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EPA IRM REQUIREMENTS FOR BOLLGARD™ II COTTON

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Abstract

The United States Environmental Protection Agency (EPA) required a refuge-based insect resistance management (IRM) program for Bollgard™ II cotton (Cry2Ab2 and Cry1Ac plant-incorporated protectants (PIPs) as expressed in cotton) which is virtually the same as for Bollgard™ cotton. There are three structured refuge options with specific deployment requirements: 5% external, unsprayed structured refuge, 5% embedded refuge, and 20% external, sprayed structured refuge. There is also an optional community refuge program. Other requirements are for annual resistance monitoring, grower education, compliance assurance, research, and reporting. There is also a requirement for a remedial action plan should insect resistance develop in the field. The registration for Bollgard™ II cotton was granted on December 23, 2002 and expires May 1, 2004 due to the expiration of the temporary tolerance exemption for the Cry2Ab2 protein.

Introduction

Bollgard™ II cotton expresses both the *Bacillus thuringiensis* Cry2Ab2 and Cry1Ac proteins (Cry1Ac is the protein found currently in Bollgard™, EPA Reg. No. 524-478). Bollgard™ II cotton is intended to protect cotton from feeding by tobacco budworm (*Heliothis virescens,* TBW), pink bollworm (*Pectinophora gossypiella*, PBW), cotton bollworm (*Helicoverpa zea*, CBW), cabbage looper (*Trichoplusia ni*, CL), saltmarsh caterpillar (*Estigmene acrea,* SC), cotton leaf perforator (*Bucculatrix thurbeiella,* CLP), soybean looper (*Pseudoplusia includens,* SL), beet armyworm (*Spodoptera exigua,* BAW), fall armyworm (*Spodoptera frugiperda*, FAW) and yellowstriped armyworm (*Spodoptera ornithogolli*, YSA). By comparison, Bollgard™ cotton is intended to protect primarily against TBW, CBW, and PBW. Based on cotton insect loss data from 1991-2000, the three primary pests, TBW, CBW, and PBW, account for more than 77% of the yield lost and 84% of the insecticide use due to lepidopteran infestation in cotton (Williams 1996 -2000).

The Monsanto Company transformed Bollgard™ DP50 lines with a linearized fragment of vector B1579, also known as PV-GHBK11, using particle bombardment. In addition to the *cry1Ac* gene contained in the original Bollgard™ line 531, the new Bollgard™ II lines also contain the *cry2Ab2* gene from *Bacillus thuringiensis* var. *kurstaki* and the *uidA* gene encoding for the β-D-glucuronidase from *Escherichia coli* protein as a scorable marker. Bollgard™ II cotton, in combination with a refuge and other components of an insect resistance management plan (IRM), may significantly delay the development of insect resistance to cotton containing the Cry1Ac protein (Bollgard™ cotton). The IRM requirements for Bollgard™ cotton are found in EPA's 2001 *Bt* Plant-Incorporated Protectants Biopesticides Registration Action Document (EPA 2001, see Section III. "*Bt* Cotton Confirmatory Data and Terms and Conditions of the Amendment"). An IRM plan has been required and implemented for Bollgard™ cotton since its commercial introduction in 1996.

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EPA Review of IRM Strategy for Bollgard™ II Cotton

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 insects.

TBW, CBW, and PBW differ in their impact on cotton. 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 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 a 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. PBW has limited mobility and dispersal (although it has extensive spring flights) and limited host range. Additional research 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. Such 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 or Cry2Ab2 proteins. Therefore, for Bollgard™ cotton, the Agency made the determination that some additional data are needed to improve the IRM strategy. Specifically, the Agency required research to better understand the relevance of alternate hosts as potential refuge, the impact of supplemental insecticide treatments on refuge effectiveness, and potential north-south movement of CBW (from corn regions to cotton regions) (USEPA 2001, see Section III. "*Bt* Cotton Confirmatory Data and Terms and Conditions of the Amendment"). These same data requirements will also apply to Bollgard™ II cotton.

Insecticidal Activity and High Dose Determination

Insecticidal Activity Against Lepidopteran Pests. The results of *in vitro* and *in planta* studies indicate that both the Cry1Ac and Cry2Ab 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). FAW and BAW are more sensitive to Cry2Ab than Cry1Ac, but TBW and CBW are more sensitive to Cry1Ac.

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 (Appendix 4 in Head and Reding 2001) 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 in Bollgard™ II cotton are present at consistently high levels across all plant parts for the duration of the growing season, although there is some drop off in expression later in the season. EPA defines a high dose as twenty-five times the protein concentration necessary to kill susceptible larvae based on the recommendation of the 1998 FIFRA Scientific Advisory Panels (SAP) (SAP 1998). The 1998 SAP identified five possible approaches that could be used to verify whether a *Bt* plant-incorporated protectant could be considered to provide a high dose for a particular target insect.

Bollgard™ II High Dose Determination for TBW, CBW, and PBW. Data were evaluated to demonstrate that the Cry2Ab protein alone and the combination of Cry2Ab + Cry1Ac proteins as expressed in Bollgard™ II cotton produce a functional (or close to) high dose for control of CBW, TBW, and PBW. These studies will be discussed below. EPA has previously concluded that a 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 Appendix 4, Figure 6 in Head and Reding, 2001). 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.

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 Appendix 4, Figures 1-5 in Head and Reding, 2001). 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 is LC_{50} equal to 0.006 and of Cry2Ab is LC_{50} equal to 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 functional 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 non-high dose against CBW. Monsanto submitted three separate sets of laboratory studies to demonstrate that the Cry2Ab protein alone and the Cry2Ab + Cry1Ac proteins are expressed at a functional high dose (or nearly high dose) in Bollgard™ II cotton for control of CBW. These three methods were: a reconstituted diet bioassay using various dilutions of lyophilized cotton tissues of either nontransgenic or transgenic tissue, a diet overlay bioassay in which plant tissue was overlaid on lepidopteran insect diet at various dilutions (method as described by Greenplate 1999), and lastly, artificial infestation of nontransgenic or transgenic tissue using late instar larvae. These three methods taken together provide a strong case that the Cry2Ab protein represents, at least in the laboratory, a high dose (or very close to a high dose) against CBW.

Results of the diet reconstitution bioassay examining Bollgard™ II cotton, which contains both the Cry2Ab and Cry1Ac proteins, and the Bollgard™ II segregant, which contains only the Cry2Ab protein, indicate that CBW is controlled even at a 10- to 25-fold dilution of the transgenic cotton tissues. Replication was somewhat variable between replications of the experiment; thus, Bollgard™ II cotton produces at least a 10X concentration, and perhaps 25X concentration, to kill all of the susceptible insects. However, even in the circumstances where there wasn't 100% mortality at the 25X dilution, none of the surviving larvae in the reconstituted diet overlay completed development.

Results of the diet overlay bioassay indicate that the percent CBW population arrested at or before the second instar caused by plant material from either Bollgard™ II cotton or from the Bollgard™ II segregant was comparable to the developmental arrest caused by Bollgard™ (Cry1Ac) cotton tissue with TBW. By deductive reasoning, this means that there is a functional high dose of Cry2Ab produced to control CBW since it has already been shown that there is a high dose of Cry1Ac produced to control TBW.

A third laboratory study indicates that there is 100% mortality of late instar CBW larvae on both the cotton squares and the bolls of Bollgard™ II cotton. Therefore, it appears that Bollgard™ II cotton expresses a functional "high dose" (or very near a high dose) of the Cry2Ab protein against CBW based on these three laboratory studies. Based on the data, it is likely the inheritance of Cry2Ab protein will be recessive. This means that a homozygous resistant individual would be rare. Until resistance develops in the field, it is not possible to determine the heterozygote dominance, the inheritance of resistance, and the mechanism of resistance to the Cry2Ab protein.

Cross-Resistance Potential and Mode of Action

Cross-resistance occurs when a pest becomes resistant to one *Bt* protein that then allows the pest to resist other, separate *Bt* proteins. Efforts are underway to assess whether cotton insects show cross-resistance to various *Bt* proteins. Some pests of cotton are also pests of other crops for which *Bt* transgenic varieties or microbial *Bt* insecticides are available (e.g., CBW on cotton, fall armyworm (*Spodoptera frugiperda* J. E. Smith) on tomato). Future resistance monitoring methods may incorporate such information because cross-resistance is an area of major concern for resistance management and poses risks to both transgenic *Bt* crops and microbial *Bt* insecticides. There are currently registered *Bt* microbial pesticide products that contain both Cry1A and Cry2A toxins. Cross-resistance also poses a risk to pyramid strategies, in which multiple proteins are deployed simultaneously in the same hybrid. To date, the development of cross-resistance has not been shown in insect pests exposed in the field to *Bt* crops producing different *Bt* proteins. A detailed discussion of cross resistance as related to the registered *Bt* plant-incorporated protectants is found in USEPA (2001, Section IID.).

Discussions of cross-resistance are complicated due to the fact that the exact nature and genetics of *Bt* resistance are not fully understood. Resistance may vary substantially from pest to pest, adding to the unpredictability of the system. In general, it is

possible for resistance to *Bt* proteins to occur through several different mechanisms, some of which may result in cross-resistance to other proteins. The most recent review of the biochemistry and genetics of insect resistance to *Bacillus thuringiensis* is by Ferré and Van Rie (2002). The most understood mechanism of resistance is reduced (midgut) binding affinity to *Bt* proteins. Different Cry proteins may bind to distinct receptors in an insect gut. Modifications to these insect crystalline protein receptors have been implicated in resistance to Cry proteins. An example of a possible shared binding site resulting in cross-resistance was observed with TBW. In this case, a laboratory strain of TBW selected for resistance to Cry1Ac was also found to be resistant to the Cry1Aa, Cry1Ab, and Cry1F proteins (Gould et al. 1995).

Because of the complexity and uncertainty associated with predicting cross-resistance, the Agency has taken measures to evaluate the cross-resistance of pest species to the Cry proteins expressed in *Bt* plants. EPA requested that Monsanto submit data and/or literature evaluating the cross-resistance potential of various insect pests to the Cry1Ac and Cry2Ab proteins expressed in Bollgard™ II cotton. The following biochemical information was used to evaluate cross-resistance potential: sequence homology of Cry1A versus Cry2A proteins, structural comparison of Cry1Ac and Cry2Ab proteins, mechanism of action and binding characteristics, activity of Cry2Ab against Cry1A-resistant colonies (summarized in Head and Reding, 2001; Reed, 2002). Based on the evidence presented below, there is a low likelihood of high levels of cross-resistance in the target insect pests for the Cry2Ab and Cry1Ac proteins.

Sequence Homology of Cry1A Versus Cry2A Proteins. Based on the information evaluated, 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. There were two compelling pieces of information to support the hypothesis that the low likelihood of substantial sequence similarity between the Cry1Ac and Cry2Ab proteins suggests that there is a difference in their tertiary structure. Morse et al (2001) determined the three-dimensional crystal structure of the Cry2Aa toxin and defined the putative receptor binding epitope on the toxin. This 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). This 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 one of more receptors, the likelihood of cross-resistance increases. Information from the available literature supports 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 (not concentration dependent) regardless of the amount of toxin added. Additional experiments demonstrated that no specific binding was observed between the full-length Cry2Ab protein and any brush border membrane vesicles (BBMV) from CBW, TBW, and PBW (Reed 2002). 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 insecticidal 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. The Agency reviewed a series of studies examining the activity of Cry2Ab against Cry1A-resistant colonies (Head and Reding 2001). This evidence indicates that when Cry1A-resistant colonies are challenged with Cry2Ab that the potential for cross-resistance is low in TBW, in CBW, and in PBW. Gould (Appendix 1 in Head and Reding 2001) 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 colony (>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, that is, there will only relatively low levels of resistance to several different Cry proteins. When these insects were placed 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 in Head and Reding 2001) 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 in Head and Reding 2001) 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 (Caprio 1998; Roush 1998; Hurley 2000; Livingston et al. 2002).

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, Cry1Ab and Cry2Ab, which have different modes of action, there is a very low likelihood of cross-resistance to Cry1Ab 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). Roush's 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 (or very close to 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.

Even without a high dose for CBW, as in the case of Bollgard™ (Cry1Ac alone), 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 relative to the single gene varieties (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 4.3×10^{-4} (Burd et al. 2001). Burd et al. (2001) also estimated the resistance allele frequency for Cry2Ab to be 3.9×10^{-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,).

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. These 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. 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 cotton 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 time to resistance to the second protein will be greater than the decrease in time to resistance to the initial protein. Thus, extending this argument to Bollgard™ II cotton, Cry2Ab is more effective than Cry1Ac, then 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.

Both Livingston et al. (2002) and Hurley (2000) provide bioeconomic 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. That is, the overall durability of Bollgard™ II cotton will be greater than if Bollgard™ cotton varieties producing only the Cry1Ac protein or Bollgard™ II cotton varieties producing only the Cry2Ab protein were introduced sequentially or in a mosaic. These 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 that 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 (independent introductions in different hybrid lines).

Structured Refuge

The currently required refuge options for Bollgard™ cotton are: 1) 5% external, unsprayed structured refuge (must be within ½ mile of Bollgard™ fields and at least 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) options for Bollgard™ cotton (USEPA 2001, see Section III. "*Bt* Cotton Confirmatory Data and Terms and Conditions of the Amendment"). Based on the modeling predictions discussed above, the currently required IRM program for Bollgard™ cotton is sufficient for Bollgard™ II cotton. That is, all three refuge options are more protective against insect resistance for the three target pests, TBW, CBW, and PBW, using Bollgard™ II cotton 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 cotton 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™ will both be deployed commercially for some overlapping period of time of at least five years, it would be prudent, conservative, practical and provide a uniform message regarding IRM, for Bollgard™ II cotton and Bollgard™ cotton to have the same structured refuge requirements. In addition, unless there is evidence that other hosts are proven to be suitable, only non-*Bt* cotton should be relied upon as refuge.

Resistance Monitoring

EPA has required that Monsanto develop a Bollgard™ II cotton monitoring plan as an extension of the current Bollgard™ cotton monitoring plan for the TBW/CBW and PBW programs (see USEPA 2001, see Section III. "*Bt* Cotton Confirmatory Data and Terms and Conditions of the Amendment"). Baseline susceptibility data to the Cry2Ab (specifically the Cry2Ab2) toxin for the key pests, TBW, CBW, and PBW are being collected for the 2002 and 2003 growing seasons. A report of the baseline susceptibility data is required to be submitted to EPA. Monsanto must also establish diagnostic concentrations for testing for resistance to Cry2Ab2, and provide a detailed resistance monitoring plan for both the Cry1Ac and Cry2Ab2 toxins.

The need for proactive resistance detection and monitoring is critical to the survival of *Bt* technology. For Bollgard™ cotton, Monsanto is required to monitor for insect resistance 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. 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 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 F_2 screen (Andow and Alstad, 1998, Andow et al. 1998) and DNA markers (Gahan et al. 2001) 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. EPA has required that appropriate diagnostic concentration assays be developed to evaluate resistance development to the Cry2Ab toxin.

Remedial Action Plan

EPA required a remedial action plan for Bollgard™ cotton be available in the unfortunate situation that resistance is suspected or actually does develop (USEPA 2001). EPA also required remedial action plans for Bollgard™ II cotton for the purpose of containing resistance and, perhaps, eliminating resistance if it develops. These plans define not only suspected and confirmed resistance, but also the key steps and actions needed if and when resistance develops. The Arizona *Bt* Cotton Working Group has produced "A Remedial Action Plan for PBW Resistance to *Bt* Cotton in Arizona" (see USEPA 2001, Appendix 1). An interim

remedial action plan is currently required to address TBW and CBW resistance to *Bt* cotton, key economic pests of cotton in the mid-South and Southeastern US (see USEPA 2001, Appendix 2). Monsanto has submitted to EPA a revised remedial action plan in May 2002 for Bollgard™ cotton to address TBW and CBW, but this plan is still under review. A key attribute of these plans is having the farmer's involvement in the plan's development.

Generally, if resistance is confirmed, the farmers involved will be required to treat their *Bt* crop with alternative pest control measures. This might be a chemical pesticide known to be highly effective against the insect or it might mean measures such as crop destruction. In addition, the sales and distribution of the *Bt* crop would be suspended in that area and the surrounding area until it can be determined that insects in that area have regained their susceptibility to the *Bt* protein. There would also need to be increased monitoring to define the remedial action area(s). Other remedial action strategies include increasing refuge size, changing dispersal properties, use of sterile of insects, or use other pest control methods (chemical, biological, or transgenic) with other modes of action. Geospatial surveys would help define the scale of remedial action and where to intensify monitoring. However, because no field resistance has yet been found, all of these tactics are untested.

Grower Education and Compliance

Grower education and compliance are essential to the sustainability of any IRM strategy. EPA required Monsanto 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 cotton. The grower education 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"). Because of the importance of grower education, these same type of grower education and compliance assurance requirements will be 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"). 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™ II cotton (or any pesticide technology) is tied into the belief that new technologies will reduce the risk of resistance. To date, Monsanto's grower surveys indicate that greater than 91% of growers surveyed complied with the refuge size requirements for Bollgard™ cotton since 1996 (USEPA 2001, Section IID.).

The compliance assurance program for Bollgard™ II cotton, just as for Bollgard™ cotton, must contain the following general elements that are implemented each grower season: grower education programs, grower affirmation of IRM requirements, an annual grower survey conducted by an independent third party, and penalties for non-compliance (e.g., lack of access to the technology for deviations from the refuge requirements). An annual compliance report is required to be submitted by Monsanto to EPA following each growing season.

Discussion

Even though the Cry2Ab and Cry1Ac proteins are pyramided, a structured refuge is still necessary for Bollgard™ II (Cry1Ac and Cry2Ab) cotton to delay insect resistance. Based on modeling simulations, assuming no-cross resistance and 70% mortality of the RS heterozygotes for each protein, then the use of proteins jointly in a pyramided variety is approximately six-fold better in delaying insect resistance than the use of each protein sequentially. In addition, the size of the refuge may be smaller for the two proteins deployed in a pyramid, such as like Bollgard™ II cotton, to produce a similar delay than when the two proteins are deployed sequentially or as a mosaic.

Based on the Agency's review of the laboratory bioassay data, Bollgard™ II cotton appears to express a functional high dose (or nearly high dose) of the Cry2Ab protein to control TBW, CBW, and PBW. The durability of Bollgard™ II cotton is driven by the Cry2Ab protein. This means that the Cry2Ab protein is far more important than the Cry1Ac in overall resistance management. In addition, it is unlikely there will be any significant cross-resistance between Cry1Ac and Cry2Ab. Cry1Ac and Cry2Ab share very little amino acid homology or tertiary structure similarities and have different binding kinetics and mode of action. When Cry1A-resistant TBW, CBW, and PBW colonies were challenged with Cry2Ab, Cry1Ac appeared to confer only low levels (TBW and CBW) of resistance to Cry2Ab or no resistance to Cry2Ab at all (PBW).

Because Bollgard™ cotton varieties and Bollgard™ II cotton varieties will overlap in the Cotton Belt for at least five years or more, the current refuge options required for Bollgard™ cotton should are the same for Bollgard™ II cotton (USEPA 2001, see Section III. "*Bt* Cotton Confirmatory Data and Terms and Conditions of the Amendment"). This decision provides an increased degree of protection against resistance and is consistent and practical for the growers who are the key to the success of an IRM strategy. In addition, the same research data (i.e., alternate host, overspray, and north-south movement data), resistance monitoring, remedial action plans, grower education, compliance assurance, and annual reporting requirements for Bollgard™ cotton are also required for Bollgard™ II cotton (USEPA 2001, see Section III. "*Bt* Cotton Confirmatory Data and Terms and Conditions of the Amendment").

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

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Disclaimer

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Table 1. Relative insecticidal activity of Cry1Ac and Cry2Ab. (modified from Table 1, p. 15, Head and Reding 2001).

Family/Species	**Cry1Ac (LC_{50} in ppm)**	**Cry2Ab (LC_{50} in ppm)**
PBW	0.006	0.1
CBW	1.56	15.26
TBW	0.035	0.62
FAW	>100	47.5
BAW	>100	19.4
SL	0.725	0.752