demonstrated. Cry1Aa, Cry1Ab, and Cry1Ac also have cadherin-like receptors that have been shown to mediate Cry1A toxicity. Other putative receptors, i.e., alkaline phosphatases, glycolipids, BTR-270, P252, may also play a role in Cry1A toxicity, but further study is needed. While there has been progress in what is known about Cry1A toxicity, little is known about other Cry families, such as the Cry2A family. How pore formation confers toxicity requires further study.

Monsanto characterized binding of Cry1A.105 and Cry1Ab proteins to ECB brush border membrane vesicles using Biacore. These studies (Li and English 2006) indicated that Cry1A.105 and Cry1Ab occupy different binding sites on the ECB midgut epithelial membrane and therefore have distinct membrane binding mechanisms. Cry1Ab binding data suggest that the binding patterns are much more complex for Cry1Ab than for Cry1A.105 despite these two proteins having 90% amino acid sequence homology. BPPD agrees with Monsanto that differences in binding mechanisms lessen the likelihood of Cry1A.105 and Cry1Ab cross-resistance.

Monsanto also summarized the laboratory studies examining ECB colonies selected for resistance to the Cry1Ab protein. While these colonies are imperfect tools for predicting what will happen in the field, they are the best tools available for looking at potential resistance mechanisms. In particular, Monsanto discussed a series of studies conducted on three ECB colonies selected for resistance to Cry1Ab by Blair Siegfried at the University of Nebraska (Siegfried and Spencer 2001). Two of the colonies were created by laboratory selection, the

Europe colony was established from larvae collected in Lombardia region of northern Italy and a second colony was created from larval collected in Nebraska. A third colony was created from survivors of diagnostic bioassays from both the Europe and Nebraska populations. All three colonies, along with two susceptible colonies, were assayed for their response to purified Cry1Ab, Cry1Ac, Cry2Ab2, and a version of the Cry1A.105 protein. Results of the bioassays indicated that all three Cry1Ab-resistant colonies were resistant to Cry1Ab and Cry1Ac, but remained susceptible to Cry1A.105 and Cry2Ab2 with no evidence of cross-resistance.

As noted earlier, Cry1A.105 is a chimeric protein consisting of domains I and II and the C-terminus of Cry1Ac and domain III of Cry1Fa. Several pieces of evidence suggest that there is at least some likelihood of cross-resistance of Cry1Fa and Cry1Ac/Cry1Ab. Denolf et al. (1993) conducted Cry1Ab, Cry1Ac, and Cry1B proteins binding experiments with isolated brush border membrane vesicles (BBMV) and gut tissue sections from ECB. These studies indicated that Cry1Ab and Cry1Ac proteins recognized the same membrane receptor with different binding affinities while the Cry1B protein recognized a separate receptor. More recent binding studies with BBMV from ECB conducted by Hua et al. (2001) indicated that there was limited shared binding between Cry1Fa and Cry1Ab/Cry1Ac proteins. Pereira et al. (200) showed that there was little cross-resistance to Cry1Ac (6.9-fold) in a Cry1Fa-resistant line of ECB (>3,000-fold). Jurat-Fuentes and Adang (2001) demonstrated that Cry1Fa (and Cry1Ja) share the Receptor A binding site with the Cry1A toxins in *Heliothis virescens* (tobacco budworm), but they also have unique binding sites. These researchers proposed a model that suggests that Cry1Fa, Cry1Ab, and Cry1Ac all bind to the Cry1Aa binding site (called Receptor A, although with different binding affinities) as well as to unique binding sites. An altered Cry1Aa binding site may cause resistance to Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Fa proteins, but the unique binding sites also play a role in toxicity. Competition binding experiments performed by Hernández and Ferré (2005) showed the occurrence of a common receptor for Cry1Ac, Cry1Fa, and Cry1Ja in *Helicoverpa armigera*, *H. virescens*, and *Spodoptera exigua*. So far, all available information on binding site competition suggests that Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja share a common binding site in most, if not all, insects in the order of Lepidoptera. These authors suggest that Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja protein binding to a common site explains, perhaps, the biochemical basis of multiple resistance and cross-resistances among these five proteins in some insect species. Jurat-Fuentes and Adang (2006) recently demonstrated that a cadherin-like protein, HevCaLP, is the functional receptor for Cry1Ac binding in a highly-resistant (>300,000-fold) tobacco budworm colony (YHD2 while it is not a receptor for Cry1Fa (130-fold resistant). These results suggest that the Cry1Fa and Cry1Ac shared binding site is not a cadherin-like protein and that cross-resistance would be due to modification of some other receptor. Collectively, the availability information indicates that there is some likelihood of cross-resistance to both the Cry1Fa and Cry1Ab/Cry1Ac proteins through modification of a single shared receptor site. Hernández and Ferré (2005) suggest that transgenic plants expressing stacked combinations of Cry1Ac (by extension Cry1Ab), Cry1Fa, and Cry1Ja nor rotations of Bt crops containing single genes of these three (four) proteins would be a good resistance management strategy. In the case of corn, primary pests susceptible to Cry1Ab and Cry1Fa, such as ECB (and SWCB and CEW), would necessitate the importance of establishing the binding site model for this species in order to develop an appropriate resistance management strategy.

Monsanto has shown, using the weight-of-evidence approach, that there is a low likelihood of cross-resistance between Cry1A.105 and Cry2Ab2 (see Section 1, **"Assessment of the Probability of Cross-Resistance to the Cry1A.105 and Cry2Ab2 Proteins"** of this review). It is assumed that primary mechanism of resistance will be that of binding site modification, a reasonable assumption based on studies with other Bt-resistant insect populations (laboratory and field) (see Ferré and Van Rie 2002). Similarly, Monsanto has adequately demonstrated that there is a low likelihood of cross-resistance of Cry1A.105 and Cry1Ab. On the other hand, Monsanto has not addressed the likelihood of cross-resistance of Cry1A.105 and Cry1Fa and Cry1Ac. The Cry1A.105 protein is a chimeric protein consisting of Domains I and II and the C-terminus of Cry1Ac and Domain III of Cry1Fa.

It is recommended that Monsanto provide BPPD with additional information on cross-resistance of Cry1A.105 and Cry1Fa and Cry1Ac (including binding site models and use of resistant colonies) for the target pests and determine how such cross-resistance may impact the durability of MON 89034.

4. Proposed IRM Plan for MON 89034

Monsanto contends that the introduction of MON 89034 will significantly decrease the risk of lepidopteran pests evolving resistance to Bt corn. Monsanto's Insect Resistance Management (IRM) plan for MON 89034 focuses on three key assumptions: (1) resistance to Cry1A.105 and Cry2Ab2 is expected to be at least partially recessive; (2) the probability of cross-resistance between Cry1A.105 and Cry2Ab2 is low; and (3) the level of both Cry1A.105 and Cry2Ab2 produced in MON 89034 confer high level of control of susceptible target pests (high dose defined as at least 90% and preferably >95% control). Should these assumptions be met, MON 89034 will have significantly more durability than all existing single-gene products for lepidopteran control in the U.S., including MON 810 (Cry1Ab), BT11 (Cry1Ab), Herculex I (Cry1Fa), and their respective stacked products. The primary focus of the MON 89034 IRM plan is on management of ECB resistance and to a lesser extent, SWCB and CEW in regions where these pests are economically important. FAW and sugarcane (SCB) are not a focus of Monsanto's IRM for MON 89034.

Monsanto's proposed IRM plan for MON 89034 consists of the following elements.

1. A 5% structured non-lepidopteran Bt corn refuge for the Corn Belt based on two independent (minimal cross-resistance), highly effective modes of action of Cry1A.105 and Cry2Ab2;
2. A 20% structured non-lepidopteran Bt corn refuge for cotton-growing areas;
3. Annual resistance monitoring, grower education, and compliance monitoring programs; and
4. A remedial action plan that describes a series of action to investigate suspected resistance, confirms actual resistance, and mitigates the resistant population(s).

Each of these elements will be discussed below.

5% Structured Refuge for Field Corn Uses of MON 89034 in the Corn Belt

The critical question is whether Monsanto has provided sufficient data/information to indicate that the durability of a 5% structured refuge (as Monsanto has proposed) is equal to or greater than durability of a 20% structured refuge (the current structured requirement for lepidopteran-protected Bt corn products) for management of resistance to MON 89034. Monsanto's MON 89034 IRM plan for field corn uses focuses on ECB. Does MON 89034 have consistently high mortality of susceptible homozygotes for all of the primary target species?

In the case of MON 89034, two Bt genes, *cry1A.105* and *cry2Ab2*, were engineered into Bt corn plants to provide even better control (than first-generation, single Bt protein products) of ECB, CEW, SWCB, and FAW. Two proteins are expressed in MON 89034 corn plants: Cry1A.105, a chimeric protein consisting of domains of Cry1Ac and Cry1Fa; and Cry2Ab2, the same protein that is expressed in Bollgard II cotton (a Monsanto product). Monsanto has provided sufficient efficacy data to demonstrate that MON 89034 provides good control of ECB, CEW, SWCB, FAW, and SCB (see Matten, 2007 for BPPD's review of Monsanto's submission, Headrick et al. 2006, MRID# 469514-15). The level of control of MON 89034 for these pests was equal to or greater than YieldGard (MON 810, Monsanto's single Bt (Cry1Ab) trait corn product).

Monsanto's first assumption is that Cry2Ab2 and Cry1A.105 have different modes of action and therefore the potential for cross-resistance is low. As discussed earlier in this review, Cry2Ab2 and Cry1A.105 have low sequence homology (14%), different activation mechanisms and binding characteristics, unique antibody binding sites, and resistant insects to one protein will be controlled by the other protein. Therefore, it can be concluded that Cry2Ab2 and Cry1A.105 have different modes of action and therefore it is expected that there will be a low likelihood of cross-resistance (see earlier discussion in Section 1 **"Assessment of the Probability of Cross-Resistance to the Cry1A.105 and Cry2Ab2 Proteins"**). Lack of cross-resistance would increase the durability of MON 89034. These two proteins, Cry2Ab2 and Cry1A.105, therefore, seem to be good candidate proteins for pyramiding. BPPD agrees with Monsanto that the probability of cross-resistance between Cry1A.105 and Cry2Ab2 is low and these two proteins have different modes of action.

On the other hand, Roush (1998) cautions that proteins that have already shown significant levels of cross-resistance in resistant insect strains (e.g., *H. virescens*, *H. armigera*, *S. exigua*, *O. nubilalis*, *Plutella xylostella*), such as between Cry1A, Cry1Fa and Cry1J proteins, should not be used in pyramiding. This same warning was also given by Hernández and Ferré (2005). Cry1A.105 is a chimera that consists of binding domains of Cry1Ac and Cry1Fa. There are commercial Bt crops that express Cry1Ac, Cry1Ab, Cry1Fa proteins and these products have been in the marketplace for nearly a decade. Should there be insect populations resistant to Cry1Ac, Cry1Ab, and/or Cry1Fa that are cross-resistant to Cry1A.105 then the durability of MON 89034 would be significantly reduced and a 5% structured refuge would be insufficient to maintain high levels of durability.

Given that there is very high amino acid similarity between Cry1Ab, Cry1Ac, and Cry1A.105 proteins then the potential for cross-resistance between Cry1Ab, Cry1Ac and Cry1A.105 is an important consideration. Cry1A.105 and Cry1Fa have about 76% amino acid similarity. However, what is really important is the similarity of the binding domain III of Cry1Fa and Cry1A.105 which is presumed to be very high. Cross-resistance is a real possibility for these two proteins. There are several lines of evidence that indicate that Cry1Fa and

Cry1Ab/Cry1Ac share at common binding receptor although each of these proteins has unique binding receptors as well (Denolf 1993, Hua 2001, Jurat-Fuentes and Adang 2001; Hernández and Ferré, 2005). Evidence for a shared binding receptor would increase the likelihood of cross resistance should resistance evolve through modification of the shared binding receptor.

Evidence provided by Monsanto indicates that there is little cross-resistance of Cry1A.105 and Cry1Ab. One cannot, however, infer much about the likelihood of cross-resistance of Cry1A.105, Cry1Fa, and Cry1Ac based on the binding patterns of Cry1A.105 and Cry1Ab because binding patterns are unique to each species (e.g., ECB, SWCB, and CEW) and each protein. Monsanto did not address the likelihood of cross-resistance of Cry1A.105 and Cry1Fa, a protein already in existing Bt corn and Bt cotton products, and what impact cross-resistance would have on the durability of MON 89034. BPPD recommends that Monsanto provide additional information on cross-resistance of Cry1A.105 and Cry1Fa and Cry1Ac (including binding site models and use of resistant colonies) for the target pests and determine how such cross-resistance may impact the durability of MON 89034.

Monsanto's second assumption is that resistance will be recessive. Ten years of resistance monitoring data indicate that the frequency of Cry1Ab alleles in ECB is very low ($<4 \times 10^{-4}$) and that this frequency has not changed significantly during this time. The 20% structured refuge requirement for single-gene Bt corn products has been in place for over a decade and, as noted earlier in this review, there is no evidence of field resistance to Cry1Ab and Cry1Fa in ECB, SWCB, and CEW during that period in the continental U.S. There is also no evidence of Cry2Ab2 resistance (CEW) after five years of widespread use of Bollgard II cotton. The absence of any cases of field resistance to Bt crops after a decade of use indicates that any relatively common Bt-resistant alleles must be recessive (Tabashnik et al., 2003). This evidence provides a strong indicator that resistance to the Bt proteins expressed in MON 89034 would also be recessive. BPPD agrees with this line of reasoning. Pyramids are considerably more effective when resistance frequencies are low provided that the susceptible homozygotes are all killed by each of the toxins used separately (Roush 1998; Figure 4.).

Monsanto's third assumption for its proposed 5% structured refuge depends on whether the level of both Cry1A.105 and Cry2Ab2 produced in MON 89034 confers a high level of control of susceptible target pests (defined as at least 90% and preferably >95% mortality). It is this third assumption that is the most difficult to prove.

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). Pyramiding two or more proteins increases the chance that at least one of the proteins will be especially favorable to resistance management. Modeling simulations predict that pyramids (without cross-resistance) can reduce the need for larger refuges (Roush 1998).

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.

Monsanto relies on the Roush (1998) model to support the need for a 5% structured refuge rather than a 20% structured refuge in the Corn Belt. Roush's model (1998; Figure 2) indicates that a 5% structured refuge is equal to or greater than a 20% structured refuge for a highly effective, high dose single-gene product when a two-gene product (MON 89034 in this case) achieves at least 95% control of susceptible homozygotes **and** 70% control of heterozygotes assuming there is no cross-resistance. Monsanto's dose studies, as discussed earlier, present a mixed picture for MON 89034 (see Section 2, **"Dose"**). Dose and efficacy data indicate that MON 89034 has a high level of control against the four major target pests (as described in Head 2006): "(1) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provide essentially 100% control of ECB; (2) the Cry1A.105 protein in MON 89034 provides approximately 95% control of SWCB, while the Cry2Ab2 protein provides 80-90% control; (3) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provide >95% control of FAW; and (4) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provide 90-95% control of CEW." The actual level of control may be even higher due to growth inhibition among survivors that would likely preclude developmental completion. The target pests appear to be somewhat more sensitive to Cry1A.105 than to Cry2Ab2.

Monsanto's dose testing indicates that MON 89034 has a high level of control (greater than 90%) of susceptible homozygotes (ECB, SWCB, CEW, FAW), one of two thresholds needed to support the durability of a 5% structured refuge for a two-gene pyramided Bt corn product (as equal to or better than that of a single-gene Bt corn product expressing a high dose of control against the target pests). However, it is not easily discernable as to whether each individual toxin kills greater than 95% of susceptible individuals. This is important for prediction of the durability of MON 89034: Roush's simulations (1998; Figure 3) showed that the greatest gains of pyramiding two proteins are when the mortality of susceptible insects is considerably greater than 95%, especially if resistance allele frequencies are quite low.

On the other hand, Monsanto's dose studies cannot directly evaluate whether MON 89034 kills at least 70% of the heterozygotes, the other threshold needed to support a 5% structured refuge for a two-gene pyramided Bt corn product. Monsanto has not provided enough information to determine the mortality of susceptible (SS) homozygotes and heterozygotes (RS) on MON 89034 plants. It is important to know whether Cry1A.105 and Cry2Ab2 are: 1) both high dose proteins; 2) one high dose and one moderate dose protein (and which one is high and which one is moderate); or 3) two moderate dose proteins to control ECB (and SWCB) in the Corn Belt. In other words, one has to establish whether each protein can kill potentially resistant individuals to the other protein. To evaluate MON 98034 in the context of Roush's model, it must be determined whether Cry1A.105 and Cry2Ab2 are produced at high levels to kill at least 95% of susceptible homozygotes and 70% of the heterozygotes. Because of this, BPPD recommends that Monsanto further investigate heterozygote mortality for MON 89034. BPPD recognizes that direct evaluations of heterozygote effects can be difficult, particularly if resistant colonies for the target pests are unavailable. However, the 1998 SAP suggested several ways to estimate mortality for less susceptible larvae (i.e. heterozygotes). These techniques included testing larger, later instar larvae that may be less susceptible to the either the Cry1A.105 or Cry2Ab2 proteins or with PIPs expressing less protein (less Cry1A.105 or Cry2Ab2) than MON 89034. As Roush (1998) cautions, "...small

refuges remain risky..." when mortalities of heterozygotes are lower than expected. For MON 89034, it has only been assumed (but not verified) that the heterozygote mortality will be at least 70% for each protein.

Cross-resistance between Cry1A.105 and Cry1Ac and Cry1Fa is not known, but published studies indicate that there is at least some potential for cross-resistance between Cry1A and Cry1Fa proteins in a number of insect species (see earlier discussion). What impact this cross-resistance will have on the durability of MON 89034 is not known.

Monsanto's use of the Roush (1998) model as a guide to predict the durability of MON 89034 is very useful, but it is only a first step. Roush encouraged readers to further investigate the points raised in his 1998 paper with additional modeling and experiments (see Roush 1998). This was not done by Monsanto. Additional modeling using a species-specific (e.g., ECB and SWCB for the Corn Belt), spatially-explicit, preferably stochastic, landscape model of available Bt crops expressing many different Cry proteins (needs to be a multiple gene model, a more complex model) needs to be used to more precisely predict the evolution of ECB resistance (or SWCB) to MON 89034. This new model would need to consider the impact of other Bt proteins in which there may be some cross-resistance. This is analogous to the species-specific simulation modeling that EPA required Monsanto do to support the use of natural refuge (instead of a structured refuge) for management of *H. virescens* and *H. zea* to the Cry1Ac and Cry2Ab2 proteins expressed in Bollgard II cotton. In conclusion, Monsanto's data and modeling do not support a 5% structured refuge for MON 89034 for field corn uses in the Corn Belt.

Given the uncertainties in the dose determination for ECB and SWCB (SS and RS mortality) (note: CEW and FAW are lesser pests in the Corn Belt), cross-resistance likelihood of Cry1A.105, Cry1Ac, and Cry1Fa, and limitations of the simulation modeling, BPPD recommends that the current 20% structured refuge requirement for field corn uses of MON 89034 in the Corn Belt be maintained until such time as Monsanto can address these uncertainties associated with the durability of a 5% structured refuge. There are many Bt corn and Bt cotton products in the landscape. Cross-resistance conferred by any of these proteins may negatively affect the durability of MON 89034. Studies indicate that there is at least some potential for cross-resistance between Cry1A.105, Cry1Fa, and Cry1Ac proteins in a number of insect species (see earlier discussion). Monsanto needs to examine the potential of Cry1A.105, Cry1Ac, and Cry1Fa cross-resistance and what impact it has on the durability of MON 89034.

20% Structured Refuge for Field Corn Uses of MON 89034 in Cotton-Growing Areas

Monsanto has proposed that a 20% structured refuge rather than the current 50% structure refuge requirement for single-gene lepidopteran-control products be used to manage insect resistance to MON 89034 in cotton-growing areas. The major pest of concern for Bt corn in cotton-growing areas is CEW (also known as cotton bollworm when it feeds on cotton), although ECB, FAW, SCB (sugar cane borer) are also sporadic corn pests in cotton-growing areas. As described earlier in this review (Section 2 **"Dose"**), Cry1A.105 and CryAb2 proteins have at least 90% control of CEW. Previous studies submitted by Monsanto (Head and Reding 2001; reviewed in EPA 2007) demonstrated the low likelihood of cross-resistance between the Cry2Ab2 and Cry1Ac proteins. Both Cry1A.105 and Cry2Ab2 have a low

likelihood of cross-resistance with Cry1Ab (see earlier discussion in Section 3, "**Impact of Prior Use of Cry1Ab-Expressing Bt Corn Products on MON 89034**").

Monsanto used its deterministic, non-spatial model (Gustafson and Head 2005) to examine whether planting a 20% structured non-Bt corn refuge with MON 89034 was sufficient to manage the risk of resistance evolution to Bt corn and Bt cotton products. In this model, it was assumed that all cotton planted consisted of Bollgard II cotton, with no non-Bt cotton in the system, and that 80% of the corn planted in the region consisted of MON 89034 and 20% non-Bt corn. The modeling was focused on estimation of the likelihood of CEW resistance in the Mississippi region because of the relatively higher risk of CEW resistance evolution in this review. Monsanto estimated the effective (all non-Bt hosts of CEW, including current levels of non-Bt cotton and 20% non-Bt corn refuge associated with MON 89034) and natural refuge (only non-cotton hosts of CEW, including 20% structured non-Bt corn refuge associated with MON 89034 and other unmanaged hosts) available for CEW in this region as described in Gustafson and Head (2005). These estimates were used as parameter values in the model. One scenario modeled assumed that MON 89034 is fully cross-resistant with Bollgard II cotton (i.e., Cry1A.105 and Cry1Ac are fully cross-resistant). Resistance was assumed to be complete with no associated fitness costs. Using these assumptions, the simulation modeling predicted that a 20% non-Bt corn refuge for MON 89034 in the southern cotton-growing areas would be sufficient to manage the risk of resistance evolution to Bt corn and Bt cotton products. Resistance to Cry2Ab2 protein evolved first and took >24 modeling years to evolve (modeling time was 25 years). It is not clear from Monsanto's discussion whether Cry1A.105 and Cry1Fa cross-resistance was included in the modeling. The current landscape has both Cry1Fa- and Cry1Ab-corn and Cry1Fa- and Cry1Ac- and Cry2Ab2 + Cry1Ac-cotton products. Should there be substantial cross-resistance then the value of MON 89034 would be dramatically reduced.

A 20% non-Bt corn refuge for MON 89034 in the southern cotton-growing areas would be sufficient to manage the risk of resistance evolution to Bt corn and Bt cotton products assuming there is no cross-resistance. However, Monsanto did not sufficiently address the cross-resistance of Cry1A.105, Cry1Fa, and Cry1Ac in the cotton-growing landscape and how such cross-resistance may impact the durability of MON 89034. Should cross-resistance be of concern then the durability of MON 89034 in the southern cotton-growing areas might be compromised. Monsanto needs to address this potential in subsequent simulation modeling.

Sweet Corn Uses

As described in Monsanto's submission (Head 2006): "In the U.S., sweet corn is grown on approximately 500,000 acres, with California, Florida, Georgia, New York, Ohio, and Pennsylvania accounting for 62% of the acres. The insecticide use per acre on sweet corn is approximately 35-fold that of field corn (2.7 lb/A versus 0.76 lb/A) (USDA, 2006) and typically, 12 - 40 applications of insecticides may be applied to a single crop of sweet corn in the southern U.S (Adams 1996). Therefore, planting of MON 89034 has the ability to drastically reduce the amount of synthetic insecticides used for sweet corn production."

Monsanto has proposed the use of MON 89034 as a sweet corn product to control certain lepidopteran insect pests in conjunction with no structured refuge. While sweet corn has a similar pest spectrum to field corn, agronomic practices differ between sweet corn and field corn. This makes pest management different between the two crops. As described in Monsanto's submission (Head 2006): "Sweet corn is harvested approximately 18 to 23 days

after silk emergence, compared to field corn in which the grain is allowed to mature and dry in the field. For sweet corn, the ears are harvested while still wet and placed in cold storage for fresh market corn or processed immediately. Shortly after harvest, corn stalks are typically destroyed in the field by disking, chopping or plowing. Previous work by Lynch et al. (1999), show that these harvest and post-harvest practices make it unlikely that any surviving/resistant larvae could survive, complete its development, and contribute any resistant allele to the next generation in sweet corn. Even if a larva was to survive, sweet corn farmers, including home gardeners, typically grow sweet corn in small plots along with many other vegetables that serve as alternative hosts for these polyphagous lepidopteran pests. Therefore, sufficient non-corn refuge should be present due to the typical practices of planting multiple host crops."

BPPD requested in its January 17, 2007 that Monsanto provide additional (dose) data to support the sweet corn use. Monsanto responded to BPPD's request for supplemental data on March 9, 2007. Monsanto provided data that compared the estimated Cry1A.105 and Cry2Ab2 protein levels in leaf tissues collected from MON 89034 sweet corn varieties with field corn varieties (see Table 4 in Bogdanova, 2007; MRID# 470794-02). Sweet corn data came from one site and field corn data came from five sites. The mean levels of the Cry1A.105 and Cry2Ab2 proteins were comparable between field and sweet corn MON 89034 hybrids.

BPPD agrees that no structured refuge is needed in conjunction with the sweet corn use based on the destruction of potential resistant larvae through cultivation practices.

Popcorn Use of MON 89034

Monsanto proposes to use the same IRM plan described for field corn for popcorn. Monsanto states that there are approximately 291,000 acres of popcorn grown annually in the U.S., with Illinois, Indiana, Iowa, Nebraska, and Ohio accounting for 86% of the planted acres (Pike, 2003). Popcorn, like field corn, is allowed to mature and dry in the field, and the pest spectra are essentially identical in popcorn and field corn.

Monsanto provided no additional dose and/or efficacy data to what was provided for field corn to support the use of MON 89034 on popcorn. Without these data, the popcorn use cannot be supported.

Other Elements of IRM for MON 89034

Monsanto proposes to have resistance monitoring, grower education and compliance monitoring as necessary parts of the IRM program. They propose to implement a program similar to what is currently carried out for MON 810 and other single-gene Bt corn products. In particular, the educational and compliance assurance programs for MON 89034 will follow the structure established through consultations between EPA and the industry, and will involve working closely with NCGA and other interested stakeholders.

Similarly, post-commercial resistance monitoring programs will be established as an extension of existing programs to track the susceptibility of the key lepidopteran corn pests to the Cry1A.105 and Cry2Ab2 proteins. In the monitoring program, insect populations will be collected and each protein will be tested separately, rather than a mixture of the two proteins,

because resistance to one protein could be masked by the activity of the other. As part of this program, baseline susceptibility studies are planned for the Cry1A.105 protein against ECB (through Dr. Blair Siegfried at the University of Nebraska), and for the Cry1A.105 and Cry2Ab2 proteins against SWCB (through Dr. Qisheng Song at the University of Missouri), and CEW (through Bruce Lang of Custom Bio-Products). The baseline susceptibility of ECB to Cry2Ab2 has already been assessed over a two year period (see Appendix 4 - Siegfried and Spencer, 2001 in Monsanto's submission, Head, 2006; MRID# 469514-30). In the case of CEW, baseline studies and annual monitoring have been conducted for Cry2Ab2 protein as part of the Bollgard II cotton IRM program, and the resulting data will be useful for MON 89034. In addition to the formal monitoring program, any unusual damage from lepidopteran pests will be monitored by the routine scouting of corn fields and be reported to Monsanto or local extension agents.

A remedial action plan has been developed and approved by EPA for MON 810 and other single-gene Bt corn products (EPA, 2001). This plan describes a series of actions to investigate suspected resistance, confirm actual resistance, and mitigate the resistant population. The basis of this plan also is appropriate for MON 89034. However, because MON 89034 contains both the Cry1A.105 and Cry2Ab2 proteins, this product has the advantage of having a "built-in" mitigation program if resistance evolves to one of the Cry proteins but not the other. Therefore, Monsanto indicates that the remedial action plan should only be implemented for MON 89034 if a field population evolves resistance to both the Cry1A.105 and Cry2Ab2 proteins.

Monsanto's proposed program for resistance monitoring, grower education and compliance monitoring as part of the MON 89034 IRM program is "acceptable." No Cry1A.105 baseline susceptibility studies have been conducted, but are planned by Monsanto. Monsanto has indicated that baseline susceptibility information for ECB to Cry2Ab2 has been collected over a two-year period (summarized in Monsanto's submission). For each protein, a discriminatory concentration (diagnostic dose) will have to be determined for use in the annual resistance monitoring program. Annual reporting to the Agency of the results of the resistance monitoring, grower education, and compliance monitoring is needed (as is required for all other Bt PIPs). If there is confirmed resistance to either protein then it must be reported to the Agency (see FIFRA 6(a) incident reporting requirements and the requirements as part of the Remedial Action plan).

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Chemical:	Bacillus thuringiensis Cry1A.105 protein and genetic material necessary (vector PV-ZMIR245) for its production in corn Bacillus thuringiensis Cry2Ab2 protein and the genetic material necessary (vector PV-ZMIR245) for its production in corn
	PC Code: 006514 006515
HED File Code:	41400 BPPD IRM
Memo Date:	11/6/2007
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