UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D C. 20460

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OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

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SUBJECT: Review of Insect Resistance Management (IRM) in *Bacillus thuringiensis* var. *aizawai* Cry1F insect control protein as expressed in maize as a part of Pioneer Hi-Bred and Dow AgroSciences application for a full commercial use registration. EPA Reg. No. 68467-E; Barcode Nos D263761 & D267075; Case No 066174; Submission Nos S574903 & S581619; MRID Nos 451311-01, 450201-01, 450201-14, 450201-16, & 450201-15.

CLASSIFICATION: Supplemental to addressing recommendations listed below

BPPD IRM TEAM RECOMMENDATIONS:

The following recommendations for Cry1F event 1507 are based on the Agency's requirements for Cry1Ab expressing corn. This is due to the possibility of cross-resistance between Cry1Ab and Cry1F. Modifications of these requirements may result following the Agency's reassessment of the Cry1Ab expressing corn products.

1. The proposed Bt Cry1F field corn label states that this product is "for control of the European corn borer and other lepidopteran pests." It is unacceptable to use the vague description "other lepidopteran pests." Target pest should be listed on the label.

2. For Bt field corn grown outside cotton-growing areas (e.g., the Corn Belt), grower agreements (stewardship agreements) will specify that growers must adhere to the refuge requirements as described in the grower guide/product use guide and/or in supplements to the grower guide/product use guide. Specifically, growers must plant a minimum structured refuge of at least 20% non-Bt corn on their farm. Insecticide treatments for control of ECB, CEW, SWCB, FAW, and/or BCW may be applied only if economic thresholds are reached for one or more of these target pests. Economic thresholds will be determined using methods recommended by local or regional professionals (e.g., Extension Service agents, crop consultants). Instructions to growers will specify that microbial Bt insecticides must not be applied to non-*Bt* corn refuges.

3. For the 2001 growing season, grower agreements (stewardship agreements) for Cry1F Bt field corn grown in cotton-growing areas specified that growers must adhere to the refuge requirements as described in the grower guide/product use guide and/or in supplements to the grower/product use guide. Specifically, growers in these areas must plant a minimum structured refuge of 50% non-Bt corn on their farm. Cotton-growing areas include the following states: Alabama, Arkansas, Georgia, Florida, Louisiana, North Carolina, Mississippi, South Carolina, Oklahoma (only the counties of Bryan, Caddo, Canadian, Garvin, and Grady), Tennessee (only the counties of Carroll, Chester, Crockett, Fayette, Franklin, Gibson, Hardeman, Hardin, Haywood, Hendersen, Lake, Lauderdale, Lawrence, Lincoln, McNairy, Madison, Obion, Rutherford, Shelby, and Tipton), Texas (except the counties of Carson, Dallam, Hansford, Hartley, Hutchinson, Lipscomb, Moore, Ochiltree, Roberts, and Sherman), Virginia (only the counties of Greensville, Isle of Wight, Northampton, Southampton, Sussex, Suffolk) and Missouri (only the counties of Butler, Dunkin, Mississippi, New Madrid, Pemiscot, Scott, Stoddard).

4. Requirements for refuge deployment will be described in the Grower Guides/Product Use Guides as described in Section D of the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) IRM Plan submitted to EPA on April 19, 1999. Growers must continue to plant only non-Bt corn in the refuge and to plant the refuge within ½ mile of their Bt corn acreage and encourage growers to plant the refuge within 1/4 mile. In regions of the corn belt where conventional insecticides have historically been used to

control ECB and SWCB, growers wanting the option to treat these pests must plant the refuge within ¼ mile of their Bt corn. Refuge planting options include: separate fields, blocks within fields (e.g., along the edges or headlands), and strips across the field. When planting the refuge in strips across the field, growers must be instructed to plant multiple non-Bt rows that extend the length of the field.

5. The registrants will monitor for the development of resistance using baseline susceptibility data and/or a discriminating concentration assay when such an assay is available. The registrants will proceed with efforts to develop a discriminating concentration assay. The registrants will ensure that monitoring studies are conducted annually to determine the susceptibility of ECB, SWCB, and CEW populations to the Cry1F protein. This resistance monitoring program will be developed to measure increased tolerance to Bt corn above the various regional baseline ranges.

6. Populations of ECB, SWCB, and CEW will be collected from representative distribution areas that contain the registrant's Bt corn plant-pesticide and monitored/screened for resistance, with particular focus on those areas of highest distribution. The results of monitoring studies will be communicated to the Agency on an annual basis, by January 31 of the year following the population collections for a given growing season.

7. In addition, the registrants will instruct its customers (growers and seed distributors) to contact the registrant (e.g., via a toll-free customer service number) if incidents of unexpected levels of ECB, SWCB, and/or CEW damage occur. The registrants will investigate and identify the cause for this damage by local field sampling of plant tissue from corn hybrids that contain the Cry1F Bt corn plant-pesticide and sampling of target pest populations, followed by appropriate in vitro and in planta assays. Upon the registrant's confirmation by immunoassay that the plants contain Cry1F protein, bioassays will be conducted to determine whether the collected pest population exhibits a resistant phenotype.

8. Until such time that a discriminating concentration assay is established and validated by the registrants, the registrants will utilize the following to define a confirmed instance of ECB, SWCB, and/or CEW resistance:

   Progeny from the sampled population will exhibit both of the following characteristics in bioassays initiated with neonates

   a. An LC_{50} in a standard Cry1F diet bioassay that exceeds the upper limit of the 95% confidence interval of the mean historical LC_{50} for susceptible ECB, SWCB, or CEW populations, as established by the ongoing baseline monitoring program. The source of Cry1F crystal protein standard for this bioassay will be *Bacillus thuringiensis* subsp. *aizawai*.

b. > 30% survival and > 25% leaf area damaged in a 5-day bioassay using Cry1F-positive leaf tissue under controlled laboratory conditions.

Based upon continued experience and research, this working definition of confirmed resistance may warrant further refinement. In the event that the registrants find it appropriate to alter the criteria specified in the working definition, the registrants must obtain Agency approval in establishing a more suitable definition.

The current insect monitoring program was expanded to include SWCB and CEW, in addition to ECB. The expanded program must focus monitoring in areas that typically have a high density of Bt corn or have historically been prone to high levels of corn borer pressure and where the refuge areas may more likely be treated with insecticides.

9. The current definition of confirmed insect resistance must be used as described in the ABSTC IRM Plan. Agency approval will be sought prior to implementation of any modified definition of confirmed insect resistance.

a. When resistance has been demonstrated to have occurred, the registrants must stop sale and distribution of Bt corn in the counties where the resistance has been shown until an effective local mitigation plan approved by EPA has been implemented. The registrants assume responsibility for the implementation of resistance mitigation actions undertaken in response to the occurrence of resistance during the growing season. EPA interprets "suspected resistance" to mean, in the case of reported product failure, that the corn in question has been confirmed to be Bt corn, that the seed used had the proper percentage of corn expressing Bt protein, that the relevant plant tissues are expressing the expected level of Bt protein, that it has been ruled out that species not susceptible to the protein could be responsible for the damage, that no climatic or cultural reasons could be responsible for the damage, and that other reasonable causes for the observed product failure have been ruled out. The Agency does not interpret "suspected resistance" to mean grower reports of possible control failures, nor does the Agency intend that extensive field studies and testing to fully scientifically confirm insect resistance be completed before responsive measures are undertaken.

10. The registrants will maintain a (confidential) database to track sales (units and location) of its Bt corn on a county-by-county basis. The registrants will provide annually, on a CBI basis, sales data for each state indicating the number of units of corn hybrids that contain the registrant's Bt corn plant-pesticide that were sold. As part of the overall sales report, the registrants will provide a listing of an estimate of the acreage planted with such states and counties with sales limitations. This information will be provided by

January 31 of the year following each growing season.

11. The registrants will provide grower education. The registrants will agree to include an active partnership with such parties as: university extension entomologists and agronomists, consultants, and corn grower groups. The registrants will implement a grower education program (in part, as requested by the registrants, through the Grower Agreement setting forth any resistance management requirements) directed at increasing grower awareness of resistance management, in order to promote responsible product use. Insect Resistance Management educational materials for each growing season must be provided to the Agency as they become available for distribution. Survey results and other available information must be used to identify geographic areas of non-compliance with insect resistance management plans. As described in the ABSTC IRM Plan, an intensified grower education program will be conducted in these geographic areas prior to the following growing season. If individual non-compliant growers are identified, they must be restricted from future purchases of Bt corn seed.

12. Several aspects of the IRM Plan will operate in synergy to promote grower compliance, however, the cornerstones of the compliance program must be the:

a. Grower Guides

Grower Guides and/or Product Use Guides should be submitted to the Agency prior to distribution to growers and prior to the growing season for EPA review. These guides should not be distributed until EPA's review of them is complete. These guides must be distributed to each seed customer and updated on an annual basis, as needed. The guides provide complete information for growers regarding routine IRM practices that must be employed, and will be a primary educational and reference tool.

b. Stewardship Agreement (grower agreement).

Each grower who purchases Bt field corn seed must be required to sign a stewardship agreement annually, which will obligate the grower to follow the required IRM practices as specified in the grower guide/product use guide and/or in supplements thereof.

c. A Strong and Multi-Pronged Grower Education Program.

A variety of methods must be employed to promote grower education and to continue to reinforce the need for adherence to all aspects of the IRM program.

d. Additional mechanisms must also be used to promote grower compliance. For example, training of sales personnel, seed dealers and technical support staff as

well as coordination and reinforcement of IRM requirements through other organizations (e.g., NC-205, the Cooperative Extension Service, USDA, National Corn Growers Assn. (NCGA), American Crop Protection Assn., Biotechnology Industry Organization, crop consultants and other crop professionals).

13. The registrants will confer with the EPA as the registrants develop various aspects of its resistance management research program. The registrants agree, as a condition of this registration, to submit annually, progress reports on or before January 31st each year on the following areas, as a basis for developing a long-term resistance management strategy which include:

a. Research data on CEW relative to resistance development and the registrant's plans for producing resistance predictive models to cover regional management zones in the cotton belt based on CEW biology and cotton, corn, soybeans, and other host plants. These models must be field tested and must be modified based on the field testing. EPA notes that there is some scientific work and even some models for CEW on other crops in at least NC and TX that could be used for reference. EPA wants to be in close communication with the registrants as the model development and testing is ongoing. The requirement for development of resistance predictive models may be modified if the registrants provide the results of research that demonstrates resistance to CEW would have no significant impact on the efficacy of foliar Bt products and other Bt crops. Actual usage data of Bt on crops to control specific pests as well as successes and failures and field validated research would be necessary to support such a waiver request.

b. ECB, SWCB, and CEW pest biology and behavior including adult movement and mating patterns, larval movement, survival on silks, kernels, and stalks, and overwintering survival and fecundity on non-corn hosts. A combination of a comprehensive literature review and research can fulfill this condition.

c. The feasibility of "structured" refuge options for ECB including both "block" refuge, "50-50 early/late season patchwork;" research needs to be done in both northern and southern areas on ECB as well as CEW.

d. Development of a discriminating concentration (diagnostic concentration) assay for field resistance (field screening) for ECB, SWCB, and, CEW and other lepidopteran pests of corn. Specific sampling locations will be established in each state to determine if increases in Bt protein tolerance are occurring before crop failures develop. Increased tolerance levels need to be identified before field failure occurs. In

monitoring for tunneling damage, the number of trivial tunnels may be less indicative of resistance development than the total extent of tunneling damage (e.g., length of tunnels). The extent of tunneling damage must be monitored as well as the number of tunnels.

e. Effects of corn producing the Cry1F delta endotoxin on pests other than ECB, including but not limited to CEW, BCW, FAW, and the stalk borer complex.

f. The biology of ECB resistance including receptor-mediated resistance and its potential effect on population fitness, as well as the effects on insect susceptibility to other Cry proteins. More data are needed on protein expression in various parts of the plant at different stages plant development in regard to ECB, CEW and other secondary pests of corn (i.e. stalk borer complex, FAW, and SWCB).

g. The registrants must assess the feasibility of using the F_2 screen, sentinel plots, and in-field screening kits to increase the sensitivity of resistance monitoring in 2000. By January 31, 2001, the registrants must provide the Agency with the results from these investigations.

h. The registrants must implement a survey approach similar to the Iowa State University Bt Corn Survey (e.g., Pilcher and Rice 1999). A statistically valid sample, as determined by independent market research, of Bt corn growers in key states will be surveyed by a third-party. Bt corn growers will be included based upon a proportionately stratified random sample designed to balance the survey evenly across seed companies and geographies. In addition to demographic information, the survey will include questions related to insect resistance management such as:

1) What is your primary source of information on Bt corn?

2) What percentage of your acres were planted to Bt corn this year?

3) Are you following a recommended insect resistance management strategy?

4) If you plant most of your acreage to Bt corn, are you likely to scout your non-Bt corn for economically damaging populations of corn borers?

5) Did you treat your Bt corn acres with an insecticide?

6) What planting pattern did you use for your refuge?

° Planted Bt corn as one block in one field.
° Planted Bt corn in one block in every field.
° Split seed boxes in the planter and alternated every row or several rows with Bt and non-Bt corn in every field.
° Planted Bt corn in large strips alternated with large strips of a non-*Bt* corn hybrid.
° Planted Bt corn in an entire field and planted the border around the field with non-Bt corn.
° Planted pivot corners to non-Bt corn with the irrigated area of the field planted to Bt corn.

BPPD IRM TEAM REVIEW, SUMMARY AND CONCLUSIONS:

This review is based upon the current understanding of the science. The Agency is still awaiting the October 18-20 FIFRA Scientific Advisory Panel (SAP) report and intends to do a complete a reassessment of the risks and benefits of Cry1Ab expressing corn upon publication of a final draft. This reassessment will address insect resistance management (IRM) needs based upon current information. Because of the potential cross-resistance between Cry1F and Cry1Ab expressing field corn, Cry1F will be subject to IRM requirements addressed in the Bt corn reassessment to be completed in 2001. The BPPD IRM team agrees with Dow AgroSciences and Pioneer Hi-Bred that all seed manufacturers and extension specialists should convey the same uniform IRM message to growers for all Bt field corn products in which there is possible cross-resistance. Therefore, Cry1F IRM requirements should coincide with the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) requirements and message.

Pest Biology:

Pests susceptible to Cry1F include the European corn borer (ECB, *Ostrinia nubilalis*), southwestern corn borer (SWCB, *Diatraea grandiosella*), black cutworm (BCW, *Agrotis ipsilon*), fall armyworm (FAW, *Spodoptera frugiperda*), lesser cornstalk borer (LCSB, *Elasmopalpus lignosellus*), sugarcane borer (SCB, *Diatraea saccharalis*), and to a lesser extent the corn earworm (CEW, *Helicoverpa zea*). A high dose has been demonstrated for ECB and a high level of efficacy was found for SWCB, FAW, and BCW. Unlike currently registered Cry1Ab field corn hybrids, Cry1F is highly efficacious against the BCW and FAW. Otherwise, the relative susceptibilities of the insects tested to Cry1F were LCSB>SWCB>SCB. Although the LCSB, SCB, and CEW were shown to be susceptible to Cry1F, these pests are not listed on the label. The CEW is susceptible to Cry1F at approximately the same rate it is susceptible to Cry1Ab, but the registrants have chosen not to include it on the label and may want to consider adding it. However, since CEW is susceptible to Cry1F, IRM requirements and research needs should be addressed. Current Bt corn registrations require annual submission of research data by January 31. Cry1F is subject to the same research requirements that are outlined in the table below. However, these requirements may change when all Bt corn registrations are reassessed. In addition, an LC_{50} should be determined for SWCB, CEW, FAW, and BCW if a colony can be

established

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Summary of Data Needed to Improve Insect Resistance Management Strategies for Bt Corn Products

Data	Pests
Pest Biology: e.g., larval movement, adult movement, mating behavior, pre- and post-mating dispersal, ovipositional behavior, fitness, and overwintering habitat and survival	ECB, SWCB and CEW
North to South Movement	CEW
High Dose Verification (using 1998 SAP techniques)	ECB and SWCB
Resistance Allele Frequency	ECB, SWCB and CEW
Cross-Resistance - Cry1F, Cry2A, Cry1A proteins	ECB, SWCB and CEW
Evaluation (field studies and models) of Refuge Options (20% external refuge (sprayable) v. 20% in-field) - [Issues to consider: production of susceptible insects (500:1 ratio) in insecticide treated and non-insecticide treated refuges, adequacy of size, structure, and deployment of the refuge, rotation of refuge.]	ECB, SWCB and CEW
Collection of Baseline Susceptibility Data and Validation of Discriminating/Diagnostic Dose	ECB, SWCB and CEW
Evaluation of Resistance Monitoring Techniques. e.g., discriminating v. diagnostic dose, F_2 screen, sentinel plots, gene mapping	ECB, SWCB and CEW
Grower Compliance - more detailed information on refuge (%, deployment, and management)	ECB, SWCB and CEW

Significant pest biology research has been conducted for ECB as it relates to IRM. Work with larval movement, adult movement, mating behavior, ovipositional behavior, and host range have established critical parameters for ECB refuge, although more research is needed in these areas. A less extensive pest biology knowledge base is available for CEW and SWCB. As is the case with ECB, additional IRM-related research is needed for SWCB, CEW, BCW, FAW and other secondary corn pests to increase confidence that the IRM plans will be effective at reducing the likelihood that insects will become resistant to Bt corn.

High Dose:

The February, 1998 FIFRA Scientific Advisory Panel (SAP) Subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides and Resistance Management determined that a high dose/refuge strategy is necessary to mitigate resistance of stalk boring Lepidoptera in Bt corn (meeting held on February 9-10, 1998. Docket # OPPTS-00231). The SAP determined that a high dose (defined as 25× the

dose necessary to kill all susceptible insects) should be verified by two of five techniques outlined in the final report (http://www.epa.gov/scipoly/sap/1998/february/finalfeb.pdf).

Ideally, high dose should be evaluated for all susceptible pests, so that appropriate resistance management strategies can be developed. At a minimum, high dose should be determined for the major target pests of Cry1F corn (ECB and SWCB). The registrants claim that a high dose for Cry1F event TC1507 was determined by the fourth and fifth methods described by the SAP. Method four is similar to method 3, but would use controlled infestation with a laboratory strain of the pest that had an LD50 value similar to field strains. Method 3 involves surveying large numbers of commercial plants on sentinel plots in the field (e.g., sentinel sweet corn method) to make sure that the cultivar is at the $LD_{99.99}$ or higher to assure that 95% of heterozygotes would probably be killed. With this approach *Bt* sweet corn hybrids are used to attract high densities of ECB and cotton bollworm (*Helicoverpa zea*)(Boddie)) (CBW/CEW) moths, sampling can be limited to sweet corn ears in the *Bt* plot (ca. 1/4-1/2 acre block), and a frequency of resistant phenotypes can be estimated as the ratio of density of larvae/plant in *Bt* sweet corn to density of larvae/plant in an adjacent planting of non-*Bt* sweet corn (Andow and Hutchison, 1998; Hutchison, unpublished data). With the fifth method identified by the SAP, it should be determined if an older instar of the targeted pest could be found with an LD50 that was about 25-fold higher than that of the neonate larvae. If so, that stage could be tested on the crop plants to determine if 95% or more of the older stage larvae were killed.

Although the fourth method was used, Bt field corn rather than sweet corn was planted because Cry1F sweet corn does not exist. The SAP recommended sweet corn be used since it is more attractive to ECB than field corn. It was also confusing how many and why neonates from other trials were used in the method four verification. Overall, a high dose for ECB has been demonstrated in this report for event TC1507. Additional high dose data are needed for the SWCB.

Refuge:
A refuge should be designed to produce 500 susceptible insects for every one potentially resistant insect. This Cry1F registration will be subject to the same requirements as the Agricultural Biotechnology Stewardship Committee (ABSTC) and October 2000 SAP report that will consider refuge needs in an IRM strategy. Refuges planted to mitigate resistance to Cry1F field corn should be identical to those needed for Cry1Ab corn and should consist of agronomically similar non-Bt field corn varieties. The Bt and non-Bt field corn varieties should be cared for and managed in a similar fashion. The non-Bt refuges should be planted within the Bt field or in close proximity. In general, refuges may be planted as external blocks on the edges or headlands of fields or as strips within the Bt corn field. In-field strips should include multiple rows (at least 2-6) of non-Bt field corn and extend the full length of the field. Refuges should be treated as needed to control lepidopteran stalk-boring insects with non-Bt insecticides or other appropriate IPM practices. Insecticide use should be based on scouting using economic thresholds as part of an IPM program

The issue of refuge proximity is a critical variable for resistance management. Refuges must be located so that the potential for random mating between susceptible moths (from the refuge) and possible resistant survivors (from the Bt field) is maximized. The USDA NC-205 North-Central regional research committee on ecology and management of European corn borer and other stalk-boring Lepidoptera has recommended to the Agency that all Bt corn should be placed within one half mile of the non-Bt corn refuge, but that refuge plantings within one quarter mile would be even better (NC-205 letter to Dr. Janet Andersen, 5/24/99). However, the complete picture of ECB dispersal is still unknown and among females, mating is likely to occur (relatively) close to the site of pupal eclosion. In addition, information is needed on SWCB and CEW movement and mating behavior to fully understand optimal placement of refuges. This research should be conducted and submitted to the Agency as part of the annual research report.

Based upon NC-205 recommendations made from 1998-2000, and information regarding ECB resistance management acquired from models (Onstad & Gould 1998, Onstad & Guse 1999), a minimum of 20% non-Bt corn refuge is recommended for non-cotton growing regions (e.g., Corn Belt) that do not spray insecticides on a regular basis. A 20% non-Bt corn refuge in non-cotton growing regions was recommended by the ABSTC in April 1999 and mandated by EPA in 2000.

The October 2000 SAP Subpanel is currently drafting a report that considers whether a larger refuge, e.g., 40% non-Bt corn refuge, would decrease the risk of insect resistance in non-cotton growing regions that spray insecticides on a regular basis (e.g., High Plains for SWCB). This area needs to be clearly identified on a county basis or triggers developed to indicate when higher insecticide use poses a greater risk to resistance. A larger refuge may be needed in this area because of the increased risk of resistance created when insecticides are used, thus killing insects susceptible to Bt. Currently, EPA has mandated a 20% non-Bt corn refuge in all non-cotton growing regions.

Cry1F corn grown in cotton-growing regions, including the northern cotton growing region, should plant a minimum 50% non-Bt corn refuge that may be treated only as necessary with non-Bt insecticides. This larger refuge size in cotton-growing regions is needed due to increased concerns regarding the development of CEW resistance in cotton growing regions especially those regions growing Bt cotton.

Monitoring:
The monitoring strategy submitted for this registration is not adequate because it only addresses grower monitoring for unexpected damage. A resistance monitoring strategy for Bt corn is needed to test the effectiveness of resistance management programs. Detecting shifts in the frequency of resistance genes (i.e., susceptibility changes) through resistance monitoring can be an aggressive method to detect the onset of resistance before widespread crop failure occurs. As such, the utilization of sensitive and effective resistance monitoring techniques is critical to the success of an IRM plan. Techniques such as the F_2 screen, and sentinel plots need to be thoroughly investigated for their feasibility as resistance monitoring tools in addition to the

currently used discriminating dose concentration assays. The ABSTC must submit research to the Agency regarding the feasibility of conducting the F_2 screen by March 2001. In addition, baseline susceptibility to Cry1F data should be developed for ECB, SWCB, and CEW.

The ABSTC proposed a monitoring strategy in March 2000 for ECB, CEW, and SWCB in Cry1Ab field corn. The ABSTC plan should to monitor for ECB, SWCB, and CEW resistance should also be used for Cry1F field corn in addition to the grower monitoring presented in the Dow AgroSciences/Pioneer Hi-Bred submission. The ABSTC plan focuses resistance monitoring in areas where Bt corn market penetration is highest as well as areas with the highest insecticide use. The ABSTC plan includes the identification of counties growing more than 50,000 acres of field corn (Bt and non-Bt) to focus monitoring efforts. ABSTC's proposed plan is designed to detect resistance when it reaches 1 - 5% (a level that allow for detection of resistance before field failures occur). Four corn-growing regions were identified and monitoring for each pest will occur in the regions in which the pests are prevalent. When possible, at least 200 first or second flight adults (100 females), 100 second flight egg masses, and 100 diapausing larvae per site will be collected in each region, though insect population levels may limit the number collected. The sampling strategy should be adequate, although the program would be improved if sampling locations can be separated by a sufficient distance to reflect discreet pest populations.

The BCW and FAW are polyphagous insects that are considered secondary pests of field corn. BCW feed on a wide range of cultivated crops including turfgrass, field and vegetable crops. FAW feed on over 80 host plants including corn and vegetables, but they prefer grasses. Both of these pests overwinter in the south and migrate north. FAW only overwinters in south Texas and south Florida. Due to their polyphagous nature and migratory patterns, there is a great amount of gene flow. There have not been reports of resistance to insecticides by either of these pests in the U.S. For these reasons, there is not a concern of FAW or BCW becoming resistant to Bt field corn. In addition, it would be difficult to survey field corn for unexpected damage since these pests will probably cause some damage prior to being killed by the Cry1F toxin (personal communication with Galen Dively 1/2001). Therefore, monitoring for BCW and FAW resistance is not necessary in field expressing the Cry1F protein. However, if there were to be large amounts of Cry1F field corn (>1000A) planted in the areas in which FAW overwinters (south Florida and south Texas), the Agency will reexamine whether there is significant selection pressure for FAW and BCW to develop Cry1F resistance.

Remedial Action:
Remedial Action was not adequately addressed by Dow AgroSciences and Pioneer Hi-Bred. According to the registrants, all instances of resistance will be reported to the Agency and mitigation measures will be taken. Mitigation measures include ceasing sales in the affected counties, but the registrants do not address continued monitoring in the area or the use of alternate control measures. The Cry1F remedial action plan should be identical to the ABSTC plan for Cry1Ab expressing field corn already approved by the Agency in 2000.

Remedial actions should include: informing customers and extension agents in the affected areas of suspected or confirmed resistance, increasing monitoring in the affected areas, implementing alternative means to reduce or control target pest populations in the affected areas, implementing a structured refuge in the affected areas, and cessation of sales in the affected and bordering counties until an effective local management plan approved by EPA has been implemented. During the voluntary suspension period, registrants may sell and distribute in these counties only after obtaining EPA approval to study resistance management in those counties. The implementation of such a strategy will be coordinated by the Agency with other registrants and stakeholders.

Compliance:
Dow AgroSciences and Pioneer Hi-Bred do not believe 100% compliance is necessary as long as the large majority of growers comply with IRM requirements. Growers will need to sign a technology use agreement that outlines IRM requirements and acknowledges the growers responsibility to comply with them. The agreement will also state that growers received the Product Use Guide. This agreement may be a section of the growers order sheet or some other document or format. The grower agreement may be signed annually or as needed. The registrants recommend grower surveys to estimate the level of compliance, limiting non-compliant growers access to the technology, and implement additional education efforts will target non-compliant growers.

Models used to determine the size of the non-Bt corn refuge are based on 100% compliance and 100% adoption. Because it is unknown how the level of compliance will affect IRM, 100% compliance should be the goal of any IRM plan. BPPD strongly disagrees with the registrants assertion that 100% compliance is not necessary. Since there is not 100% adoption of Bt corn, models should be developed that consider < 100% compliance and < 100% adoption. In addition, grower surveys alone are not adequate to address grower compliance. An improved method of assessing grower compliance needs to be developed. This may be done, for example, through improved grower education, site visits and mapping, certification programs, incentive programs, or other means. BPPD will also need grower information material submitted on an annual basis for review to ensure proper requirements are articulated.

Cross-Resistance:
Cross-resistance, in which one toxin confers resistance to another, is an area of major concern for resistance management and poses risks to both transgenic Bt crops and microbial Bt insecticides. The most well-documented mechanism of cross-resistance with Bt occurs when two toxins share the same binding site (receptor) in the insect midgut (Tabashnik 1994).

Competitive binding experiments provided by the registrants showed that Cry1F may share a binding site in the ECB midgut with Cry1Ab or Cry1Ac, but not with the Cry9 proteins. This demonstrates that ECB has the potential for cross-resistance between Cry1F and Cry1Ab (currently registered Bt corn) and Cry1Ac (currently registered Bt cotton). Therefore, the IRM strategy mandated for Cry1Ab field corn should also be implemented for Cry1F corn. In

addition, it is not recommended to stack Cry1Ab and Cry1F in the same corn hybrid.

Grower Education:
Dow AgroSciences and Pioneer Hi-Bred have adequately addressed grower education through training their trainers, Product Use Guides, field placards, slide presentations and publicity. The proposal they submitted to the Agency should be implemented. Growers are perhaps the most essential element for the implementation and success of any IRM plan as they will ultimately be responsible for ensuring that refuges are planted according to guidelines and that Bt fields are monitored for unexpected pest damage. Therefore, a program that educates growers as to the necessity of IRM and provides guidance as to how to deploy IRM should be an integral part of any resistance management strategy. Ideally, the educational messages presented to growers should be consistent (among different registrants) and reflect the most current resistance management guidelines for current Bt corn registrations. Specific examples of education tools for growers can include grower guides, technical bulletins, sales materials, training sessions, Internet sites, toll-free numbers for questions or further information, and educational publications.

Annual Reports:

Written reports on various aspects of IRM, submitted on an annual basis to EPA, are of great aid in the evaluation of the success of resistance management for Bt corn. Reports should be submitted to the Agency on an annual basis on Bt corn sales/market penetration, IRM-related research, and grower education. In addition to these reports, it would be particularly useful to receive reports from Bt corn registrants on grower compliance and resistance monitoring.

References:
Onstad, D.W. and F. Gould. 1998. Modeling the dynamics of adaptation to transgenic maize by European corn borer (Lepidoptera: Pyralidae). J. Econ. Entomol. 91(3): 585-593.

Onstad, D.W. and C.A. Guise. 1999. Economic analysis of transgenic maize and nontransgenic refuge managing European corn borer (Lepidoptera: Pyralidae). J. Econ. Entomol. 92(6): 1256-1265.

BACKGROUND:
Dow AgroSciences and Pioneer Hi-Bred have requested separate registrations for *Bacillus thuringiensis* var. *aizawai* Cry1F insect control protein as expressed in field corn. Testing of *Bacillus thuringiensis* var. *aizawai* (Bt) Cry1F insect control protein as expressed in maize began in 1996 under USDA permits. In 1999 and 2000 additional field tests were conducted under Mycogen's EPA Experimental Use Permit No. 68467-EUP-2. There were no adverse effects observed during these field trials or any tests conducted to support this product's registration.

A request was submitted to the Agency for the registration of all progeny lines derived from Event TC1507 produced through a standard breeding program. Although submitted data

addresses Events TC1507 and TC1360, only Event TC1507 will be addressed in this review. The proposed Bt Cry1F field corn label states that this product is "for control of the European corn borer and other lepidopteran pests." The label claims that the black cutworm, European corn borer, fall armyworm, and Southwestern corn borer are controlled in field corn. In addition "[g]rowers are instructed to read information on insect resistance management."

This review addresses DowAgroSciences/Pioneer Hi-Bred insect resistance management (IRM) strategy for Cry1F Event 1507 corn. Insect resistance management involves a high dose/refuge strategy. A high dose was defined by the February 1998 Scientific Advisory Panel Subpanel (SAP) as 25 times the dose needed to kill all susceptible insects. The SAP also determined that a refuge should be designed to produce 500 susceptible insects for every one potentially resistant insect.

DATA EVALUATION REPORT

Reviewed by: Robyn Rose, Entomologist, BPPD 12/9/01
Secondary Reviewers: Alan Reynolds, Entomologist, BPPD, and Sharlene Matten, Ph.D, Biologist, BPPD 1/24/01

PRODUCT ID NUMBERS:	EPA Reg. No. 68467-E; Barcode D267075; Case No 066174; Submission No S581619
STUDY TYPE:	Demonstration of a high dose for the European corn borer
MRID NOS.:	451311-01 and 453077-01
TEST MATERIAL:	*Bacillus thuringiensis* var. *aizawai* Event 1507 Cry1F insect control protein as expressed in maize
STUDY NO.:	GH-C 5072
SPONSOR:	Dow AgroSciences LLC 9330 Zionsville Rd, Indianapolis, IN 46268-1054 and Pioneer Hi-Bred International Inc., 7250 N.W. 62nd Ave, P.O. Box 552, Johnston IA 50131
TESTING FACILITY:	Mycogen Seeds, 301 Campus Dr, Huxley, IA 50124
TITLE OF REPORT:	High Dose Demonstration For Cry1F Events TC1360 and TC1507: European Corn Borer.
AUTHORS:	Paul Bystrak, Laura Higgins, Daniel Moellenbeck
STUDY COMPLETED:	5/3/00
CLASSIFICATION:	Acceptable

Study Summary:

Objective:
To demonstrate that Event 1507 expressing Cry1F in maize meet the high dose standard by the use of Method 4 and Method 5 outlined by the February 1998 FIFRA Scientific Advisory Panel (SAP) for the European corn borer (ECB; *Ostrinia nubilalis*).

Methods:
Method Four

The fourth method of verifying high dose recommended by the SAP involves controlled infestations on the cultivar with insects of a known LC_{50}. In Slater, IA, Mycogen seeds planted *five replicates of Cry1F corn with no susceptible corn between blocks. Susceptible corn was* planted on the south edge of the field to reduce potential impacts of pollen expressing Bt. Four large blocks of Cry1F corn separated by susceptible plants were planted in Johnston, IA by Pioneer Hi-Bred. Plants were tested to determine susceptibility to glufosinate to confirm that the hybrids were segregating for the transgenic traits and for elimination of nulls.

ECB were infested in the trial plots by using a "bazooka" to deliver approximately 25 neonates

per shot at Johnston and 50 in Slater at the same time first and second generation were present. At the Johnston location, two shots of 25 neonates per plant were applied on four dates during the first and second generations. Two shots of 50 neonates were applied to each plant on three dates during the first generation and on two dates during the second generation; six shot of 50 larvae were applied at the Slater location. Second generation applications in Slater were made on the silks, shank, and on sheath collars above and below the ear. At the Johnston location, 1224 plants were each infested with approximately 200 1st generation and 200 2nd generation ECB. In Slater. 2456 plants were infested with 284 1st generation and 600 2nd generation ECB. A total of 489,000 ECB larvae were used to infest in the Johnston trial and 2,171,104 in the Slater trial.

Slater neonates were in culture for 11 generations prior to infestation and Johnston neonates were in culture for ten generations. Both colonies were collected the previous fall and were considered as similar in susceptibility to the wild type as possible for a lab reared colony. Additional ECB neonates were added to the field from other trials. The number of neonates was not counted but were estimated to be less than the initial infestation. Neonates applied before glufosinate selection were subtracted.

Since neonates initial bite of the plant is too small to be visible, no scar on leaf tissue was considered mortality. It was assumed that if a neonates were to survive, feeding damage would be visible. Damage was evaluated based upon an ECB1 (1st generation) and ECB2 (2rd generation) damage scale. ECB1 is a foliar damage rating based upon 9 as the best and 1 as the worst. ECB1 Cav represents the number of 1st generation pupae found in stalks. ECB2 rates damage from second generation larvae. The number of tunnels and length in inches was determined. All Cry1F plants were evaluated for ECB2 foliar damage and entrance holes. One hundred stalks from each treatment were split to evaluate tunnel damage to the nearest half -inch (Table 1).

Method Five

The fifth method of verifying high dose recommended by the February 1998 FIFRA Scientific Advisory Panel (SAP) involves bioassays on Bt plants with older instar larvae that are 25 times less susceptible to verify that 95% or greater are killed by the cultivar. A laboratory diet bioassay of purified, truncated Cry1F against first, third, and fourth instar ECB was conducted. Sensitivity of each instar was evaluated by determining the LC_{50}. ECB used in the experiment were from a Pioneer Hi-Bred colony that had been cultured for approximately ten generations.

A laboratory diet bioassay consisted of incorporating 0, 0.001, 0.01, 0.1, 1, 10, 20 and 50 µg of Cry1F per gram of diet was conducted. The LC_{50} value for first, third, and fourth instars was determined via Probit analyses. A plant feeding bioassay was conducted using 20 replicates of each ECB instar (one through five). Positive plants were chosen by applying neonates to the V6 corn stage. Leaves were taken from each of the four entries at the V10 stage. In the laboratory, leaves were rinsed with de-ionized water and discs were cut with a 2.4 cm leaf punch. Leaf discs and one larvae were placed in a well of a rearing tray containing an agar base covered with filter

paper. Trays were kept at 28°C, 80% relative humidity, and 24 hours of darkness. Mortality was recorded every 24 hours until all larvae died.

Results:

Method Four

Table 1. Results of data for 100 split plants.

Location	Hybrid	ECB1	ECB1 Cav	ECB2 Tunnels	ECB2 Inches	ECB2 Larvae
Johnston	TC 1507	9	0	3	2	0
Johnston	Isoline	5.35	0	n.d.	330	45
Slater	TC1507	9	0	4	2	0
Slater	Isoline	4.73	5	58	79.5	19

There was 100% mortality of all 1st and 2nd generation ECB and no feeding damage found in both plants; therefore, no colonies were able to be started. A small number of 2nd generation ECB started tunnels. These tunnels were attributed to older larvae moving into Bt plots and attempting to tunnel into stalks or shanks. These starter tunnels were less than 0.5 in. deep and occurred adjacent to the non-Bt border.

Method Five

According to reported results, mortality data from the diet bioassay showed a decrease in mortality from the first to the third and fourth instars. There was a 63-fold higher LC_{50} for first instar larvae than for fourth instars. This suggests that fourth instar larvae are the appropriate life stage to test for high dose verification.

LC_{50} values for Cry1F insecticidal crystal protein for 1st, 3rd, and 4th instar ECB larvae (MRID No 453077-01)

Instar	LC_{50} (95% Fiducial Limits)*	Fold Increase
1st	0.17 (0.079 - 0.301)	-
3rd	2.57 (1.39 - 4.44)	15
4th	10.67 (1.04 - 86.6)	63

First instar LC_{50} is 0.04 µg Cry1F/g diet, third instar LC_{50} is 0.10 µg Cry1F/g diet, and fourth instar LC_{50} is 10.7 µg Cry1F/g diet. There was a 2.5 fold difference in susceptibility between first and third instars and a 267.5 fold decrease between first and fourth instars. The fourth instar results show a great enough level of susceptibility to satisfy the high dose criterion #5.

Summary of results from the plant feeding bioassay

Treatment	Instar ECB	Time Post Trt	Percent Mortality
Bt	first	72 hours	100%
Bt	second	72 hours	100%
Bt	third	96 hours	100%
Bt	fourth	120 hours	90%
Bt	fourth	144 hours	100%
Bt	fifth	216 hours	100%
Bt	fifth	216 hours	100%
Control	first	24 hours	10%
Control	first	72 hours	20%
Control	second	72 hours	10%
Control	third	96 hours	5 %
Control	fourth	168 hours	15%
Control	fifth	216 hours	65%

Registrant's Conclusions:
Cry1F hybrids give 100% control of all ECB larval stages. It took three days for first and second instar larvae, four days for third instars, and nine days for fifth instars to achieve 100% mortality. This increased time for older larvae to reach 100% mortality indicates an increased LC_{50} for later instars. Although it took longer for larger larvae to die, no larvae were able to survive prolonged exposure to the Cry1F expressed in TC1507. A high dose was demonstrated according to method 5 by the LC_{50} for fourth instars which was 250-fold greater than the LC_{50} for neonates.

BPPD Conclusions:
This review only addresses event 1507 since the registrants are not currently pursuing registration of event 1360. Overall, a high dose for ECB has been demonstrated in this report for event TC1507. No additional high dose testing is needed for ECB. However, additional high dose data are needed for the SWCB.

This study was not conducted according to good laboratory practices (GLP). The study sponsor claimed that "no aspect of this study is subject to Good Laboratory Practice Standards." According to 40 CFR Part 160 all studies submitted to the Agency in support of a Section 3 registration are subject to GLP standards.

A high dose was defined by the February 1998 Scientific Advisory Panel Subpanel (SAP) as 25 times the dose needed to kill all susceptible insects. The SAP determined that a high dose (defined as 25× the dose necessary to kill all susceptible insects) should be verified by two of five techniques outlined in the final report (http://www.epa.gov/scipoly/sap/1998/february/finalfeb.pdf). Ideally, high dose should be evaluated for all susceptible pests, so that appropriate resistance management strategies can be developed. At a minimum, high dose should be determined for the major target pests of Cry1F corn (ECB, SWCB, BCW, and FAW). The registrants claim that a high dose for Cry1F event TC1507 was determined by the fourth and fifth methods described by the SAP. Method four is similar to method 3, but would use controlled infestation with a laboratory strain of the pest that had an LD50 value similar to field strains. Method 3 involves surveying large numbers of commercial plants on sentinel plots in the field (e.g., sentinel sweet corn method) to make sure that the cultivar is at the $LD_{99.99}$ or higher to assure that 95% of heterozygotes would probably be killed. With the fifth method identified by the SAP, it should be determined if an older 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, that stage could be tested on the crop plants to determine if 95% or more of the older stage larvae were killed.

Method Four

With this method of verifying high dose, if plants aren't expressing a high dose of Cry1F, then there will be a significant number of surviving heterozygotes. Although the registrants point out legitimate drawbacks of the fourth method of verifying high dose, this study demonstrated that Event 1507 has a high dose for ECB according to the fourth method provided by the February 1998 SAP. The test using the fourth verification method showed none of the >2.5 million ECB used to infest the two trial locations survived. There was some tunneling found in Bt corn adjacent to the non-Bt borders. The registrants are probably correct in their assumption that this damage is caused by late instars moving to these plants from the non-Bt plants. It is known that ECB larvae may move up to six plants in a field.

It is unclear from the materials and methods why neonates applied prior to glufosinate selection were subtracted and what affect this would have on overall results. It is also unclear why and how many ECB from other trials were "dumped" in the test plot without being counted and what affect this would have on the test results. The authors of this submission also did not define what a "large block" consists of (e.g. 1 acre or 10 rows × 10 rows).

Since drawbacks and difficulties to the five test methods exist, verifying high dose by at least two methods is necessary.

Method Five

The diet bioassay showed a decrease in mortality from the first to the third and fourth instars. There was a 63-fold higher LC_{50} for first instar larvae than for fourth instars. This suggests that

fourth instar larvae are the appropriate life stage to test for high dose verification.

The plant feeding bioassay showed that ECB feeding on corn leaves containing Cry1F will result in 100% mortality within nine days. First and second instars die within three days, third instars die within four days, and 100% mortality of fifth instars occurs within six days of feeding on Cry1F. First and second instars are much more susceptible to Cry1F than fourth and fifth instars which is demonstrated by the increased LC_{50}. Although, the early instars are more susceptible to Cry1F, no ECB larvae were able to survive feeding on the Bt plant tissue. The LC_{50} for fourth instars was 250 times greater than first instars and there was 100% mortality of the fourth instars before any of the fourth instars feeding on the control plants died.

Data from method five diet bioassay were not submitted and will be needed to complete this submission. From the data submitted thus far, it can be concluded that according to the February 1998 SAP method 5, there is a high dose of Cry1F found in Bt corn to control ECB.

DATA EVALUATION REPORT

Reviewed by: Robyn Rose, Entomologist. BPPD 1/24/01
Secondary Reviewers: Alan Reynolds, Entomologist, BPPD, and Sharlene Matten, Ph.D, Biologist, BPPD 1/24/01

PRODUCT ID NUMBERS:	EPA Reg. No. 68467-E; Barcode D263761; Case No 066174; Submission No S574903
STUDY TYPE:	Susceptibility Study
MRID NO.:	450201-01
TEST MATERIAL:	*Bacillus thuringiensis* var. *aizawai* Event 1507 Cry1F insect control protein as expressed in maize
STUDY NO.:	990029
SPONSOR	Dow AgroSciences LLC 9330 Zionsville Rd, Indianapolis, IN 46268-1054. and Pioneer Hi-Bred International Inc., 7250 N.W. 62nd Ave, P.O. Box 552, Johnston IA 50131
TESTING FACILITY:	Global Environmental Chemistry Laboratory - Indianapolis Lab, Dow AgroSciences LLC, 9330 Zionsville Rd, Indianapolis, IN 46268-1054.
TITLE OF REPORT:	Microbial Bt Cry1F (truncated) Delta-Endotoxin: Maize-Insect-Pest Susceptibility Study
AUTHORS:	R.A. Herman and V.A. Korjagin
STUDY COMPLETED:	October 12, 1999
CLASSIFICATION:	Acceptable

Study Summary:

Objective:
To measure the biological activity of the Cry1F (truncated) protein to a range of insects that feed on corn plants

Methods:
The sensitivity of six insect species to an aqueous microbial formulation of Cry1F containing 11.4% Cry1F delta-endotoxin was evaluated. Insects tested were the lesser cornstalk borer (LCSB, *Elasmopalpus lignosellus*), sugarcane borer (SCB, *Diatraea saccharalis*), southwestern corn borer (SWCB, *Diatraea grandiosella*), western corn rootworm (WCR, *Diabrotica virgifera virgifera*), corn leaf aphid (CLA, *Rhopalosiphum maidis*), and the corn leafhopper (CLH, *Dalbulus maidis*).

The LCSB, SCB, SWCB, and WCR are chewing insects. Neonates of these insects were fed various concentrations of Cry1F in diet applied to the surface of 128-well bioassay trays. Each well contained approximately 500 µL of diet with a surface area of 1.5 cm^2. This diet also

contained 10 mM potassium phosphate buffer (pH 7.5). On each bioassay date, 40 µL of treatment was added to 16 wells. Sucking insects, CLA nymphs and CLH adults, were fed an aqueous liquid diet of a single concentration of Cry1F with 13% sucrose and ~10 µg/mL yellow dye (tartrazine to enhance feeding). This diet was covered with a membrane for insects to insert their mouthparts through. Five test arenas containing five CLA or three CLH were prepared on each test date. Stability of the test substance was determined before and after the bioassays. Spinosad (0.267 µg ai/cm^2) was used in bioassays as a positive control for the chewing insects and imidacloprid (10 µg ai/mL was the positive control for the sucking insect assays.

There were three bioassays for LSCB and SCB and two bioassays for all other insects tested. Mortality data for lepidopteran insects including LCSB, SCB, and SWCB were evaluated seven days after test initiation, WCR (Coleoptera) were evaluated after five days and CLH and CLA were evaluated after three days. Potency estimates for microbial Cry1F delta-endotoxin were calculated. The LC_{50} and 95% fudicial limits determined for sensitive species using Probit Analysis. Fisher's Exact Test was used to compare mortality of insensitive species at the highest concentration tested to negative controls.

Results:

Potency of Microbial Cry1F Protein Against Six Corn Insect Pests

Insect	LC_{50} (95% Fiducial Limits)
LCSB	0.108 µg ai/cm^2 (0.019-0.294)
SWCB	0.701 µg ai/cm^2 (0.489-1.000)
SCB	1.457 µg ai/cm^2 (1.068-2.019)
WCR	>53.8 µg ai/cm^2 (no mortality observed)
CLA	>70 µg ai/mL (2% more mortality than neg. control)
CLH	>70 µg ai/mL (less mortality than neg. control)

Results showed no statistical difference between WCR, CLA, and CLH mortality at the highest concentration tested and the negative controls. Positive controls, spinosad and imidacloprid, demonstrated >93% efficacy in each bioassay except for WCR which received 62% to 81% control. These results verify that test insects were exposed to treatments. The relative susceptibilities of the insects found in field corn to Cry1F were LCSB>SWCB>SCB>>WCR, CLA, CLH.

Registrant's Conclusions:

This study reports results of six insects that feed on field corn sensitivity to the microbial Cry1F delta-endotoxin. Cry1F was not found to be active against WCR, CLH, or CLA. Of the insects tested, LCSB was the most susceptible to Cry1f followed by SWCB and SCB.

BPPD Conclusions:
According to 40 CFR Part 160 all studies submitted to the Agency in support of a Section 3 registration are subject to good laboratory practice (GLP) standards. This study did not adhere to all aspects of GLP.

The susceptibility of six field corn insect pests to microbial Cry1F delta-endotoxin was evaluated. This study showed that Cry1F is not active against WCR, CLH, or CLA. LCSB was the most susceptible insect tested followed by SWCB and SCB. Although the LCSB, SWCB, and SCB were shown to be susceptible to Cry1F, an LC_{50} was not reported. Therefore, the level of susceptibility can not be determined.

DATA EVALUATION REPORT

Reviewed by: Robyn Rose, Entomologist, BPPD
Secondary Reviewers: Alan Reynolds, Entomologist, BPPD, and Sharlene Matten, Ph.D, Biologist, BPPD

PRODUCT ID NUMBERS:	EPA Reg. No. 68467-E; Barcode D263761; Case No 066174; Submission No S574903
STUDY TYPE:	Efficacy
MRID NO.:	450201-14
TEST MATERIAL:	*Bacillus thuringiensis* var. *aizawai* Event 1507 Cry1F insect control protein as expressed in maize
STUDY NO.:	PHI99-024
SPONSOR:	Dow AgroSciences LLC 9330 Zionsville Rd, Indianapolis, IN 46268-1054 and Pioneer Hi-Bred International Inc., 7250 N.W. 62nd Ave, P.O. Box 552, Johnston IA 50131
TESTING FACILITY:	Pioneer Hi-Bred International, Inc., 7250 NW 62nd Ave. Johnston, IA 50131.
TITLE OF REPORT:	Efficacy of Cry1F Events TC1360 and TC1507
AUTHORS:	Daniel Moellenbeck and Melvin Peters
STUDY COMPLETED:	November 20, 1999
CLASSIFICATION:	Acceptable

Study Summary:

Objective:
To evaluate the efficacy of Cry1F event TC1507 corn against the European corn borer, southwestern corn borer, black cutworm, corn earworm, and fall armyworm.

Methods:
Efficacy of Cry1F event TC1507 corn was evaluated against five major lepidopteran pests of corn including European corn borer (ECB, *Ostrinia nubilalis*), southwestern corn borer (SWCB, *Diatraea grandiosella*), black cutworm (BCW, *Agrotis ipsolon*), corn earworm (CEW, *Helicoverpa zea*), and fall armyworm (FAW, *Spodoptera frugiperda*). The Bt hybrids were compared to non-Bt isolines. Test plots consisted of a 15 ft single row bordered on each end by 2.5 ft alleyways and 30 in row spacing.

European Corn Borer

Three replicates of the European corn borer test plot was planted in Johnston, IA in a randomized block design. Plants were verified for the presence of Cry1F at the V6 stage by applying Liberty herbicide since these plants were also resistant to Liberty. Plants susceptible to Liberty were

removed. At the V6 stage, 50 neonate larvae were applied to the whorl of plants every two to three days until four successful (no rainfall within 12 hours of application) infestations occurred. Leaf feeding damage was rated after 21 days according to a 1-9 scale (Table 1). Plants were infested with 50 second generation neonate ECB during 50% pollen shed to the leaf axil from three leaves above and one leaf below the primary ear and on its tip every two to three days until four successful infestations occurred. Shank and stalk tunnel damage was evaluated approximately 60 days after the last infestation. Stalks were split from the primary ear to the ground and tunnel length was measured to the nearest inch (<1 in = 1 in). Shanks were split from ear to stalk and were measured to the nearest tenth of an inch. Damage from up to ten plants was evaluated and averaged. Data was analyzed as a randomized block by SAS and mean separations were conducted by calculating 2X mean standard error confidence intervals.

Table 1. Pioneer ECB Leaf Feeding Scoring System

RATING	DESCRIPTION
9	No visible leaf injury or a small amount of pin or fine shot-hole type injury on a few leaves
8	Small amount on shot-hole type lesions on a few leaves
7	Shot-hole injury common on several leaves
6	Several leaves with shot-hole and elongated lesions
5	Several leaves with shot-hole and elongated lesions (ca. ½")
4	Several leaves with shot-hole and elongated lesions (ca. 1")
3	Long lesions common on about ½ of leaves
2	Long lesions common on about 2/3 of leaves
1	Most of leaves with long lesions

Southwestern Corn Borer

Three replicate plots were planted at Union City, TX and Plainview, TX after the normal time to plant corn to ensure a natural SWCB infestation. Liberty herbicide was applied to eliminate non-Cry1F plants. Up to ten plants were split and evaluated for average feeding damage during the peak SWCB damage period. The bottom six nodes of the plant were cut and length of tunnels were recorded to the nearest inch (<1 in = 1 in). Data was analyzed as a randomized block by SAS and mean separations were conducted by calculating 2X mean standard error confidence intervals.

Corn Earworm

Efficacy of Cry1F against corn earworm was evaluated in two locations, Waimea and Koloa, Hawaii, and on two dates, April, 1999 and July, 1999 respectively. Plots were replicated three times and planted to ensure heavy CEW infestations. Non-transgenic plants were removed from

test plots by spraying Liberty herbicide. Average CEW ear damage was evaluated during the dough stage according to a 1-9 rating scale (Table 2). The number and instar of CEW were also counted to determine if Cry1F reduced the number or delayed development. Data was analyzed as a two location randomized block by SAS and mean separations were conducted by calculating 2X mean standard error confidence intervals.

RATING	DESCRIPTION
9	No damage to eartips or kernels, slight damage to silks or husks
8	Slight damage to silks, husks, or eartips but no kernel damage
7	Small damage to silks, husks, or eartips and slight damage to kernels (1-2 kernels damaged or lost)
6	Small damage to silks, husks, or eartips, and 0.1-1.0 cm (>2) kernels damaged or lost
5	Moderate damage to silks, husks, or eartips, with 1.1-2.0 cm kernels lost
4	Moderate damage to silks, husks, or eartips, with 2.1-3.0 cm kernels lost
3	Heavy damage to silks, husks, or eartips, with 3.1-4.0 cm kernels lost
2	Heavy damage to silks, husks, or eartips, with 4.1-5.0 cm kernels lost
1	Heavy damage to silks, husks, or eartips, with 5.1(+) cm kernels lost

Black Cutworm

Efficacy of Cry1F was evaluated at three locations, Huxley, IA, Johnston, IA, and Fowler, IN and replicated three times at each location. Since there was no damage found at the Fowler, IN location, it was dropped from the data analysis. Experimental plots consisted of single 10 ft row plots surrounded by metal sheeting to keep the black cutworms enclosed. Plants were tested via an ELISA for presence of Cry1F at the VE leaf stage. Non-transgenic plants were eliminated from the trial. Plots were then thinned to eight plants per enclosed row and each seedling was infested with 3rd and 4th instar BCW at the V1 stage. The progression of BCW feeding was recorded daily until day 13 by evaluating the number of destroyed plants. Data was analyzed as a two location randomized block by SAS and mean separations were conducted by calculating least significant difference.

Fall Armyworm

Plots were planted during two separate growing cycles (April, 1999 and July, 1999) in Salinas, Puerto Rico. Plots were planted after most of the other corn in the area to ensure an heavy natural infestation of FAW. Trials were replicated three times on each date. Non-transgenic plants were eliminated by spraying with Liberty herbicide. Leaf feeding damage was evaluated during pollen shed using a 1-9 rating scale (Table 3) based on a 14-day leaf feeding scale. Each test plot received a value from 1-9. Data from the plot planted in July was not analyzed because of impacts of leaf disease. Data from the April planting was analyzed as a randomized block by

SAS and mean separations were conducted by calculating 2X mean standard error confidence intervals.

Table 3. Pioneer FAW Leaf Feeding Scoring System

RATING	DESCRIPTION
9	No visible leaf damage or only pinhole lesions present on whorl leaves
8	Pinhole and small circular lesions present on whorl leaves
7	Small circular lesions and a few small elongated (rectangular-shaped) lesions of up to 1.3 cm (½") in length present on whorl and furl leaves
6	Several small to mid-size 1.3 to 2.5 (½" to 1") in length elongated lesions present on a few whorl and furl leaves
5	Several large elongated lesions greater than 2.5 cm (1") in length present on a few whorl and furl leaves and/or a few small- to mid-sized uniform to irregular-shaped holes (basement membrane consumed) eaten from the whorl and/or furl leaves
4	Several large elongated lesions present on several whorl and furl leaves and/or several large uniform to irregular-shaped holes eaten from furl and whorl leaves
3	Many elongated lesions of all sizes preset on several whorl and furl leaves plus several large uniform to irregular-shaped holes eaten from furl and whorl leaves
2	Many elongated lesions of all sizes present on most whorl and furl leaves plus many mid- to large-sized uniform to irregular shaped holes eaten from the whorl and furl leaves
1	Whorl and furl leaves almost totally destroyed

Results:

European Corn Borer

According to the registrants, "The artificial infestation level in this trial was not exceptional, but it was adequate to show the efficacy of the events against both first and second generation European corn borer." All of the event TC1507 corn plants received no shank tunneling (0 inches) and an ECB1 leaf damage rating of 9 which meant there was "no visible leaf injury or a small amount of pin or fine shot-hole type injury on a few leaves." A few tunnels found in the stalk were thought to be from late instar ECB that moved from control plants to Cry1F plants. All tunnels were less than 1 in and there were no surviving larvae found.

Southwestern Corn Borer

There was 0 to 0.1 inches of tunneling per plant in the TC1507 plants versus the 3.5 to 4.5 inches of tunneling in the non-Bt corn. The high level of damage in the non-Bt corn demonstrated that there was adequate insect pressure during the trial. Any small tunneling found in the Cry1F corn was assumed to be from older larvae moving to these plants from control plants. No live SWCB

larvae were found.

Corn Earworm

The control group consisting of non-Bt corn received 3.4-3.8 (moderate to heavy damage on silks, husks, or ear tips, with 2.1 to 4.0 cm of kernels lost) on the rating scale, thus demonstrating a high level of insect pressure during the trial. Cry1F event TC1507 rated significantly ($P<0.05$) greater than the non-Bt hybrid and equal to MON810 corn. There was, however, some CEW damage in the Bt corn. Ear damage rating in event TC1507 ranged from 5.0 (moderate damage to silks, husks, or eartips, with 1.1-2.0 cm kernels lost) to 5.8 (small damage to silks, husks, or eartips, and 0.1 - 1.0 cm (>2) kernels damaged or lost). Results indicate that, although some larvae will survive to pupation, there is some reduction of CEW survival and larval development.

Black Cutworm

There was significantly more of the Cry1F plants surviving than the non-Bt isolines. Event TC1507 demonstrated at least 88% plant survival when exposed to BCW.

Fall Armyworm

Results indicate that event TC1507 provides 100% control of FAW. In each case, leaf damage was rated at ≥8 (pinhole and small circular lesions present on whorl leaves or no visible leaf damage or only pinhole lesions present on whorl leaves. The limited damage that occurred on Cry1F leaves was attributed to FAW larvae moving from plot to plot.

Registrant's Conclusions:
Efficacy data presented in this study showed a high level of control of ECB, SWCB, BCW, and FAW. Instance of extremely small levels of ECB, SWCB, BCW, or FAW damage was attributed to movement of older larvae from non-transgenic to Bt corn. Although infestations of ECB were somewhat low, there is still evidence that event TC1507 is highly efficacious against ECB. There is also evidence of CEW suppression. In all instances, the Cry1F corn was significantly more efficacious than the non-Bt plants. In addition, Cry1F corn appears to have a broader spectrum of lepidopteran control than MON810.

BPPD Conclusions:
According to 40 CFR Part 160 all studies submitted to the Agency in support of a Section 3 registration are subject to good laboratory practice (GLP) standards. This study was not conducted according to GLP. Since the registrants are not pursuing registration of event TC1360, it was not addressed in this review.

Results of this study showed Cry1F expressed in field corn prevented significant ECB, SWCB, and FAW damage and, therefore, a high level of efficacy against these pests can be assumed. Although there were low infestations of ECB in this study, a high dose was demonstrated in

another study (MRID No.451311-01). A high dose still needs to be demonstrated for SWCB. There was a moderate level of BCW control (88% plant survival) and Cry1F provided some control of CEW. It can be assumed that field corn expressing Cry1F will suppress CEW. However, an LC_{50} for Cry1F should be established for each of the pests.

DATA EVALUATION REPORT

Reviewed by: Robyn Rose, Entomologist, BPPD 12/4/01
Secondary Reviewers: Alan Reynolds, Entomologist, BPPD, and Sharlene Matten, Ph.D., Biologist, BPPD

PRODUCT ID NUMBERS:	EPA Reg. No. 68467-E; Barcode D263761; Case No 066174; Submission No S574903
STUDY TYPE:	Binding Study
MRID NO.:	450201-15
TEST MATERIAL:	*Bacillus thuringiensis* var. *aizawai* Event 1507 Cry1F insect control protein as expressed in maize
STUDY NO.:	GH-C 5008
SPONSOR:	Dow AgroSciences LLC 9330 Zionsville Rd, Indianapolis, IN 46268-1054 and Pioneer Hi-Bred International Inc., 7250 N.W. 62nd Ave, P.O. Box 552, Johnston IA 50131
TESTING FACILITY:	NRC-BRI, 6100 Royalmount Avenue, Montreal, Quebec, Canada H4P 2R2 and University of Georgia, Dept. of Entomology, Biological Sciences Building, 125 Cedar St., Athens, GA 30602
TITLE OF REPORT:	Cry1F Binding Studies
AUTHORS:	Luke Masson, Michael Adang, and George Schwab
STUDY COMPLETED:	September 30, 1999
CLASSIFICATION:	Acceptable

Study Summary:

Objective:
"There is evidence that the evolution of resistance to a particular Bt toxin may develop through mutation of one or more midgut proteins that bind the toxin (*c.f.*, Gould, 1998). The purpose of these studies was to determine whether the Bt toxins currently produced in commercial transgenics, Cry1Ab, Cry1Ac and Cry9C, compete for the same binding site as the gene expression product of Cry1F, Dow AgroScience's candidate maize transgene."

Methods:
Surface plasmon resonance (SPR) and radioligand binding are two complementary techniques used to assess and characterize Cry1F binding. These assays utilize brush border membrane vesicles (BBMV) prepared from ECB midguts as the source of midgut binding proteins. Activity of Cry1F, Cry1Ab, Cry1Ac, Cry9C, and Cry9E toxins were evaluated. The Wolfsberger et al. (1987) method that was modified by Ferre et al. (1991) that utilizes the $MgCl_2$ precipitation method to prepare each BBMV was used. At the end of the BBMV purification process, a sample was taken and assayed for leucine aminopeptidase activity (a marker enzyme for midgut epithelial brush border membranes) (Tuppy et al. 1962, Wolfersberger et al. 1987). "Specific

activity was calculated to be 17-20 units (O>D> 405nM/min)/mg BBMV protein." The law of mass action (Cry Toxin + BBMV ⇌Cry Toxin – BBMV) is the simple model these bioassays are based on.

The SPR technique couples a Cry protein to the surface of a sensor chip. The standard BIAcore™ amine coupling protocol provided with the Pharmacia coupling kit were used for all protein chemical immobilizations. The same Cry protein (homologous) or a different one from that coupled to a sensor chip (heterologous) are preincubated with BBMV. "In either homologous or heterologous competition experiments, BBMV were preincubated with 5-6 μM of toxin. Considering literature derived dissociation values for Bt toxins fall in the moderate to high affinity range of 10 nM to 0.1 nM, 6 μM represents 600 to 10,000-fold excess of competitor toxin." "To immobilize toxins to the carboxymethylated dextran (CM5) sensor chip surface, standard amine coupling was used."

An increased signal, or association, is achieved when the BBMV complex is injected across the chip surface. The increased signal is caused by protein binding to the surface of the chip, causing a density change that translates into a change in refractive index at that surface. The resonance changes, thus resulting in a difference in resonance angle. There is a linear correlation between resonance angel shift and protein surface concentration. As the ECB BBMV-toxin complex reacts with the surface and the instrument's software transforms the changing resonance angle into resonance units (kRU), measurements in seconds are taken over time. The injection switches form the BBMV solution to a flow of buffer. There is a decrease in signal which reflects dissociation of the bound Cry toxin-BBMV complex from the Cry toxin-coupled sensor chip surface.

The molecular sizes of Cry1Ab, Cry1Ac, and Cry1F proteins in BBMV isolated from ECB is determined by ligand blots. A qualitative binding experiment was done to identify a concentration of BBMV from ECB suitable for competition binding experiments. BBMV were run on SDS-7% PAGE for ligand blot and immunoblot analyses. "Ligand blotting was done using either 5×10^4 or 5×10^5 cpm ^{125}I-labeled Cry1Ab and cry1Ac per ml. Primary antibody was either anti-Cry1Ac or anti-Cry1F toxin sera from rabbits diluted 1:3000 in 3% BSA-PBS. Immunoblotting was done using the same procedure except primary antibody was prepared against an *E. coli* expressed portion of *Manduca sexta* 115-kDa aminopeptidase (Luo et al 1999b)." "Competition binding experiments were performed with 300 μg vesicle protein per ml for all competing toxins, except for Cry1Ac competition assays (200 μg vesicle protein per ml)."

"In radioligand binding experiments, ECB BBMV are incubated with constant amounts of ^{125}I-Cry toxin alone or in combination with increasing amounts of unlabeled toxin (competition experiments). Following incubation, ECB BBMV and total bound toxin are collected and the amount of ^{125}I-Cry toxin is quantitated. Saturable, steady-state binding data is analyzed utilizing binding isotherms. This data can be further transformed by Scatchard analysis or analyzed directly by non-linear fitting procedures to derive binding constants as well as to determine the presence of multiple binding interactions."

Cry1Ab, Cry1Ac, Cry1F binding proteins in BBMV isolated from ECB midgut tissue were identified via ligand blots. There was a cluster of at least three proteins ranging in size from 140- to 170-kDa visible on stained SDS-polyacrylamide gels. In several lepidopteran species, amino peptidases serve as Cry1 protein receptors. Therefore, antiserum against *Manduca sexta* aminopepetidase was used to probe blots of ECB BBMV. Since the anti-aminopeptidase serum detected proteins of 142-kDa and 160-kDa, the registrants hypothesized that isoforms of aminopeptidase in the brush membrane serve as Cry1Ab, Cry1Ac and Cry1F binding proteins.

Results:

Radioligand Competition Binding

Results showed that when ^{125}I-labeled Cry1Ab was incubated with varying concentrations of BBMV, maximum binding occurred at concentrations >200 of vesicle protein/mL. This binding value is comparable to the maximum Cry1Ab value previously determined by Lambert et al. (1993). Individual competition binging experiments showed the maximum bound ^{125}I-Cry1Ab range to be from 6% to 16%. Results demonstrated that ^{125}I-Cry1Ab bound to ECB BBMV with high affinity. Cry1Ac prevented Cry1Ab from binding to BBMV and Cry1F reduced the amount of ^{125}I-Cry1Ab bound at the highest concentration tested. ^{125}I-Cry1Ab binding was 49% of the maximum in the presence of 1000 nM Cry1F. There was no competition by Cry9C and Cry9E for Cry1Ab binding sites.

Ligand Blotting

"Cry1Ab recognized a 153-kDa protein and Cry1F recognized a protein of slightly larger molecular size. Cry1Ab also recognized a 139-kDa protein and to a lesser extent 244-kDa and 74-kDa proteins. Blots of *O. nubilalis* BBMV were also probed with ^{125}I-Cry1Ab and ^{125}I-Cry1Ac. A 145-kDa protein bound ^{125}I-Cry1Ab. ^{125}I-Cry1Ac recognized proteins of 177-kDa, 152-kDa and 139-kDa. The distinct pattern of Cry1Ab, Cry1Ac, and Cry1F binding to brush border proteins is evidence that each toxin has unique binding determinants." Based upon reports of toxin-binding aminopeptidases in other insects, it may be that proteins detected by Cry1Ab, Cry1Ac, and Cry1F are aminopeptidases.

Surface Plasmon Resonance

Control experiments- Cry1Ab surface: Experiments were performed to determine any anomalous interactions with the preincubated Cry toxin with immobilized toxin or alternatively, the BBMV and the surface. These experiments showed that Cry1Ab does not stick to immobilized Cry1Ab or the dextran surface of the chip. ECB BBMV also stick poorly to immobilized BSA or the dextran surface of the chip.

Competition experiments - Cry1Ab surface: There was no significant competition observed in all three competitive experiments. This result indicates that Cry1Ab, Cry1F, and Cry9C may

recognize separate receptors on the ECB BBMV surface. This was further evidenced from all heterologous sensograms in which decreasing slopes of competitor and noncompetitor curves were almost superimposable, thus showing that mass accumulation on the surface occurs at the same rate. In addition, the slopes of the homologous competition curves are different from the heterologous curves. When homologous competition occurs, the slope of the curve containing the competitor is dramatically decreased when compared to uncompeted ECB BBMV.

Control experiments - Cry 1F surface: Sixty seconds after the beginning of wash off, approximately 71.7% inhibition or 28.3% non-specific binding was observed which is relatively similar to previously reported Cry1Ab competition numbers.

Competition experiments - Cry1F surface: Cry9C and Cry9E competitive experiments resulted in no significant competition with Cry1F (immobilized surface). This result suggests that Cry9 proteins bind to a separate receptor than Cry1F on the ECB BBMV surface. There was competition observed for the Cry1Ab pre-incubated vesicles. However, there was no competition observed in the reverse configuration for instance when Cry1Ab was immobilized. "The 50kDa form of Cry9E and Cry9c can compete for the same receptor on the ECB BBMV. The same holds true for the 65 kDa form of the toxins."

Registrant's Conclusions:
Cry1F does not seem to compete for binding when it is preincubated with ECB BBMV and Cry1Ab is the immobilized partner. However, inhibition occurs when the immobilized partner is reversed. This is probably due to the fact that Cry1Ab recognizes multiple receptor sites, one of which is shared by Cry1F. Therefore, when Cry1F site or sites are saturated, Cry1Ab may still be able to recognize and bind BBMV at a different site. The ability of Cry1f to recognize a site appears to be eliminated by Cry1Ab. Complementary methods to SPR such as using labeled toxins need to be employed in order to obtain a more complete picture even though SPR can easily evaluate the overall competition qualitatively. Labeled toxins (radio/biotin) may help assess whether there may be a separate Cry1F receptor independent of Cry1Ab binding through competition binding or perhaps ligand blotting of BBMV proteins. Ligand blots of whole BBMV proteins may also be able to determine multiple binding partners since the BBMV proteins are separated according to molecular weight.

BPPD Conclusions:
According to 40 CFR Part 160 all studies submitted to the Agency in support of a Section 3 registration are subject to good laboratory practice (GLP) standards. This study was conducted according to GLP.

Results of this study indicate that Cry1Ab may share one or more binding sites with Cry1F in the insects midgut. Therefore, there is a potential for ECB cross-resistance between Cry1Ab (currently registered Cry protein expressed in field corn) and Cry1F. Due to the risk of cross-resistance between Cry1Ab and Cry1F, Cry1F Bt field corn should adhere to the same IRM strategy as Cry1Ab corn. In addition, it is not recommended to stack Cry1Ab and Cry1F in the

same corn hybrid

It is also probable that Cry1F and Cry1Ac share a binding site, therefore, raising concern of cross-resistance. However, Cry1F does not share a binding site with Cry9C or Cry9E so cross-resistance between these Cry proteins is not a concern.

References:

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DATA EVALUATION REPORT

Reviewed by: Robyn Rose, Entomologist, BPPD RR 1/24/01
Secondary Reviewers: Alan Reynolds, Entomologist, BPPD, and Sharlene Matten, Ph.D, Biologist, BPPD SM 1/24/01

PRODUCT ID NUMBERS:	EPA Reg. No. 68467-E; Barcode D263761; Case No 066174; Submission No S574903
STUDY TYPE:	Insect Resistance Management Plan
MRID NO.	450201-16
TEST MATERIAL:	*Bacillus thuringiensis* var. *aizawai* Event 1507 Cry1F insect control protein as expressed in maize
STUDY NO.:	GH-C 5037
SPONSOR:	Dow AgroSciences LLC 9330 Zionsville Rd, Indianapolis, IN 46268-1054 and Pioneer Hi-Bred International Inc., 7250 N.W. 62nd Ave. P.O. Box 552, Johnston IA 50131
TESTING FACILITY:	Trait Development and Regulatory Support, Mycogen Seeds, 301 Campus Dr, Huxley, IA 50124.
TITLE OF REPORT:	Resistance Management Plan for Transgenic Maize Expressing the Cry1F Insecticidal Protein from *Bacillus thuringiensis* var. aizawai
AUTHORS:	Paul Bystrak and Daniel Moellenbeck
STUDY COMPLETED:	December 10, 1999
CLASSIFICATION:	Supplemental to addressing the BBPD IRM comments and concerns presented in the cover memo of this review.

Study Summary:

Objective:
This study outlines Dow AgroSciences and Pioneer Hi-Bred's resistance management plan for the Cry1F delta endotoxin and the genetic material (PHI8999) necessary for its production in corn. Key target pests include: ECB, SWCB, FAW, and BCW.

Introduction:
Cry1F event TC1507 is the first Cry protein expressed in corn derived from *Bacillus thuringiensis* var. *aizawai*. Since it is a different Bt var. then currently registered Bt corns which express the *kurstaki* variety, a somewhat different spectrum of activity is expected. Cry1F is considered to be functionally equivalent to the currently registered Btk varieties. In addition, Cry1F has activity against FAW and BCW and is marginally efficacious against CEW.

Cross-Resistance:
Cross-resistance between Cry1A and Cry1F have been demonstrated in the diamondback moth (*Plutella xylostella*) and the tobacco budworm (*Heliothis virescens*) (Tabashnik et al 1997, Gould

et al. 1995). Since an ECB population resistant to Cry1F does not exist, binding studies were conducted (see MRID No. 450201-15) to see if Cry1F shares a binding site with Cry1Ab. Results of this study showed that Cry1Ab and Cry1F have different preferred binding sites or share a common binding site with different binding properties such as binding affinity or rate. In this study, there was only Cry1F competition for Cry1Ab binding sites when there was a surplus of Cry1F protein approximately equivalent to 1000X the normal levels for binding saturation of the Cry1Ab protein. This shows that Cry1Ab and Cry1F have at least one unique high affinity binding site each and a lower affinity binding site shared in common, or that Cry1Ab and Cry1F share a common receptor with different levels of affinity or rate. A personal communication between the study authors and Dr. Blair Seigfried (University of Nebraska) concluded that laboratory toxicity data show ECB colonies with a moderate level of tolerance to Cry1Ab (~6X) were highly susceptible to Cry1F. The study found in MRID No. 450201-15 also showed that Cry1F and Cry9C as well as Cry1Ab and Cry9C do not compete for binding sites.

Target Pests for Event TC1507:
The primary target pests of event TC1507 are the ECB and SWCB so the IRM plan concentrates on these stalk-boring Lepidoptera. Although event 1507 also controls FAW and BCW, an IRM plan is not proposed for these pests. There are several reasons an IRM plan is not proposed for BCW and/or FAW. First, neither the BCW or FAW is a permanent resident of the corn belt. Rather, they overwinter in gulf states and migrate to the Corn Belt in the spring and summer with minimal, if any, return migration. Second, BCW and FAW are polyphagous insects and corn is not a major host. Grass is the primary host for FAW and BCW predominantly feed on broadleaf weeds before moving in to corn. The authors believe these areas may provide refuge although no data was submitted to support this theory. Third, both the BCW and FAW cause sporadic damage in the Corn Belt and do not drive grower practices. Therefore, their control is considered a secondary benefit of Bt corn. Fourth, the IRM program designed for ECB and SWCB, in addition to natural habitat, should protect BCW and FAW from resistance.

Cry1F also provides moderate protection against the CEW. Event TC1507 provides comparable CEW control to the currently registered Cry1Ab events MON810 and Bt11. These events are considered to "suppress" rather than control CEW. This lack of a high dose for CEW effects the IRM plan in areas where it is a potential pest."

Proposed Steps for Resistance Management:

Reduction of Selective Pressure

High Dose: A high dose for ECB was verified for event TC1507 by the February 1998 FIFRA Scientific Advisory Panel (SAP) methods 4 and 5. "the event used to generate the commercial hybrids will use a promoter system that expresses the toxin throughout the growing season, in all tissues, at relatively high levels.

Refuge Percentages: The proposed refuge is based upon the minority opinion presented in the

ILSI-HESI Panel decision table. It suggests that in the northern corn growing region, high dose product fall into the moderate risk category. Therefore, a 10% untreated or 20% treated refuge will sufficiently protect against resistance (ILSI/HESI 1998). For this proposal, the minority opinion was chosen "because information developed since the Panel met shows that the value for the **h** (the degree of dominance, as defined therein), although not accurately determined, has to be much smaller than 0.1 (the majority opinion) and is almost certainly smaller than 0.01 (the minority opinion)." Models developed by Onstad and Gould (1998), Onstad and Guise (1999) and Huang et al. (1999) support the refuge sized recommended by ILSI. Since homozygous resistant ECB demonstrate 65X resistance to Bt, it can be assumed that currently registered events provide a high dose. This IRM proposal claims that the 10% unsprayed and 20% sprayed refuges are adequate to mitigate ECB and SWCB resistance to Bt and should also be sufficient for BCW and FAW since these pests are at a much less risk.

However, the registrants advocate a single refuge size be recommended to growers since they do not always know if they will be treating the fields prior to planting. Since the size of the sprayed refuge can't be reduced, the registrants recommend all refuges, sprayed or unsprayed, be 20%. This recommendation would coincide with the ABSTC (Agriculture Biotechnology Stewardship Technical Committee) IRM Plan (Vlachos et al 1999).

The Southern Cotton Growing Region is described the ILSI report (ILSI 1998). Since cotton and corn are grown in this region, there is an increased risk of CEW (also known as the cotton bollworm in cotton) resistance. Therefore, a 50% refuge, which is consistent with the ABSTC Plan, is recommended.

Refuge Proximity: Due to the mobility of adult and larval stages of all target pests, the registrants determined that a refuge planted in very close proximity to the Bt field leads to a higher risk of resistance than a moderate proximity. Growers will be instructed to plant their unsprayed refuges within 0.5 miles of the Bt field and 0.25 miles if they want the option to spray. Seed mixes will not be sold and growers will be discouraged from mixing seed to avoid sub-lethal exposure to Cry1F.

Non-Target Pests: Since Cry1F does not have a long history of use, it's spectrum of activity is not completely known. Therefore, in-house and contracted studies are underway. As planting restrictions are lifted, additional studies will be conducted. These studies will determine which pests will and will not be controlled by Cry1F corn. The registrants intend to report finding to the Agency annually.

IPM: The registrants intend to promote the use of IPM through University Extension Agents, Crop Consultants, USDA experts, and other company representatives. Information given to growers regarding IRM will encourage only spraying refuges when economic thresholds are reached and to effectively implement refuges.

Monitoring of Insect Pest Susceptibility

Annual Susceptibility Monitoring: Dr. Blair Seigfried (University of Nebraska) had determined the sensitivity of wild ECB populations throughout the corn growing areas of the U.S. to a sample of truncated Cry1F as it is expressed in the plant. When enough information is gathered, a discriminating dose will be determined. Monitoring for changes in susceptibility will continue after registration. Monitoring for resistance to other susceptible Lepidoptera will not be conducted due to the high cost and small risk of resistance.

Grower Monitoring: It is likely growers will be the first ones to observe a resistant population in the field. Growers will, therefore, be instructed to scout their TC1507 fields and reported unexpected ECB, SWCB, or CEW damage to their seed company representative. The registrants will sample tissue from the damaged plant to determine the cause. Once a discriminating dose has been established, it will be used to determine if the insect population exhibits a resistant phenotype All instances of resistance will be reported to the Agency and mitigation measures will be taken.

Resistant Colonies: There is currently and attempt to produce a colony of Cry1F resistant ECB so it can be determined if there is cross-resistance with Btk. A resistant colony will also be used to determine the exact high dose of Cry1F for ECB.

Communication and Education

Grower education is intended to make IRM a regular part of farm operations so growers view it as part of their good stewardship. Grower education includes mid-summer plot tours as well as fall and winter meetings and sales calls. In addition, information can be obtained form newsletters, mailings, and the company's websites. To reduce the potential for confusion or mistakes, the registrants recommend no major changes be made from current IRM programs. All seed manufacturers and extension specialists are attempting to convey the same uniform message outlined below.

Train the Trainers: Since grower education often comes from the seed salesman, it is important that they are properly trained in IRM. Sales representatives are trained by technical representatives who also need to be taught. There are already IRM requirements and education in place that should be continued.

Product Use Guides: All TC1507 growers will be given a Product Use Guide that will outline the need and options for implementing an IRM plan. Part of the signed grower agreement will include a statement that the Product Use Guide was received. This guide will also be available on the company's website.

Field Placards. Two types of field placards are generally used; one used as a teaching tool at grower field meetings that addresses IRM and another indicating the company name and hybrid.

Future field placards may have a logo identifying the field as a refuge. This will demonstrate that the grower is practicing good stewardship.

Slide Talk: Fall and winter grower meetings may utilize slide presentations to outline IRM principles. These slide presentations may also be used by sales and technical representatives.

Publicity: Articles regarding IRM will be in company newsletters and from press to reinforce the education received from other means.

Compliance and Compliance Monitoring

The registrants do not believe 100% compliance is necessary as long as the large majority of growers comply with IRM requirements. The following steps will be implemented to ensure growers are aware of their IRM responsibilities.

Consistent IRM Programs: To avoid confusing or discouraging growers, new IRM programs will be kept simple and consistent with existing programs. The registrants are concerned that discouraged growers will not properly implement IRM or will not grow transgenic crops.

Technology Use Agreement: Growers will be asked to sign a technology use agreement that outlines IRM requirements and acknowledges the growers responsibility to comply with them. The agreement will also state that growers received the Product Use Guide. This agreement may be a section of the growers order sheet or some other document or format. The grower agreement may be signed annually or as needed.

Compliance Monitoring: An annual industry-supported survey will be used to monitor grower compliance. Additional education efforts will target non-compliant growers.

Noncompliance: Access to the technology will be limited for growers found to be non-compliant.

Mitigation Plans:

Alternate Modes of Action

Transgenic corn with alternate modes of action and stacked genes are being developed. Stacking non-cross resistant genes may reduce the potential for resistance to develop.

Plan Failure Mitigation

If resistance of a target pest is confirmed, the registrants will:

1. Inform the EPA and relevant university personnel of the situation.
2. Immediately cease sales of transgenic products containing te Cry1F ICP in the

counties where the resistance is confirmed or suspected.
3. Begin research to determine which aspect(s) of the IRM Plan lead to the failure.
4. In the surrounding counties, increase refuge size, decrease proximity limits, and improve compliance mechanisms until the results of the failure research establish the cause of the failure; then modify the IRM plan to correct that deficiency.
5. Use resistant insects to test various premises of IRM theories.

Registrant's Conclusions:
No overall conclusions for this submission were presented by the registrants. The BPPD IRM Team conclusions and recommendations are presented in the cover memo of this review.

BPPD Conclusions:
According to 40 CFR Part 160 all studies submitted to the Agency in support of a Section 3 registration are subject to good laboratory practice (GLP) standards. This study was not conducted according to GLP.

Pest Biology

The primary target pests of event TC1507 (the event to be commercialized) are ECB, SWCB, FAW, and BCW. The IRM strategy is based upon ECB biology. This is acceptable based upon current scientific research. However, additional research regarding dose and pest biology is necessary for SWCB in particular as well as FAW, BCW, and CEW.

High Dose

The February, 1998 FIFRA Scientific Advisory Panel (SAP) Subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides and Resistance Management determined that a high dose/refuge strategy is necessary to mitigate resistance of stalk boring Lepidoptera in Bt corn (meeting held on February 9-10, 1998. Docket # OPPTS-00231). The SAP determined that a high dose (defined as 25× the dose necessary to kill all susceptible insects) should be verified by two of five techniques outlined in the final report (http://www.epa.gov/scipoly/sap/1998/february/finalfeb.pdf).

Ideally, high dose should be evaluated for all susceptible pests, so that appropriate resistance management strategies can be developed. At a minimum, high dose should be determined for the major target pests of Cry1F corn (ECB and SWCB). The registrants verified a high dose for Cry1F event TC1507 was by the fourth and fifth methods described by the SAP.

Method four is similar to method 3, but would use controlled infestation with a laboratory strain of the pest that had an LD_{50} value similar to field strains. This is used to determine if there would be a significant number of surviving heterozygotes. Method 3 involves surveying large numbers of commercial plants on sentinel plots in the field (e.g., sentinel sweet corn method) to make sure that the cultivar is at the $LD_{99.99}$ or higher to assure that 95% of heterozygotes would probably be killed. With this approach Bt sweet corn hybrids are used to

attract high densities of ECB and cotton bollworm (*Helicoverpa zea*)(Boddie)) (CBW/CEW) moths, sampling can be limited to sweet corn ears in the *Bt* plot (ca. 1/4-1/2 acre block), and a frequency of resistant phenotypes can be estimated as the ratio of density of larvae/plant in *Bt* sweet corn to density of larvae/plant in an adjacent planting of non-*Bt* sweet corn (Andow and Hutchison, 1998; Hutchison, unpublished data). With the fifth method identified by the SAP, it should be determined if an older instar of the targeted pest could be found with an LD50 that was about 25-fold higher than that of the neonate larvae. If so, that stage could be tested on the crop plants to determine if 95% or more of the older stage larvae were killed.

Overall, a high dose for ECB has been demonstrated in this report for event TC1507. Additional high dose data are needed for the SWCB.

Refuge

A refuge should be designed to produce 500 susceptible insects for every one potentially resistant insect. This Cry1F registration will be subject to the same requirements as the Agricultural Biotechnology Stewardship Committee (ABSTC) and the October 2000 SAP report that will consider refuge needs in an IRM strategy. Refuges planted to mitigate resistance to Cry1F field corn should be identical to those needed for Cry1Ab corn and should consist of agronomically similar non-Bt field corn varieties. The Bt and non-Bt field corn varieties should be cared for and managed in a similar fashion. The non-Bt refuges should be planted within the Bt field or in close proximity. In general, refuges may be planted as external blocks on the edges or headlands of fields or as strips within the Bt corn field. In-field strips should include multiple rows (at least 2-6) of non-Bt field corn and extend the full length of the field. Refuges should be treated as needed to control lepidopteran stalk-boring insects with non-Bt insecticides or other appropriate IPM practices. Insecticide use should be based on scouting using economic thresholds as part of an IPM program.

The issue of refuge proximity is a critical variable for resistance management. Refuges must be located so that the potential for random mating between susceptible moths (from the refuge) and possible resistant survivors (from the Bt field) is maximized. The USDA NC-205 North-Central regional research committee on ecology and management of European corn borer and other stalk-boring Lepidoptera has recommended to the Agency that all Bt corn should be placed within one half mile of the non-Bt corn refuge, but that refuge plantings within one quarter mile would be even better (NC-205 letter to Dr. Janet Andersen, 5/24/99). However, the complete picture of ECB dispersal is still unknown and among females, mating is likely to occur (relatively) close to the site of pupal eclosion. In addition, information is needed on SWCB, CEW, BCW and FAW movement and mating behavior to fully understand optimal placement of refuges. This research should be conducted and submitted to the Agency as part of the annual research report.

Based upon NC-205 recommendations made from 1998-2000, and information regarding ECB

resistance management acquired from models (Onstad & Gould 1998, Onstad & Guse 1999), a minimum of 20% non-Bt corn refuge is recommended for non-cotton growing regions (e.g., Corn Belt) that do not spray insecticides on a regular basis. A 20% non-Bt corn refuge in non-cotton growing regions was subsequently recommended by the ABSTC in April 1999 and mandated by EPA in 2000.

The October 2000 SAP Subpanel is currently drafting a report that considers whether larger refuges, e.g., a 40% non-Bt corn refuge, would decrease the risk of insect resistance in non-cotton growing regions that spray insecticides on a regular basis (e.g., High Plains for SWCB). This area needs to be clearly identified on a county basis or triggers developed to indicate when higher insecticide use poses a greater risk to resistance. A larger refuge may be needed in this area because of the increased risk of resistance created when insecticides are used, thus killing insects susceptible to Bt. Currently, EPA has mandated a 20% non-Bt corn refuge in all non-cotton growing regions.

Cry1F corn grown in cotton-growing regions, including the northern and southern cotton growing regions, should plant a minimum 50% non-Bt corn refuge that may be treated only as necessary with non-Bt insecticides. This submission did not include the northern cotton growing region that is also subject to a 50% refuge. This larger refuge size in cotton-growing regions is needed due to increased concerns regarding the development of CEW resistance in cotton growing regions especially those regions growing Bt cotton.

Monitoring

The monitoring strategy submitted for this registration is not adequate because it only addresses grower monitoring for unexpected damage. A resistance monitoring strategy for Bt corn is needed to test the effectiveness of resistance management programs. Detecting shifts in the frequency of resistance genes (i.e., susceptibility changes) through resistance monitoring can be an aggressive method to detect the onset of resistance before widespread crop failure occurs. As such, the utilization of sensitive and effective resistance monitoring techniques is critical to the success of an IRM plan. Techniques such as the F_2 screen, and sentinel plots need to be thoroughly investigated for their feasibility as resistance monitoring tools in addition to the currently used discriminating dose concentration assays. The ABSTC must submit research to the Agency regarding the feasibility of conducting the F_2 screen by March 2001. In addition, baseline susceptibility to Cry1F data should be developed for ECB, SWCB, and CEW.

The ABSTC proposed a monitoring strategy in March 2000 for ECB, CEW, and SWCB in Cry1Ab field corn. The ABSTC plan to monitor for ECB, SWCB, and CEW resistance should also be used for Cry1F field corn in addition to the grower monitoring presented in the Dow AgroSciences/Pioneer Hi-Bred submission. The ABSTC plan focuses resistance monitoring in areas where Bt corn market penetration is highest as well as areas with the highest insecticide use. The ABSTC plan includes the identification of counties growing more than 50,000 acres of field corn (Bt and non-Bt) to focus monitoring efforts. ABSTC's proposed plan is designed to

detect resistance when it reaches 1 - 5% (a level that allow for detection of resistance before field failures occur). Four corn-growing regions were identified and monitoring for each pest will occur in the regions in which the pests are prevalent. When possible, at least 200 first or second flight adults (100 females), 100 second flight egg masses, and 100 diapausing larvae per site will be collected in each region, though insect population levels may limit the number collected. The sampling strategy should be adequate, although the program would be improved if sampling locations can be separated by a sufficient distance to reflect discreet pest populations.

Remedial Action

Remedial Action was not adequately addressed by Dow AgroSciences and Pioneer Hi-Bred. According to the registrants, all instances of resistance will be reported to the Agency and mitigation measures will be taken. Mitigation measures include ceasing sales in the affected counties, but the registrants does not address continued monitoring in the area or the use of alternate control measures. The Cry1F remedial action plan should be identical to the ABSTC plan for Cry1Ab expressing field corn already approved by the Agency in 2000.

Remedial actions should include: informing customers and extension agents in the affected areas of suspected or confirmed resistance, increasing monitoring in the affected areas, implementing alternative means to reduce or control target pest populations in the affected areas, implementing a structured refuge in the affected areas, and cessation of sales in the affected and bordering counties until an effective local management plan approved by EPA has been implemented. During the voluntary suspension period, registrants may sell and distribute in these counties only after obtaining EPA approval to study resistance management in those counties. The implementation of such a strategy will be coordinated by the Agency with other registrants and stakeholders.

Compliance

Dow AgroSciences and Pioneer Hi-Bred do not believe 100% compliance is necessary as long as the large majority of growers comply with IRM requirements. Growers will need to sign a technology use agreement that outlines IRM requirements and acknowledges the growers responsibility to comply with them. The agreement will also state that growers received the Product Use Guide. This agreement may be a section of the growers order sheet or some other document or format. The grower agreement may be signed annually or as needed. The registrants recommend grower surveys to estimate the level of compliance, limiting non-compliant growers access to the technology, and implement additional education efforts will target non-compliant growers.

Models used to determine the size of the non-Bt corn refuge are based on 100% compliance and 100% adoption. Because it is unknown how the level of compliance will affect IRM, 100% compliance should be the goal of any IRM plan. BPPD strongly disagrees with the registrants assertion that 100% compliance is not necessary. Since there is not 100% adoption of Bt corn,

models should be developed that consider < 100% compliance and < 100% adoption. In addition, grower surveys alone are not adequate to address grower compliance. An improved method of assessing grower compliance needs to be developed. This may be done, for example, through improved grower education, site visits and mapping, certification programs, incentive programs, or other means. BPPD will also need grower information material submitted on an annual basis for review to ensure proper requirements are articulated.

Cross-Resistance

Cross-resistance, in which one toxin confers resistance to another, is an area of major concern for resistance management and poses risks to both transgenic Bt crops and microbial Bt insecticides. The most well-documented mechanism of cross-resistance with Bt occurs when two toxins share the same binding site (receptor) in the insect midgut (Tabashnik 1994).

Competitive binding experiments provided by the registrants showed that Cry1F may share a binding site in the ECB midgut with Cry1Ab or Cry1Ac, but not with the Cry9 proteins. This demonstrates that ECB has the potential for cross-resistance between Cry1F and Cry1Ab (currently registered Bt corn) and Cry1Ac (currently registered Bt cotton). Therefore, the IRM strategy mandated for Cry1Ab field corn should also be implemented for Cry1F corn. In addition, it is not recommended to stack Cry1Ab and Cry1F in the same corn hybrid.

Grower Education

Dow AgroSciences and Pioneer Hi-Bred have adequately addressed grower education through training their trainers, Product Use Guides, field placards, slide presentations and publicity. The proposal they submitted to the Agency should be implemented. Growers are perhaps the most essential element for the implementation and success of any IRM plan as they will ultimately be responsible for ensuring that refuges are planted according to guidelines and that Bt fields are monitored for unexpected pest damage. Therefore, a program that educates growers as to the necessity of IRM and provides guidance as to how to deploy IRM should be an integral part of any resistance management strategy. Ideally, the educational messages presented to growers should be consistent (among different registrants) and reflect the most current resistance management guidelines for current Bt corn registrations. Specific examples of education tools for growers can include grower guides, technical bulletins, sales materials, training sessions, Internet sites, toll-free numbers for questions or further information, and educational publications

Annual Reports:

Written reports on various aspects of IRM, submitted on an annual basis to EPA, are of great aid in the evaluation of the success of resistance management for Bt corn. Reports should be submitted to the Agency on an annual basis on Bt corn sales/market penetration, IRM-related research, and grower education. In addition to these reports, it would be particularly useful to receive reports from Bt corn registrants on grower compliance and resistance monitoring.

References:

Gould, F., A. Anderson, A. Reynolds, L. Bumgarner, and W. Moar. 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J. Econ. Entomol. 88(6): 1545-1559.

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ILSI-HESI. 1998. An Evaluation of Insect Resistance Management in Bt Field Corn: A Science-Based Framework for Risk Assessment and Risk Management. International Life Science Institute. Washington, DC.

Onstad, D.W. and F. Gould. 1998. Modeling the dynamics of adaptation to transgenic maize by European corn borer (Lepidoptera: Pyralidae). J. Econ. Entomol. 91(3): 585-593.

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Tabashnik, B.E., Y.B. Liu, N. Finson, L. Masson, D.G. Heckel. 199. One gene in diamondback moth confers resistance to four *Bacillus thuringiensis* toxins. Proc. Ntl. Acad. Sci. 94: 1640-1644.

Vlachos, D., S. Brody, P. Bystrak, P. Davis, e. Debus, E. Sachs, D. Shanahan, J. Stein, R. Townsend, and G. Wandrey. 1999. Industry Insect Resistance Management Plan for Cry1A Plant-Expressed Protectants in Field Corn. National Corn Growers Assoc. http://www/ncga.com/02profits/insectMgmtPlan/main.htm.

Reviewer: Gail Tomimatsu, Ph.D. Date: 1/29/2001
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Secondary Reviewer: Zigfridas Vaituzis, Ph.D. Date: 2/9/2001
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Phil Hutton, Chief
Biopesticides and Pollution Prevention Division (7511C)

DATA EVALUATION REPORT

STUDY TYPE: Nontarget Fish Testing (OPPTS Series 885.4200)

CITATION: Mayes, M. (1999). Waiver Request: Fish Toxicity Test with Transgenic Maize (Corn) Containing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry 1F Delta-Endotoxin. Global Toxicology Laboratory. Dow AgroSciences LLC, 9330 Zionsville Rd., Indianapolis, IN 46268-1054 MRID 450442-01.

DP BARCODE: D263758 **CASE**: 066174
REG./FILE#:068467-E **CHEMICAL/BIOL#**: 006481 *Bacillus thuringiensis* CRY1F protein

COMPANY/SPONSOR: Dow AgroSciences, LLC

TEST MATERIAL: (Bt) Cry 1F delta-endotoxin producing corn meal in fish food.

REVIEW SUMMARY & CONCLUSION: The registrant is requesting a waiver from testing freshwater fish to toxicity by transgenic maize containing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry 1F delta-endotoxin. The basis of the waiver is the presumed lack of exposure to fish (in aquafarms) to the low content of Cry1F endotoxin in corn kernels in commercially manufactured fish diets.

ADEQUACY OF STUDY: Acceptable. The submitted data and results are sufficient to conclude that the amounts of Cry1F endotoxin in corn kernels and the lack of measurable concentrations of Cry1F endotoxin in commercial fish diets are unlikely to present hazardous exposures to fish. Accordingly the registrant's request to waive farmed fish toxicity studies is granted.

BACKGROUND: The Agency previously has waived static renewal toxicity tests for freshwater fish due to the lack of substantial exposure (Bt Crops Assessment, 2000). Therefore, the primary target of concern is the possible toxicity of commercial fish diets prepared with grain from transgenic corn expressing Bt delta-endotoxin(s). Agency scientists have therefore required registrants to conduct toxicity tests with fish fed a diet prepared with corn kernels expressing the specific Bt endotoxin, but have waived testing when submitted data have shown the Bt protein was denatured or inactivated during a typical commercial fish diet manufacturing process.

METHODOLOGY

Dow AgroSciences/Mycogen contracted Purina Mills, a major manufacturer of fish diets to produce a diet manufactured with kernels from corn plants expressing the Bt Cry 1F delta-endotoxin.

The experimental fish food was processed by extrusion, the most common method for the preparation of diets for warm water species, and using the maximum corn content found in fish diets (34 to 49 %). Corn content of diets for cold water species, such as trout, generally contain 10 to 15% corn, because corn is not as efficiently metabolized by these species (National Research Council. 1993 Nutrient Requirements of Fish. Committee on Animal Nutrition. National Research Council; National Academy Press, Washington, DC. 114 p). Processed diets (those containing corn expressing the Bt Cry 1F endotoxin, and non-transgenic corn) were shipped for ELISA analyses and for bioassays with first instar tobacco budworm (TBW).

In the extrusion process, mixed mill is ground through a screen followed by steam conditioning (180 to 212° F) for about 30 sec. The meal is moved to an extruder barrel and processed under pressure and heat (up to 350 ° F). Water is then added to both the conditioned product and to the barrel of the extruder. The product is extruded through a die and cut into ¼ " pellets and is dried to less than 11% moisture in a batch dryer (about 15 to 30 min). After cooling to ambient temperature, it is bagged for shipment. A copy of Purina's process was provided in the submission The major ingredients included: 50% soybean meal, 38.72% corn meal. 8% fish meal and 1.5% animal fat.

Four separate bioassays on tobacco budworm (TBW) were conducted; each used the following treatments: fish diet prepared with corn expressing the Bt Cry 1F endotoxin, control corn fish diet with spinosad (positive control), a spinosad carrier control (2:1 mix of acetone:water); and an agar control. A total of 64 larvae of TBW were exposed to each treatment in small plastic cups (16 larvae per cup) containing an agar-based TBW diet to which the test substance was applied and allowed to dry. Mortality and growth were assessed after 7 days of exposure.

REPORTED RESULTS

Although results of the bioassays were variable, the biological activity associated with the diet containing corn meal expressing Cry 1F delta endotoxin was similar to that of the 3 other treatments.

The Cry 1F endotoxin protein content of kernels before processing into feed was 2.2-3.5 ng/mg (however, Table 3 states, 2.2 - 3.2 ng/mg). Analysis of the fish diet samples by ELISA demonstrated that Cry 1F endotoxin was not detectable in the feed samples (5 mg sample) with a detection limit of 0.04 ng/mg. Original data are contained in another referenced report: Young, D. L. and R.A. Herman. 1999. Characterization of Expressed Cry 1F Protein in Maize Tissues (Pollen, Grain, Grain Containing Feed, and Purified Maize-Expressed Cry 1F Protein) and Microbial Expressed Cry 1F delta Endotoxin by Biological and Biochemical Procedures. Dow AgroSciences, LLC unpublished report GH-C 5006, dated 18 Nov. 1999.

The registrant requests that the fish testing requirement be waived because fish fed commercial diets containing corn meal from kernels expressing Cry 1F endotoxin will not be exposed to biologically active, or to detectable amounts of Cry 1F endotoxin.

REVIEWER'S COMMENTS:

A. **Test Procedures:** There is no validated protocol for this test. **Processing grain for commercial fish food is considered CBI** .

B. **Reported Results and Statistical Analysis:** Table 2 of the report shows that 3/64 larvae died in the group fed Bt maize; 2 of 64 larvae died in the group fed spinosad-incorporated agar. No mortalities were reported in groups fed agar incorporated with the acetone carrier or agar alone. The study author reported variability in the results concerning bioactivity but these do not affect the assessment or the overall conclusion. Statistical analyses were reported for larval weight. The means were not statistically different.

C. **Discussion/Risk Assessment:** The study is scientifically sound and though there was some variation in the bioactivity of the test substances, the overall conclusion is not affected. These data show that there will be no statistically significant biological activity from Bt Cry 1F transgenic corn grain. In addition, there were low levels of the Bt Cry 1F protein in corn kernels; following processing there were undetectable levels in fish food containing Cry 1F maize. Accordingly, the request to waive testing requirements for fish toxicity is acceptable.

D. **Adequacy of the Study:**

1. Validation Category: Acceptable. Request to waive farmed fish testing is granted.
2. Rationale: This study is an acceptable substitute for toxicity testing on farmed fish. EPA Guideline requirements for toxicity testing of bluegill sunfish and rainbow trout are waived because of low environmental exposure to Bt corn pollen in the aquatic environment.

Reviewer: Gail Tomimatsu, Ph.D. Date: Jan. 31.2001
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Secondary Reviewer: Zigfridas Vaituzis, Ph.D. Date: 2/9/2001
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Phil Hutton, Chief
Biopesticides and Pollution Prevention Division (7511C)

DATA EVALUATION REPORT

STUDY TYPE: Nontarget Earthworm Testing (OECD Guideline 207)

CITATIONS: Hoxter, K. A., J.R. Porch, H. O. Krueger (1999) Cry 1F Bacillus thuringiensis var. aizawai delta Endotoxin: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate. Wildlife International Ltd. Project Number 354-112, December 8, 1999. MRID 450201-06 and Mayes, M.A. (2001) Supplement to MRID 45020106: Cry1F Bacillus thuringiensis var. aizawai Delta Endotoxin: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate. Mycogen Seeds c/o Dow AgroSciences LLC, 9330 Zionsville Rd., Indianapolis, IN 46268-1054 Laboratory Study ID: GH-C5171. January 16, 2001. MRID 453078-04.

DP BARCODE: D263758 **CASE**: 066174
REG./FILE#:068467-E **CHEMICAL/BIOL#**: 006481 *Bacillus thuringiensis* CRY1F protein

COMPANY/SPONSOR: Mycogen c/o Dow AgroSciences LLC Corporation, 5501 Oberlin Dr., San Diego, CA 92121

TEST MATERIAL: Off-white powder, identified as microbial (truncated) Cry1F delta-endotoxin from *Bacillus thuringiensis* var. *aizawai* (Bt)

REVIEW CONCLUSION: The one limit test concentration of 2.26 mg Cry1F/kg dry soil represented up to 100X the estimated concentration present in the top six inches of an acre of soil following the incorporation of 25,000 senescent corn plants. Based on the lack of adverse effects on earthworms in this study, it is unlikely that Cry1F transgenic corn plantings will have adverse effects on earthworms.

ADEQUACY OF STUDY:
Validation category: Acceptable (Supplemental).
Rationale: Although the study was conducted according to GLP standards, the report is not in total compliance with GLP test material analysis requirements.

MATERIALS & METHODS: The methods used in this study are based upon procedures outlined in the Organization for Economic Cooperation and Development (OECD) Guideline No. 207, Guideline for Testing of Chemicals, Earthworm, Acute Toxicity Tests.

The test material, was an off-white powder, identified as Cry1F microbial (truncated); Lot no. 1599-45. The reported purity of the test substance was 11.4% active ingredient, as determined by

ELISA. A summary of the GLP characterization (bioassay and ELISA testing) of the test substance was included in the study.

The test organisms, adult earthworms (*Eisenia fetida*) were supplied by Willingham Worm Farm, Butler, GA. In the laboratory, the worms were maintained in culture chambers containing a mixture of moist peat and manure. Twenty four hours prior to the test, 115 worms were selected and placed in a container of prepared artificial soil substrate adjusted to a moisture content of approximately 33%, for the acclimation period. On the day of test initiation, the worms were rinsed briefly with deionized water, and impartially distributed by pairs into groups of 10 worms each. Each group of worms was weighted and placed on the soil in the appropriate test chamber. Worms were not fed during the 14 day test period.

The test chambers were one liter glass beakers covered with plastic wrap which was perforated for air exchange. There were four test chambers (replicates) each for the control and test soil groups.

Artificial soil was prepared by blending 70% sand, 20% Kaolin clay, and 10% sphagnum peat. The pH of the bulk soil was adjusted to 5.8 using calcium carbonate.

The test soil substrate was prepared by dissolving the test substance in deionized water and mixing directly into moistened artificial soil. Sufficient water was added to the soil to achieve a moisture content of approximately 33%. The soil was mixed for a total of fifteen minutes. The control soil was mixed similarly, but without the addition of test substance. Seven hundred fifty grams of prepared soil were added to each of four test chambers per treatment or control group. Soil samples were collected from each treatment and control groups to measure the initial and final pHs and moisture contents of the soils, before and after testing.

Environmental conditions, during the study were based on conditions in environmental chambers. Soil temperature was measured in each treatment and control group on Days 0 and 14 of the test. The photoperiod during the test was 24 hr continuous overhead light. Light intensity was measured once.

Reference toxicity test, was conducted under a separate protocol to determine the LC50 value for earthworms exposed to a reference toxicant, chloroacetamide, in the soil. The test was conducted using worms from the same source and under conditions similar to those in the test with Cry1F *Bacillus thuringiensis* var. *aizawai* delta endotoxin in order to monitor the techniques used and the sensitivity of the test population. The worms were exposed to chloroacetamide in the soil at concentrations of 7.5, 15, 30 and 60 mg a.i./kg dry soil.

REPORTED RESULTS/CONCLUSION: There were no apparent treatment-related effects on mortality or body weight of worms in the test. At the end of the 14-day study, 3 (of 40 initially) worms died in the control replicates; only 1 worm died in test soil. All other control worms were normal in appearance and behavior throughout the test. Surviving worms showed no signs of behavioral abnormalities on Days 0, 7, or 14. No aversion to the test soil was observed on Day 0 or 7. Body weights were averaged from Day 0 and Day 7 measurements for individual worms. When compared to the control group, there were no apparent treatment-related effects upon body weight in the 1.7 mg Cry1F/kg treatment group. Both the control and the treatment group lost an average of 0.04 g per worm over the course of the 14-day test. The NOEC value was determined

to be equal to 1.7 mg Cry1F/kg dry soil and LC_{50} value was determined to be greater than the test concentration of 1.7 mg Cry1F/kg dry soil. [Actually 2.26 mg (Reviewer's comment: 33% moisture content appears to have been subtracted twice to obtain the 1.7 mg figure)]

The 14-day LC_{50} value for earthworms exposed to chloroacetamide in an artificial soil substrate was determined to be approximately 15.7 mg a.i./kg dry soil, with a 95% confidence interval of 7.5 to 30 mg a.i./kg soil. These results are consistent with those observed in previous studies, and verify the adequacy and consistency of the methods used in the present test concerning B.t. Cry1F delta endotoxin.

Biological analysis of the purified maize-expressed Cry1F protein, the bacterially derived Cry1F and maize pollen test substances demonstrated that the Cry1F protein present in all test substances was active against the European corn borer (ECB) at all time points tested. The LC_{50} value for purified Maize-expressed Cry1F protein (1568-022B) was reported less than 0.03 μg Cry1F/mL diet; and the LC_{50} value for Microbial Cry 1F powder (101788) was reported in the range of 0.06 to < 0.02 μg Cry1F/mL of diet.

REVIEWER'S COMMENTS:

A. **Test Procedures:** The procedures used follow those outlined in the Organization for Economic Cooperation and Development (OECD) Guideline No. 207, *Guideline for Testing of Chemicals, Earthworm, Acute Toxicity Tests.* The study was conducted in compliance with Good Laboratory Practice Standards as published in 40 CFR Part 160 by the U.S. EPA; ENV/MC/CHEM (98)17, Paris 1998; and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, with the following exceptions:

1) Verification of the test concentrations, stability and homogeneity of the test substance in the soil were not determined.

2) The stability of the test substance under storage conditions at the test site was not conducted in accordance with GLPs.

B. **Statistical Analysis:** The LC_{50} value could not be statistically defined because only one concentration was tested. The non-mortality and no-observed-effect concentrations were determined using a visual examination of the mortality and clinical observation data.

C. **Discussion/Risk Assessment:** The one limit test concentration of 2.26 mg Cry1F/kg dry soil represented up to 100X the estimated concentration present in the top six inches of an acre of soil following the incorporation of 25,000 senescent corn plants. This concentration is higher than any amount of Cry protein that may be present in the soil during any stage of the growing season (such as from root exudation). Based on the results of this study, it is not likely that Cry1F transgenic corn plantings will have adverse effects on earthworms.

Nota Bene: The supplemental MRID (453078-04) reports data (non-GLP) different from that of the original submission (MRID 45020106) and is not evaluated further in the present record. The supplemental submission is incomplete to justify additional review at this time.

D. **Adequacy of the Study:**

1. Validation Category: Acceptable (Supplemental).

2. Rationale: Although the study was conducted according to GLP standards, the report is not in total compliance with GLP test material analysis requirements.

Reviewer: Gail Tomimatsu, Ph.D. Date: _____
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Secondary Reviewer: Zigfridas Vaituzis, Ph.D. Date: 2/14/2001
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DATA EVALUATION REPORT

STUDY TYPE: Nontarget Aquatic Invertebrate Testing (OPPTS Series 885.4240)

CITATION: Drottar, K. R., and H. O. Krueger (1999) Bt Cry1F Delta-Endotoxin: A 48-Hr Static Renewal Acute Toxicity Test with the Cladoceran (*Daphnia magna*) Using Bacterially-Expressed Bt Cry 1F Delta-Endotoxin and Pollen from Maize Expressing Bt Cry 1F. Wildlife International Ltd., 8598 Commerce Drive, Easton, MD 21601. Wildlife International Ltd. Project Number 354A-111, September 3, 1999 MRID 450201-08.

DP BARCODE: D263758 **CASE**: 066174
REG./FILE#:068467-E **CHEMICAL/BIOL#**: 006481 *Bacillus thuringiensis* CRY1F protein

COMPANY/SPONSOR: Mycogen c/o Dow AgroSciences LLC Corporation, 5501 Oberlin Dr., San Diego, CA 92121

TEST MATERIALS: Bacterially Expressed Bt Cry 1F Delta Endotoxin, and Maize Pollen containing Bt Cry1F

REVIEW CONCLUSION: This study was conducted according to approved EPA guideline procedures. The 48-hr EC50 for *Daphnia magna* exposed to Bt Cry 1F delta-endotoxin was > 100 mg a.i./L. The no-mortality concentration and NOEC were estimated to be >100 mg a.i./L. There were no overt signs of toxicity to daphnids exposed to 100 mg *Bt*-pollen/L - (maize pollen containing the *Bt* Cry1F delta-endotoxin). These data show that there will be no adverse effects on daphnia from incidental field exposure to transgenic corn pollen containing Cry1F.

ADEQUACY OF STUDY: Validation category: Acceptable. This study meets EPA Guideline requirements for assessing acute pesticidal risks to *Daphnia*, a highly sensitive representative of aquatic invertebrate species.

MATERIALS & METHODS: The methods used in this study are based upon procedures specified in U.S. Environmental Protection Agency Series 72 of the Pesticide Assessment Guidelines. *FIFRA Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms*; Series 154 of the U.S. Environmental Protection Agency's FIFRA Subdivision M, *Hazard Evaluation, Biorational Pesticides; OECD Guideline for Testing of Chemicals*, 202: FIFRA Subdivision E, Series 72-2, and FIFRA Subdivision M, Series 154-9 of the U.S. Environmental Protection Agency's Registration Guidelines, Pesticide Assessment Guidelines, and upon OECD Guideline 202.

The test materials, included bacterially expressed *Bacillus thuringiensis* (Bt) Cry 1F delta-endotoxin, maize pollen expressing the endotoxin and maize pollen without the delta endotoxin. Bacterially-expressed Bt Cry1F delta-endotoxin was produced by a recombinant strain of *Pseudomonas fluorescens* and was identified as Cry 1F Microbial Protein (TR), from lot number 1599-45, TSN 101788 with 11.4% Bt Cry 1F delta-endotoxin. This microbial test substance was stored in a refrigerator at 4° C. Maize pollen expressing (Bt) Cry 1F delta-endotoxin was yellow in appearance, and identified as Bt Cry 1F maize pollen (TC 1507). The Sponsor indicated a purity of 32 ng/mg Bt. Bt maize pollen was stored in a freezer at approximately -80° C. An assay control substance was a yellow pollen from maize lacking the Cry 1F delta-endotoxin from lot number 5XH751. Maize control pollen was stored in a freezer at approximately -80 °C .

The test organisms, *Daphnia magna*, a freshwater aquatic invertebrate, were used for this study. Neonates were less than 24-hr old and were obtained from cultures maintained by Wildlife International Ltd., Easton, MD. Taxonomic identification of the original brood stock was verified by The Academy of Natural Sciences, Philadelphia, PA. Juveniles were obtained from adult daphnids and held for at least 16 days in cultures; adults showed no signs of disease or stress during this holding period. Neonate daphnids were obtained from 8 adult daphnids which were observed to have no neonates present less than 24 hours prior to test initiation. At test initiation (30 min after start of aeration), test daphnids were collected and indiscriminately transferred to 10-mL glass beakers. The daphnids then were transferred from the beakers to the test chambers. Daphnids in the cultures were fed a mixture of yeast, and trout chow, and a suspension of *Selenastrum capricornutum*, a freshwater green alga. Neonates were not fed during the test. Water temperatures ranged from 20.0 to 20.3 °C. The pH of the water ranged from 8.3 to 8.5 and dissolved oxygen ranged from 8.3 to 8.8 mg/l.

The test chambers consisted of 600-mL glass beakers containing 300 mL of test solution. The depth of test solution in a representative test chamber was 5.5 cm. Test chambers were indiscriminately placed in a temperature-controlled environmental chamber set to maintain a temperature of 20±1 °C. Test chambers were aerated throughout the exposure period. For the 2 test and 2 control groups, three replicate test chambers with 10 neonate daphnids per chamber were established; a total of 30 neonate daphnids were tested for each group.

Environmental conditions and measurements. Lighting was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone®). The photoperiod was set at 16 hr of light and 8 hr of dark, controlled with an automatic timer. To avoid sudden changes in lighting, a 30 minute transition period of low light intensity was provided when lights went on and off. The light intensity was 615 lux at the start of the test and was measured using a SPER Scientific model 840006C light meter. Temperature was measured in each test chamber at the beginning and end of the test and prior to and after the renewal (old and new solutions) using a liquid-in-glass thermometer. Temperature also was measured continuously in a negative control replicate using a Fulscope ER/C Recorder. The target test temperature during the study was 20 ± 1 °C. At approximately 24 hrs (prior to and after the renewal) and at the beginning and end of the test, dissolved oxygen and pH were measured using a Yellow Springs Instrument Model 51B dissolved oxygen meter and a Fisher Accumet Model 915 pH meter, respectively. Specific conductance was measured in the dilution water at test initiation using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Hardness, alkalinity measurements were made by titration (*Standard Methods for the Examination of Water and Wastewater*) in the dilution water at test initiation.

The test concentrations, The test substance solutions were prepared by adding 0.263 g of the test substance to 300 mL of dilution water in the test chambers. The nominal test concentration was 100 mg a.i./L. The Bt Cry 1F maize pollen solutions were prepared by adding 0.03 g of pollen to 300 mL of water in the test chambers. The control pollen solutions were prepared by adding 0.03 g of the maize pollen to 300 mL of dilution water in the test chambers. At test initiation and termination of the test, the pollen treatments appeared to have yellow particles suspended throughout the water column and on the bottom of the test chambers.

The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Wildlife International Ltd. site. The well water is characterized as moderately-hard water. The well water was passed through a sand filter to remove particles greater than approximately 25μm, and pumped into a 37,800-L storage tank where the water was aerated. The water was filtered again (0.45 μm) to remove microorganisms and particles just prior to use.

REPORTED RESULTS: There were no mortalities or abnormalities noted in the negative control (0 mg pollen/L), nor in the test solutions containing pollen with Bt Cry1F delta endotoxin or pollen containing no delta endotoxin after 48 hrs. The group containing 100 mg Bt Cry 1F delta-endotoxin/L, reported 23% mortality with 3 lethargic animals.

Temperatures remained within the 20 ± 1 °C range, established for the test. Measurements of pH ranged from 7.6 to 8.5. Dissolved oxygen concentrations in the negative control, pollen control and 100 mg Bt Cry 1F delta-endotoxin pollen/L treatment groups remained ≥ 8.6 mg/L (95% of saturation). Dissolved oxygen concentrations in the 100 mg a.i./L treatment group dropped as low as 2.1 mg/L (23% of saturation) after 24 hrs of exposure. Gentle aeration was supplied to each test chamber throughout the exposure, however the dissolved oxygen concentrations could not be maintained at acceptable levels in the 100 mg a.i./L treatment group (bacterial Bt Cry 1F delta-endotoxin) due to the high oxygen demand of the test substance.

DISCUSSION: This study was conducted according to approved EPA guideline procedures for acute toxicity testing of pesticidal substances to freshwater aquatic invertebrates. The 48-hr EC50 for *Daphnia magna* exposed to Bt Cry 1F delta-endotoxin (microbial test substance) was > 100 mg a.i./L. The mortality which was reported (23%) may have been due to the low dissolved oxygen concentrations: DO levels were reported higher after 48 hrs (5.2-5.5 mg/L).The no-mortality concentration and NOEC (of the test substance, microbial Cry1F) were estimated to be >100 mg a.i./L.

REVIEWER'S COMMENTS:

A. **Test Procedures:** The procedures used follow those recommended by EPA Pesticide Testing Guidelines for Microbial and Biochemical Pest Control Agents, Subdivision M..

B. **Statistical Analysis:** The LC_{50} value could not be statistically defined because only one concentration was tested. The no-mortality and no-observed-effect concentrations were determined by visual examination of the mortality and clinical observation data.

C. **Discussion/Risk Assessment:** The study is scientifically sound; there were no overt signs of toxicity to daphnids exposed to 100 mg pollen/L -containing the Bt Cry1F delta-endotoxin. These

data show that there will be no adverse effects on daphnia from incidental field exposure to transgenic corn pollen containing Cry1F protein.

D. **Adequacy of the Study:**

1. Validation Category: Acceptable.

2. Rationale: This study meets EPA Guideline requirements for assessing acute pesticidal risks to *Daphnia*, a representative aquatic invertebrate species.

Reviewer: Gail Tomimatsu, Ph.D. Date:
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Secondary Reviewer: Zigfridas Vaituzis, Ph.D. Date: 2/14/2001
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Phil Hutton, Chief
Biopesticides and Pollution Prevention Division (7511C)

DATA EVALUATION REPORT

STUDY TYPE: Nontarget Avian Testing (OPPTS Series 885.4050)

CITATION: Gallagher, S.P., J. Grimes, and J.B. Beavers (1999) Transgenic Corn Expressing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry1F Delta-Endotoxin: A Dietary Toxicity Study with the Northern Bobwhite. Wildlife International Ltd., 8598 Commerce Drive, Easton, MD 21601. Wildlife International Ltd. Project Number 354-116, December 14, 1999. MRID 450201-12.

DP BARCODE: D263758 **CASE**: 066174
REG./FILE#:068467-E **CHEMICAL/BIOL#**: 006481 *Bacillus thuringiensis* CRY1F protein

COMPANY/SPONSOR: Mycogen c/o Dow AgroSciences LLC Corporation, 5501 Oberlin Dr., San Diego, CA 92121

TEST MATERIAL: Genetically modified corn line expressing *Bacillus thuringiensis* var. *aizawai* (Bt) Cry1F delta-endotoxin

REVIEW CONCLUSION: The dietary LC_{50} value for corn grain (meal) expressing *Bacillus thuringiensis* var. *aizawai* protein in corn grain when fed to juvenile northern bobwhite for 5 days was determined to be greater than 100,000 ppm (10% corn meal), the only concentration tested. The no-observed-effect concentration was also 100,000 ppm. These data show that there will be no adverse effects on avian wildlife from incidental field exposure to Cry1F corn. These data are, however, not sufficient to make a hazard assessment from repeated exposure(s) to higher doses of Bt corn.

ADEQUACY OF STUDY:

Validation Category: Supplemental.

Rationale: The study is insufficient to assess hazards to avian species which may be exposed continuously to high levels of Cry1F in domestic poultry feed (which normally contain 60 to 70% corn).

MATERIALS & METHODS: The methods used in this study are based upon procedures specified in Section 71-2 of the U.S. Environmental Protection Agency's Registration Guidelines, Pesticide Assessment Guidelines, FIFRA Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms and upon ASTM Standard E857-87, "Standard Practice for Conducting Subacute Dietary Toxicity Tests with Avian Species".

The test material, ground corn seed from a corn line genetically modified to express *Bacillus thuringiensis* var. *aizawai* (Bt) Cry1F delta-endotoxin to provide protection against certain insect pests. The test substance was identified as Transgenic Corn Seed (Cry1F TR) TSN 101791; Lot No. 2722; RL0016. A summary of the GLP characterization of the test substance was included in the study.

The control substance was ground corn grain from a corn line that is genetically similar to the test substance. The control material was identified as: Control Substance Corn Seed TSN 101792; Lot No. 2722. Test and control materials were stored frozen at approximately -20 °C .

The test organisms, juvenile northern bobwhite (~10 days old) and appeared to be in good health at initiation of the test. The birds were obtained from Wildlife International, Ltd. Production Flock, Easton, MD and were hatched on July 12, 1999. Birds ranged in weight from 18 to 23 g at test initiation. All birds were from the same hatch, pen-reared and phenotypically indistinguishable from wild birds. All birds were acclimated to the caging and facilities from the day of hatch until initiation of the test.

The test chambers were brooding pens manufactured by Beacon Steel Products Co. (Model No. B735Q) ; external walls, ceilings and floors were composed of galvanized steel wire and sheeting. Each pen had floor space that measured approximately 72 X 90 cm with a ceiling height of approximately 23 cm. Birds were assigned to 2 experimental groups. Each group contained 30 chicks. The immature birds, without gender differentiation characteristics were housed in brooding pens containing 5 birds each.

The test diets were based on a game bird ration formulated to Wildlife International, Ltd.'s specifications throughout acclimation and testing. The birds were given a vitamin supplement in their water from the day they were hatched until the initiation of the test. Water and feed were provided ad libitum during acclimation and during the test. The birds did not receive any antibiotic medication during acclimation or during the test.

Test diets were prepared by incorporating the appropriate test and control substances directly into basal ration using a Hobart (Model Number AS200T) mixer to obtain a 10% concentration of the test substance. An amount of diet sufficient to last the five-day exposure period was prepared on the day of the test initiation and stored frozen. The birds were presented a portion of the diet daily during the exposure period. Samples of diets for the test and control substances were collected at Days 0 and 5. One verification sample was collected from each diet at preparation on Day 0. At the end of the exposure period (Day 5), one sample was collected from each treatment group diet. The day 5 samples were collected from feed composited by treatment group remaining in the feeders.

The test duration consisted of a 5 day exposure period followed by a 3 day post-exposure observation period.

REPORTED RESULTS: There were no mortalities or overt signs of toxicity in the genetically modified corn line tested. One bird was noted limping and with foot lesions on Day 8 of the test; all other birds in this group were normal in appearance and behavior for the duration of the test. There were no mortalities in the non-genetically modified parental corn line control group. Two birds in the control group suffered foot lesions as a result of penmate aggression during the course of the test. Several other birds exhibited abnormal symptoms; these were attributed to penmate aggression, and not to dietary consumption. The dietary LC_{50} for corn grain (meal) expressing

Bacillus thuringiensis var. *aizawai* protein in corn pollen when fed to juvenile northern bobwhite for 5 days was determined to be greater than 100,000 ppm (10% of diet), the only concentration tested. The no-observed-effect concentration was also 100,000 ppm.

Biochemical analyses by ELISA of the purified maize-expressed Cry1F protein, microbial derived Cry1F protein, maize grain, feeds containing maize grain and maize pollen test substances demonstrated that the Cry1F protein was present in all Cry1F expressed test substances.

DISCUSSION: This study was conducted according to approved EPA guideline procedures. The dietary LC_{50} for Cry1F protein in corn grain when fed to juvenile northern bobwhite for 5 days was determined to be greater than 100,000 ppm diet. The no observed effect concentration was 100,000 ppm Cry1F.

REVIEWER'S COMMENTS:

A. **Test Procedures:** The procedures used follow those recommended by EPA Pesticide Testing Guidelines for Microbial and Biochemical Pest Control Agents, Subdivision M.

B. **Statistical Analysis:** The LC_{50} value could not be statistically defined because only one concentration was tested. The non-mortality and no-observed-effect concentrations were determined using a visual examination of the mortality and clinical observation data.

C. **Discussion/Risk Assessment:** The study is scientifically sound and no treatment mortality or behavior change was observed between the dosed and control replicates. These data show that there will be no adverse effects on avian wildlife from incidental field exposure to Cry1F corn.

D. **Adequacy of the Study:**

1. Validation Category: Supplemental.
2. Rationale: This study meets EPA Guideline requirements for acute toxicity testing of non-target wild birds. However the study is rated as supplemental because the concentration tested (10% corn in the diet) is too low to assess hazards to non-target birds from continuous exposure to high levels of Cry1F protein. A six week study with 60 to 70% corn in the diet is necessary to assess hazards from chronic exposure of wild and domesticated fowl.

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

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

FEB 15 2001

SUBJECT: Review of Petition from the Requirement of a Tolerance for *Bacillus thuringiensis* ssp. *aizawai* Cry1F insect control protein as expressed in all agricultural commodities.

TO: Mike Mendelsohn, Product Manager
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division

FROM: Chris A. Wozniak, Ph.D., Biologist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division

THROUGH: John L. Kough, Ph.D., Biologist
Senior Scientist, Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division

ACTION REQUESTED

To review the Notice of Filing, as to be published in the Federal Register, for the product Cry1F insect control protein from *Bacillus thuringiensis* var *aizawai* strain PS811 (NRRL B-18484), as expressed in maize.

BACKGROUND

Mycogen Corporation and Pioneer Hi-Bred, International have submitted an application for a Section 3 registration to apply their product as a means of European corn borer control. This product is based on the insecticidal protein of *B. thuringiensis* var. *aizawai*, Cry1F, in a truncated and synthetic (modified) form which represents the active ingredient of this plant-pesticide. Maize plants are also intended to express phosphinothricin acetyl transferase for tolerance to the herbicide glufosinate ammonium. Approval of the tolerance exemption will allow the registrant to sell the Cry1F Bt-maize for processing into the food supply and as animal feed.

Temporary Food Tolerance Exemption for Cry1F in Maize

I. Mammalian Toxicity Assessment:

The lack of mammalian toxicity at high levels of exposure demonstrate the safety of the product at levels above possible maximum exposure levels. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-pesticide was derived. [See 40 CFR Sec. 158.740(b).] For microbial products, further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study.

The acute oral toxicity data submitted support the prediction that the Cry1F protein would be non-toxic to humans. Male and female mice (5 of each) were dosed with 15 % (w/v) of the test substance, which consisted of *Bacillus thuringiensis* var. *aizawai* Cry1F δ-endotoxin at a net concentration of 11.4 %. Two doses were administered approximately an hour apart to achieve the dose totaling 33.7 mL / kg body weight. Outward clinical signs and body weights were observed and recorded throughout the 14 day study. Gross necropsies performed at the end of the study indicated no findings of toxicity. No mortality or clinical signs were noted either during the study. An LD_{50} was estimated at >5050 mg / kg body weight of this microbially produced test material. The actual dose administered contained 576 mg Cry1F protein / kg body weight. At this dose, no LD_{50} was demonstrated as no toxicity was observed. Cry1F maize seeds contain 0.0017 to 0.0034 mg of Cry1F / gram of corn kernel tissue.

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels [Sjoblad, Roy D., *et al.* "Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)]. Therefore, since no effects were shown to be caused by the plant-pesticides, even at relatively high dose levels, the Cry1F delta-endotoxin protein is not considered toxic.

II. Effects on the Immune System:

Since Cry1F is a protein, allergenic sensitivities were considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and proteases, may be glycosylated and present at high concentrations in the food. Data has been submitted which demonstrates that the Cry1F delta-endotoxin is rapidly degraded by gastric fluid *in vitro* and is non-glycosylated. In a solution of Cry1F:pepsin at a molar ratio of 1:100, complete degradation of Cry1F to amino acids and small peptides occurred in 5 minutes (MRID# 447148-03). A heat lability study demonstrated the loss of bioactivity of Cry1F protein to neonate tobacco budworm larvae after 30 minutes at 75 °C (MRID# 452748-01). Studies submitted to EPA done in laboratory animals have not indicated any potential for allergic reactions to *B. thuringiensis* or its components, including the δ-endotoxin of the crystal protein. Additionally, a

comparison of amino acid sequences of known allergens uncovered no evidence of any homology with Cry1F, even at the level of 8 contiguous amino acids residues. Despite decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports of immediate or delayed allergic reactions to the delta-endotoxin itself despite significant oral, dermal and inhalation exposure to the microbial product. Several reports under FIFRA § 6(a)2 have been made for various *Bacillus thuringiensis* products claiming allergic reactions. However, the Agency determined these reactions were not due to *Bacillus thuringiensis* itself or any of the Cry toxins. Thus, the potential for the Cry1F protein to be a food allergen is minimal.

Regarding toxicity to the immune system, the acute oral toxicity data submitted support the prediction that the Cry1F protein would be non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels [Sjoblad, Roy D., et al. "Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)]. Therefore, since no effects were shown to be caused by the plant-pesticides, even at relatively high dose levels, the Cry1F delta-endotoxin protein is not considered toxic.

III. Effects on the Endocrine System:

The pesticidal active ingredients are proteins, derived from sources that are not known to exert an influence on the endocrine system. Therefore, the Agency is not requiring information on the endocrine effects of these plant-pesticides at this time.

IV. Dose Response Assessment:

No toxicological endpoints are identified.

V. Dietary Risk Characterization:

A) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the Cry1F protein include information on the characterization of the expressed Cry1F δ-endotoxin in corn, the acute oral toxicity, and *in vitro* digestibility of the δ-endotoxin. The results of these studies were determined applicable to evaluate human risk and the validity, completeness, and reliability of the available data from the studies were considered.

Adequate information was submitted to show that the Cry1F test material derived from microbial cultures were biochemically and, functionally similar to the proteins produced by the plant-

pesticide ingredients in corn. Production of microbially produced protein was chosen in order to obtain sufficient material for testing.

The acute oral toxicity data submitted supports the prediction that the Cry1F protein would be non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels [Sjoblad, Roy D., et al. "Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)]. Therefore, since no effects were shown to be caused by Cry1F δ-endotoxin, even at relatively high dose levels (>5,050 mg test substance / kg body weight; 576 mg Cry1F / kg body weight), the Cry1F delta-endotoxin protein is not considered toxic (MRID# 446911-01). This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-pesticide was derived. [See 40 CFR Sec. 158.740(b).] For microbial products, further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study.

Although Cry1F expression level data was required for an environmental fate and effects assessment, residue chemistry data were not required for a human health effects assessment of the subject plant-pesticide ingredients because of the lack of mammalian toxicity.

Both (1) available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children); and (2) safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food additives, are generally recognized as appropriate for the use of animal experimentation data were not evaluated because the lack of mammalian toxicity at high levels of exposure demonstrate the safety of the product at levels well above possible maximum exposure level.

The genetic material necessary for the production of the plant-pesticides active ingredients are the nucleic acids (DNA, RNA) which comprise (1) genetic material encoding these proteins and (2) their regulatory regions. "Regulatory regions" are the genetic material that control the expression of the genetic material encoding the proteins, such as promoters, terminators, and enhancers. DNA and RNA are common to all forms of plant and animal life and the Agency knows of no instance where these nucleic acids have been associated with toxic effects related to their consumption as a component of food. These ubiquitous nucleic acids, as they appear in the subject active ingredient, have been adequately characterized by the applicant. Therefore, no mammalian toxicity is anticipated from dietary exposure to the genetic material necessary for the production of the subject active plant pesticidal ingredients.

B) Infants and Children Risk Conclusions

FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about

consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408 provides that EPA shall apply an additional tenfold margin of exposure (safety) for infants and children in the case of threshold effects to account for pre- and post-natal toxicity and the completeness of the database unless EPA determines that a different margin of exposure (safety) will be safe for infants and children.

In this instance, based on all the available information, the Agency concludes that infants and children will consume only minimal, if any, residues of this plant-pesticide and that there is a finding of no toxicity.

Thus, there are no threshold effects of concern and, as a result the provision requiring an additional margin of safety does not apply. Further, the provisions of consumption patterns, special susceptibility, and cumulative effects do not apply.

C) Aggregate Exposure (Not Including Occupational Exposure) Risk Conclusions

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-pesticide chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-pesticide is contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Oral exposure, at very low levels, may occur from ingestion of processed corn products and potentially drinking water. However a lack of mammalian toxicity and the digestibility of the plant-pesticides has been demonstrated. Study of the degradation of Cry1F in soil indicated that it is rapidly broken down, hence, contact through drinking water is very unlikely (MRID# 450201-05). The use sites for Cry1F δ-endotoxin are all agricultural for control of lepidopteran insects. Therefore, exposure via residential or lawn use to infants and children is not expected. Even if negligible exposure should occur, the Agency concludes that such exposure would present no risk due to the lack of toxicity demonstrated for Cry1F protein.

D) Occupational Exposure and Risk Characterization:

Exposure via the skin or inhalation is not likely since the plant-pesticides are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. If negligible exposure should occur, the Agency concludes that such exposure would present no risk due to the lack of toxicity.

VI. Cumulative Effects Risk Conclusions:

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

VII. Tolerance Conclusion:

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry1F protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information.

The Agency has arrived at this conclusion because, as discussed above, no toxicity to mammals has been observed for the plant-pesticides. As a result, EPA established an exemption from tolerance requirements pursuant to FFDCA section 408(j)(3) for *Bacillus thuringiensis* Cry1F δ-endotoxin and the genetic material necessary for its production in all plants.

Bacillus thuringiensis subspecies *aizawai* Cry1F δ-endotoxin and the genetic material necessary for its production in all plants are exempt from the requirement of a tolerance when used as plant-pesticides in all plant raw agricultural commodities. ``Genetic material necessary for its production" means the genetic material which comprise (1) genetic material encoding the Cry1F δ-endotoxin and (2) its regulatory regions. ``Regulatory regions" are the genetic material that control the expression of the genetic material encoding the Cry1F δ-endotoxin, such as promoters, terminators, and enhancers.

BPPD Comments:

The current Petition from the Requirement of a Tolerance for *Bacillus thuringiensis* ssp. *aizawai* Cry1F as expressed in maize, as written, is adequate to address the issues prescribed by FQPA. The non-toxic nature of the Cry1F protein, lack of evidence of allergenicity, gastric digestibility, thermolability and the small amounts of protein present in harvested grain all indicate that no harm is anticipated from consumption of Cry1F maize and products derived from it.

13544

R139631

Chemical: Bacillus thuringiensis Cry 1F protein and the genetic material necessary for its production (plasmid insert PHI 8999)in corn

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HED File Code: 41400 BPPD IRM
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