000524-00528

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

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

Subject: Insect Resistance Management Assessment for the registration of Monsanto Company's Cry3Bb1 protein expressed in event MON 863 field corn.

To: Mike Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division, 7511C

From: Robyn Rose, Entomologist Robyn Rose 2/20/03
Biopesticides and Pollution Prevention Division, 7511C

Thru: Phil Hutton, Chief
Microbial Pesticides Branch PH 2/21/03
Biopesticides and Pollution Prevention Division, 7511C

RECOMMENDATIONS

1. A 20% non-Bt corn refuge should be planted adjacent to or within MON 863 corn fields during the interim three year registration requested by Monsanto. If row strips within a corn field are implemented, then at least 6, and preferably 12 consecutive rows should be planted. Refuge acres may be treated to control CRW larvae with chemical insecticides, but insecticides should not be used on refuges to control CRW adults. Alternate hosts and seed mixes should not be utilized as refuges for CRW in MON 863 corn.
2. If growers spray their corn fields with insecticides to control pests other than CRW, then all acres (Bt and non-Bt) should be treated identically. Bt fields and the non-Bt refuge acres should be treated with identical agronomic practices such as irrigating all corn (Bt and non-Bt) at the same time. To ensure the production of similar numbers of CRW, Bt and non-Bt corn should be planted in fields with similar backgrounds.
3. Additional information is needed on various aspects of CRW pest biology as it relates to a long-term IRM strategy. More information is needed on adult and larval movement and dispersal, mating habits, ovipositional patterns, number of times a female can mate and fecundity. Additional data outlined in the pest biology section and conclusion sections below are needed to confirm the appropriateness of Monsanto's proposed IRM strategy for MON 863 field corn. All current and future research that addresses CRW biology and MON 863 IRM should be submitted to EPA.
4. Additional research is needed to determine if IRM strategies designed for WCRW and

7

NCRW are appropriate for MCRW

5. The mechanism of potential resistance of CRW to MON 863 should be determined to develop an appropriate long-term IRM strategy. Resistant colonies of WCRW, NCRW and MCRW should be developed to aid in determining selection intensity.
6. The effect of WCRW ovipositing in soybean prior to overwintering and extended diapause in NCRW on an IRM strategy needs further investigation.
7. Detailed summaries of the four data-sets identified in Monsanto's December 13, 2002 letter should be submitted to the Agency in confirmation of the conclusion that the initial resistance allele frequency is ≤.01.
8. A long-term resistance monitoring strategy for CRW-protected Bt corn is needed to test the effectiveness of resistance management programs. Baseline susceptibility studies currently underway should be continued for WCRW and initiated for NCRW and monitoring techniques such as discriminating dose concentration assays need to be thoroughly investigated for their feasibility as resistance monitoring tools.
9. Expected and confirmed resistance should be determined by the methods outlined in the monitoring section below. A remedial action plan is also necessary. If resistance is confirmed, all acres (Bt fields and non-Bt refuges) should be treated with insecticides targeted at CRW adults as well as larvae.
10. Growers should be required to sign a technology use agreement that outlines IRM requirements and acknowledges the growers responsibility to comply with them on an annual basis. The agreement should also state that growers received the Product Use Guide.

BACKGROUND

Monsanto Co. has requested a registration from EPA for *Bacillus thuringiensis* (Bt) Cry3Bb1 protein and the genetic material (Vector ZMIR13L) necessary for its production in event MON 863 field corn. MON 863 field corn provides protection against corn rootworm (CRW) species including the western corn rootworm (*Diabrotica virgifera virgifera*), northern corn rootworm (*Diabrotica barberi*) and Mexican corn rootworm (*Diabrotica virgifera zea*). In support of a MON 863 registration, Monsanto submitted a three year interim insect resistance management (IRM) plan dated January 8, 2002 and received by EPA on January 9, 2002 (MRID No 455770-01). A detailed preliminary review of Monsanto's IRM plan was documented by EPA in a memorandum from Robyn Rose to Mike Mendelsohn dated July 23, 2002.

Subsequent to EPA's review of Monsanto's IRM plan, a FIFRA Scientific Advisory Panel (SAP) was convened in August 2002. The August 2002 SAP comments regarding Monsanto's interim IRM plan were documented in a memorandum from Paul Lewis to Marcia Mulkey dated November 6, 2002 (http://www.epa.gov/scipoly/sap/2002/August/august2002final.pdf). In response to the SAP, Monsanto submitted additional information to EPA in a document from Dennis Ward to Janet Andersen dated December 13, 2002. The supplemental information provided by Monsanto supports a 20% non-Bt corn refuge planted adjacent to, or within MON 863 corn fields. Comments and recommendations made by the August 2002 SAP as well as the

additional information submitted by Monsanto are incorporated into the discussion section below. This review is intended to supplement the July 23, 2002 memorandum from Robyn Rose to Mike Mendelsohn.

DISCUSSION

Pest Biology

In order to develop an appropriate IRM strategy for MON 863 corn, as well as all insect-protected transgenic crops, it is important to consider the biology of the target pest. Knowledge of pest biology is imperative in determining optimal size and placement of refuges that will encourage random mating between Bt and non-Bt corn. Based on the movement of CRW adults, a non-Bt corn refuge should be planted adjacent to or within MON 863 fields.

Additional information is needed on various aspects of CRW pest biology as it relates to a long-term IRM strategy. Characteristics of pest biology that are relevant to IRM (e.g., movement, feeding habits and ovipositional habits) differ for WCRW and NCRW; therefore, additional information on the biology of the WCRW and NCRW are needed. According the August 2002 SAP, the NCRW and MCRW are subspecies, therefore, a lot of the data collected on biology will relate to both species. However, the Panel concluded that data on adult mating behavior, male and female migration, and reproductive biology and fecundity of females is needed to determine if the IRM plan is suitable for MCRW. Although the SAP concluded that the same IRM strategy may be appropriate for the WCRW and NCRW, the Panel recommended additional research on the NCRW and the SAP suggested collecting data from several geographic locations of the WCRW. There are behavioral differences in WCRW populations from the western and eastern regions of their distribution so studies on aspects of pest biology such as movement should be conducted in several areas. Since the biology of the SCRW is very different from the other *Diabrotica* spp. and it is not adequately controlled by MON 863, the SCRW should not be considered.

Knowledge of the maximum and average distance adult and larval CRW move is limited. In previous submissions (MRID Nos. 453484-01 and 455770-01), Monsanto listed and summarized studies currently underway that relate to the biology of CRW (Table 1). Results of the these studies should be submitted to EPA after they are completed. Additional research regarding male and female (mated and unmated) adult and larval WCRW and NCRW dispersal potential is needed to determine placement of non-Bt corn refuges. In addition, more information is needed on mating habits, ovipositional patterns, number of times a female can mate and fecundity. The effect of WCRW ovipositing in soybean prior to overwintering and extended diapause in NCRW on an IRM strategy also needs further investigation.

The August 2002 SAP identified several areas of additional research needed to fully understand CRW biology as it relates to an IRM strategy. The SAP concluded that male and female adult movement and fitness in MON 863 and non-transgenic corn should be evaluated in large-scale

field studies. Data needed on movement include, but are not limited to, the distance males and females will move over time and the rate adults leave the natal field. The SAP also recommended an evaluation of "the impact of adult density on migration patterns of adults, whether a delay in male emergence from MON 863 affects male fitness and lowers their chances of mating, and whether there are sublethal effects of MON 863 on female fecundity, offspring quality and other fitness parameters." Data are also needed on the movement of NCRW male and female adults since little is currently known.

The NCR-46 (a technical committee consisting of research and extension CRW specialists and other cooperators) submitted a letter dated May 29, 2001 to the EPA that outlines additional CRW biology research. The August 2002 SAP recommended that the EPA consider the recommendations made by the NCR-46.

Table 1. List of pest biology research currently underway sponsored by Monsanto identified in MRID Nos. 453484-01 and 455770-01

Study Title	Author(s)
Post-establishment movement of western corn rootworm larvae - three years of data under Missouri conditions	Bruce E. Hibbard
Tracking western corn rootworm premating movement	Doug Tallamy
An economic model of CRW protected transgenic corn IRM strategies	John Foster
Flight characteristics of female western corn rootworms exposed to transgenic plants	Jon Tollefson & Ted Wilson
An evaluation of CRW dispersal and mating behavior	Joseph Spencer
Larval feeding behavior of *Diabrotica virgifera virgifera*: progress report	Pete Clark & John Foster

Dose

Identifying the level of dose, as related to selection intensity, is crucial when determining size and structure of a refuge needed to delay CRW resistance to MON 863 corn. CRW feeding behavior and survival and root expression data can be used to identify the dose of MON 863. From data currently available it can be concluded that MON 863 corn does not provide a high dose for CRW control (Table 2). The August 2002 SAP suggested that it is not necessary to determine the difference between a low an moderate dose. It is adequate to differentiate between high dose and non-high dose products when determining effective refuge size. Therefore, MON 863 should be characterized as a non-high dose product.

According to the August 2002 SAP, comparing measures of fitness levels of susceptible homozygotes on MON 863 and non-Bt corn would provide a good approximation of selection

5

intensity. The SAP suggested that the first step in approximating selection intensity would be to measure efficacy of MON 863 corn against CRW larvae. However, the Panel pointed out that selection intensity based on larval efficacy may be underestimated if sublethal effects or fitness costs occur. Selection intensity based on larval survival may also be underestimated if density dependent mortality is occurring. Resistant colonies of CRW should be developed to aid in determining selection intensity.

The SAP based their determination that MON 863 is a non-high dose product on the *SS* (homozygous susceptible) survival rate. The Panel also concluded that Monsanto's artificial diet assays had deficiencies, but were adequate to determine the LC_{50} for first instar larvae, level of larval resistance and dose.

Table 2. Research to identify MON 863 dose currently underway sponsored by Monsanto identified in MRID Nos. 453484-01 and 455770-01

Study Title	Author(s)
Impact of transgenic corn rootworm protected corn on selected western corn rootworm life history traits	Lance Meinke
Efficacy of Monsanto's lead transgenic events resistant to corn rootworm larvae feeding: root damage ratings and adult emergence	Jon Tollefson *et al.*
An evaluation of Cry3Bb expression and impact on western corn rootworm from transgenic events	Vaughn *et al.*
Efficacy of advanced transgenic corn rootworm events against CRW pest species - results of year 2000 & 2001 trials	DeGooyer
1999 evaluation of Monsanto transgenic corn germplasm for corn rootworm larval feeding resistance and adult emergence	Hibbard
Impact of CRW-protected corn on silk clipping & beetle numbers	Davis, Pilcher & Spangler

Simulation Models of Resistance

In Monsanto's three year interim IRM plan, they requested planting a 20% non-Bt corn refuge to delay the potential of CRW resistance to Cry3Bb1. Monsanto's conclusion that a 20% refuge would be adequate to delay resistance to MON 863 corn was based on CRW biology, Cry3Bb1 effective dose, preliminary modeling results and agronomic considerations (Table 3). It was concluded in the July 23, 2002 memorandum from Robyn Rose to Mike Mendelsohn that a 20% non-Bt corn refuge planted within or adjacent to MON 863 corn fields is expected to adequately delay the risk of CRW developing resistance to Cry 3Bb1. Monsanto's IRM interim plan and EPA's review of Monsanto's plan were addressed by the August 2002 SAP in the transmittal document from Paul Lewis to Marcia Mulkey dated November 6, 2002.

6

Table 3. Models identified by Monsanto in MRID Nos. 455770-01

Study Title	Author(s)
Efficacy of between field refuges for resistance management of transgenic corn rootworm-insecticidal corn	David Andow & Donald Alstad
Modeling the dynamics of adaptation to transgenic corn by western corn rootworm	David Onstad
An economic model of CRW protected transgenic corn IRM strategies	Terry Hurley
Monsanto's modified version of Caprio's deterministic nonrandom mating model described in the International Life Sciences Institute Health and Environmental Sciences Institute (ILSI-HESI) report (1998)	Monsanto

According to the SAP, the current models (Monsanto's modified Caprio model, Onstad *et al.* 2001 and Andow and Alstad 2002) show that the time to resistance does not substantially differ when the refuge size ranges from 10-25%; however, a 50% refuge would be a conservative approach since resistance would be delayed substantially longer. The SAP also stated that the amount gene frequency increases during an interim period is of greater importance than years to resistance because of the potential future impact on IRM. Since MON 863 is a non-high dose product, the Panel suggested that the potential for herilable quantitative variation and rapid evolution of resistance should be considered. In addition, the models only consider monogenic (single locus) resistance, but the SAP suggested that the models consider the potential for polygenic resistance in a non-high dose product.

Additional comments were made by the Panel regarding initial resistance allele frequency. Each of the models (Andow *et al.*, Onstad *et al.*, Monsanto's modified Caprio model) submitted in support of Monsanto's IRM plan designated the initial resistance allele frequency as .001. However, the Panel suggested that the initial resistance allele frequency may be as low as 0.1 in a non-high dose product. Therefore, the Panel recommended that studies be conducted to determine if the initial resistance allele frequency is less than .01 and models should be run that investigate the full range of dominance values.

Monsanto responded to the August 2002 SAP in a ~~submission~~ letter from Dennis P. Ward (Monsanto Regulatory Affairs Manager) to Janet L. Andersen (Director, EPA's Biopesticides and Pollution Prevention Division) dated December 13, 2002. In the December 13 ~~submission~~ letter, Monsanto summarized results from four data sets from research they sponsored on the efficacy of MON 863. The first and third data sets consisted of field data collected from 1999 to 2002 by 22 scientists from 15 universities located in 15 states. The second data set included data collected by Dr. Bruce Hibbard (University of Missouri) and the fourth data set is from research conducted by Dr. Blair Seigfried (University of Nebraska). According to Monsanto, results of these four data sets demonstrate that the initial allele frequency is ≤.01. Detailed summaries of these four

7

data sets should be submitted to the Agency for confirmation.

The first data set looked at 7500 corn plants artificially infested with ≥1200 CRW eggs/plant from naturally occurring populations. If the initial resistance allele frequency is 0.01 and Hardy-Weinberg is assumed, then 24 CRW/plant ($24 = 1200*(1-(1-0.01)^2)$ would be resistant and the damage rating on the Iowa scale would be 3.1. Weiss *et al.* (1985) showed that <20 CRW = 3.1 on the Iowa scale. Since the average damage recorded in the first data set was 1.6, it can be concluded that the initial resistance allele frequency is ≤0.01.

The second data set summarized by Monsanto evaluated larval survival. In this study, ≥30 larvae were recovered per non-Bt corn plant at a wide range of egg infestation rates. If the initial resistance allele frequency is 0.01 and Hardy-Weinberg is assumed, then 0.6 resistant larvae ($0.6 = 30*(1-(1-0.01)^2)$ would occur per MON 863 corn plant. Since an average of 0.7 larvae were recovered (but not feeding normally), a ≤.01 initial resistance allele frequency can be assumed.

The third data set evaluated the number of surviving adult CRW. This data set includes several studies that infest corn plants with over 1200 eggs. Of the 1200 eggs, an average of 30 adults survived on non-transgenic corn. If the initial resistance allele frequency is 0.01 and Hardy-Weinberg is assumed, then 0.6 resistant adults ($0.6 = 30*(1-(1-0.01)^2)$ would occur per MON 863 plant and the damage rating on the Iowa scale would equal 3. Since damage averages 1.6 on the Iowa scale, a ≤.01 initial resistance allele frequency can be assumed.

The final data set (#4) examined 11 field collected adult female CRW populations reared in the lab. Between 134 and 489 larvae per population were examined for susceptibility to Cry3Bb1. These larvae demonstrated less than 6-fold difference between the most and least susceptible populations similar to populations of European corn borer and corn earworm in their susceptibilities to Cry1Ac and Cry1Ab. If the initial resistance allele frequency is 0.01 and Hardy-Weinberg is assumed, then 2% (>20) of the larvae assayed would be resistant. Monsanto asserts that since no putatively resistant large larvae were recovered at high doses which suggests no larvae survived and there was low variation (lower than with lepidopterans); therefore, a ≤.01 initial resistance allele frequency can be assumed.

Products with a resistance allele frequency ≥.01 would not have enough efficacy to justify commercialization (Bourguet *et al.* 2002, Ferre' and Van Rie 2002). If the initial resistance allele frequency were 0.1, then the efficacy of the MON 863 corn would be so poor that it would not be a marketable product. At a 0.1 initial resistance allele frequency, damage would be less than 4.6 on the Iowa root rating scale and 0.01 would result in a 3 Iowa rating. The economic threshold in corn is a 3 on the Iowa rating scale. Monsanto has demonstrated that the average damage rating is 1.6. Since MON 863 provides enough protection to result in much less than a 3 root rating, it can be concluded that the initial resistance allele frequency is ≤.01.

Monsanto modified Caprio's model to include an initial resistance allele frequency of .01 and submitted these results in their December 13, 2002 letter to Janet Andersen. Results of running

8

this model showed that a 20% refuge would delay resistance for approximately 16 years (see Fig 2 on page 13 and Fig 3 on page 14 of Monsanto's December 13, 2002 submission). For this model, SS survival was set at 0.5 and RS survival set at 0.8 which is partial dominance. Based on data collected by Monsanto and cooperators, MON 863 has been shown to control an average of 50% of the homozygous susceptible (SS) CRW. Therefore, the SS survival was designated 0.5 in the modified Caprio model.

According to Monsanto RS survival (dominance) probably equals 0.7 (communication during a 12/18/02 meeting). Therefore, basing dominance on ≥0.8 would be considered a very conservative approach. Monsanto modeled RS survival to range from 0.5 to 1. If a RS survival of 1 (absolute worst case) were to occur and the initial resistance allele frequency is assumed to be .01, then resistance would be delayed for approximately 13 years with a 20% refuge (Fig 1 on page 8 of Monsanto's December 13, 2002 submission). If RS survival is designated 0.8, then resistance will occur in approximately 16 years. According to Dr. David Andow (University of Minnesota), RS survival ranges between 0.3 and 0.8 (personal communication with Andow on 12/23/02). Therefore, a likely case assessment would be to designate RS as 0.8 which suggests that 80% of the heterozygotes survive.

Monsanto also provided the Agency with additional runs of the modified Caprio model that included consevative parameters representing a worst case scenario. These additional models included initial resistance allele frequencies of .01 and .001, RS dominance values of 0.7 and 0.8 and SS survival ranging from 0.1 to 0.8. Results of the model incorporating these conservative input parameters (e.g., initial allele frequency = .01; RS dominance value = 0.8; SS survival = 0.1) suggested that CRW resistance to Cry3Bb1 will not occur for at least seven years assuming 100% MON 863 market penetration and 100% IRM compliance (Table 4).

Table 4. Predictions for MON 863 durability

SS Survival	RS Dominance	Allele Frequency = .01	Allele Frequency = .001
0.1	0.7	7 years	9 years
0.1	0.8	7 years	9 years
0.3	0.7	11 years	15 years
0.3	0.8	10 years	13 years
0.5	0.7	20 years	30 years
0.5	0.8	16 years	23 years
0.8	0.7	> 100 years	> 100 years
0.8	0.8	> 50 years	> 50 years

Monsanto also commented on the SAP's recommendation to consider polygenic resistance in the simulation models. According to Monsanto, results of the model will not differ if polygenic

9

resistance is considered rather than monogenic resistance. Dr. Mike Caprio (Mississippi State University) agreed with Monsanto's conclusion. According to, Dr. Caprio applying monogenic or polygenic resistance to the models does not affect the outcome in the absence of refuge (personal communication with Caprio on 12/20/02, Caprio 1998). Groeters and Tabashnik (2000) concluded "that the intensity of selection , rather than the number of loci conferring resistance, is central in determining rates of resistance evolution and effectiveness of refuges." This new information provided to the Agency by Monsanto after the August 2002 SAP suggests that assuming CRW resistance to MON 863 is polygenic rather than monogenic will not affect the results of the models.

Based on the additional information submitted to the Agency by Monsanto after the August 2002 SAP and results of running Caprio's modified model with a .01 initial resistance allele frequency, it can be concluded that a 20% refuge will delay resistance for approximately 7 to 16 years and probably longer since the model also assumes 100% adoption. However, Monsanto assumes that 50% of the susceptible homozygotes (SS) will be controlled. Efficacy data submitted thus far shows 17% to 62% larval survival on MON 863 corn. If the SS input parameter were changed to a lower level of efficacy (e.g., 0.3), then the years to resistance may decrease.

Based on the results presented in Monsanto's recent submission and recommendations from national expert's, including the NCR 46, a 20% refuge should be adequate to delay resistance for 7 to 16 years. In addition, because growers are familiar with the 20% refuge required for currently registered Bt corn products, an increased in is expected based on grower feasability and presenting a consistent message to growers. A 20% refuge should be planted adjacent to or within fields. Additional research should be conducted during registration to support the continued use of a 20% refuge.

Refuge

A non-Bt corn refuge should be planted as continuous blocks adjacent to the MON 863 fields, or non-transgenic strips planted within transgenic field. A 20% non-Bt corn refuge is necessary to produce an adequate number of CRW susceptible to the Cry3Bb1 protein. Considering the limited movement of CRW larvae, planting refuges close to transgenic fields in large blocks is preferred to narrow strips (Gray 1999, Meinke *et al.* 2001). If a 20% refuge is planted as row strips within a corn field, then at least 6 to 12 consecutive rows of non-Bt corn should be planted (Onstad *et al.* 2001). According to Onstad *et al.* (2001), refuge rows should be planted at least 0.5 meters apart and Bt corn strips should be 9 to 18 meters from refuge rows (Onstad *et al.* 2001). However, additional research regarding CRW movement, dispersal, mating and ovipositional patterns is needed to verify how far apart rows should be planted and the distance non-Bt corn rows should be planted from each MON 863 row. This interim IRM plan is not intended for fields planted to increase inbred seed since these fields need to be isolated from external corn pollen sources. An in-field or adjacent non-Bt corn refuge would be inconsistent with inbred seed production practices.

Seed and granular insecticide treatments to control CRW larvae are acceptable on refuge acres.

The ability to treat refuges with larval insecticides is necessary to avoid the potential for severe damage and economic impact. However, it is not acceptable to treat refuges for adult CRW control since these treatments may diminish the effectiveness of the refuge. If growers spray their corn fields with insecticides to control pests other than CRW, then all acres (Bt and non-Bt) should be treated identically.

Bt fields and the non-Bt refuge acres should be treated with identical agronomic practices such as irrigating all corn (Bt and non-Bt) at the same time. To ensure the production of similar numbers of CRW, Bt and non-Bt corn should be planted in fields with similar backgrounds. For example, if MON 863 hybrids are planted on continuous corn fields then the non-Bt refuge should be planted on continuous corn fields or both should be planted on first-year corn acres. Non-Bt refuges should not be planted on first year corn fields if the MON 863 hybrids are planted on rotated fields.

Table 5. Research currently underway to verify refuge size and structure for MON 863 sponsored by Monsanto identified in MRID Nos. 453484-01 and 455770-01

Study Title	Author(s)
Post-establishment movement of western corn rootworm larvae - three years of data under Missouri conditions	Bruce E. Hibbard
Tracking western corn rootworm premating movement	Doug Tallamy
Efficacy of between field refuges for resistance management of transgenic corn rootworm-insecticidal corn	David Andow & Donald Alstad
Resistance management for western corn rootworm (Coleoptera: Chrysomelidae) using seed mixtures of transgenic corn expressing the Cry3B protein	Wade French
Resistance management for western corn rootworms (Coleoptera: Chrysomelidae) using seed mixtures of transgenic corn expressing the Cry3B protein	Elson Shields
Resistance management for western corn rootworms (Coleoptera: Chrysomelidae) using seed mixtures of transgenic corn expressing Cry3B protein	Dennis Calvin
Flight characteristics of female western corn rootworms exposed to transgenic plants	Jon Tollefson & Ted Wilson
An evaluation of CRW dispersal and mating behavior	Joseph Spencer
Estimating the impact of foxtail on CRW populations	Ken Ostlie Mike Gray

Monitoring for Resistance

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. Monitoring techniques such as discriminating dose concentration assays need to be thoroughly investigated for *Diabrotica* spp. for their feasibility as resistance monitoring tools.

Grower participation (e.g., reports of unexpected damage) is an important first step for resistance monitoring. Resistance monitoring is also important because it provides validation of biological parameters used in models. However, resistance detection/monitoring is a difficult and imprecise task. It requires both high sensitivity and accuracy. Good resistance monitoring should have well-established baseline susceptibility data so changes in pest susceptibility over time can be monitored. Although baseline susceptibility data is not completed at this time, research is being conducted to develop the baseline susceptibility of WCRW and NCRW to MON 863. These data are also needed for MCRW.

A comprehensive monitoring plan that targets the CRW and addresses when and where monitoring will occur is needed and should be developed within two years of commercialization. The August 2002 SAP recommended a two tiered approach to monitoring for CRW resistance to MON 863. The Panel recommended tier 1 monitoring methods should identify locations that would merit tier 2 laboratory bioassays. Early detection monitoring should be directed to areas with the highest rate of MON 863 adoption since these areas represent the highest risk of resistance occurring.

The August 2002 SAP suggested that current methods used for early detection of resistance probably do not have the necessary level of sensitivity. Therefore, the Panel recommended potential alternatives to the insect bioassay using artificial diet. For instance, susceptibility of neonate larvae to corn lines expressing varying levels of the Cry3Bb1 protein (e.g., events MON 863, MON 862, MON 853 and MON 854). Measuring larval mortality and growth data with various corn lines rather than artificial diet would be easier and may eliminate some of the problems associated with the feeding bioassay such as mold growth on the artificial diet. Susceptibility data should also be collected for the NCRW and MCRW.

The SAP also suggested that a data on root damage may be used as a monitoring tool. However, a method of using root damage ratings to monitor for resistance has not been developed or validated at this time. It also may be possible to use data on emergence patterns in the MON 863 and non-Bt corn refuges. Since more females than males from susceptible populations emerge from MON 863, evaluating the percentage of males emerging may be able to be correlated with resistance.

Monitoring will become more important after the accrual of multiple growing seasons of exposure and grower adoption increases. In addition to baseline susceptibility data, information is needed to determine how many individuals need to be sampled and in how many locations. The chance of finding a resistant larvae in a Bt crop depend on the level of pest pressure, the frequency of resistant individuals, the location and number of samples that are collected, and the sensitivity of the detection technique. Therefore, as the frequency of resistant individuals or the number of collected samples increases, the likelihood of locating a resistant individual increases (Roush and Miller 1986). If the phenotypic frequency of resistance is one in 1,000, then more than 3,000 individuals must be sampled to have a 95% probability of one resistant individual (Roush and Miller 1986).

Remedial Action

The initial observation of unexpected CRW damage or suspected resistance will likely occur by the grower. Unexpected damage will probably be observed as lodged corn plants. Growers should be required to report any unexpected CRW damage such as lodged plants to the registrant. The August 2002 SAP identified the following four steps a registrant should take to determine if further testing is needed to confirm resistance is occurring.

1. "request the grower check planting records"
2. "rule out damage from nontarget insects, weather, or other environmental factors"
3. "conduct tests to verify MON 863 was planted and that the correct percentage of plants are expressing"
4. "if plants are MON 863 and damage approaching a 0.5 (node-injury scale) is found on any expressing plant, evaluate roots from the corresponding refuge"

Resistance should be confirmed by a standard diet bioassay or evaluation of root node injury. An insect diet bioassay with the Cry3Bb1 protein that results in an LC_{50} that exceeds the upper limit of the 95% confidence interval of the LC_{50} established from baseline measurements of susceptible populations could be used to confirm resistance. Alternatively, resistance may be confirmed when one or more root nodes of at least 50% of Cry3Bb1 plants grown in the laboratory are destroyed. A discriminating concentration bioassay may also be used to confirm resistance; however, this method may take a long time to develop. The August 2002 SAP also recommended investigating the potential of using samples of populations surviving on Bt corn or an evaluation of larval root tunneling to confirm resistance.

Confirmed resistance should be reported to EPA as soon as possible and must be within 30 days. Once resistance has been confirmed, alternative control measures to reduce or control the local target pest population should be recommended to customers, extension agents, consultants, university cooperators, seed distributors, processors, state regulatory authorities, EPA regional and national authorities, and any other pertinent personnel of the incidence(s) of resistance in the affected area. Where appropriate, customers and extension agents in the affected area should apply insecticides and/or crop rotation practices to control any potentially resistant individuals.

As soon as possible following confirmation of resistance, but must be within 90 days, Monsanto should notify the Agency of the immediate mitigation measures that were implemented and submit a proposed long-term resistance management action plan for the affected area. Monsanto should work closely with the Agency in assuring that an appropriate long-term remedial action plan for the affected area is implemented. A remedial action plan that is approved by EPA should be implemented that consists of some or all the following elements, as warranted: 1) Inform customers and extension agents in the affected area of pest resistance; 2) Increase monitoring in the affected area, and ensuring that local target pest populations are sampled on an annual basis; 3) Recommend alternative measures to reduce or control target pest populations in the affected area; 4) Implement intensified local IRM measures in the affected area based on the latest research results. The implementation of such measures will be coordinated by the Agency with other registrants; and 5) Monsanto should cease sales of all MON 863 Bt corn hybrids until resistance has been shown to have been abated. During the sales suspension period, Monsanto 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 should be coordinated with the Agency.

For the growing season(s) following a confirmed resistance incident(s), Monsanto should maintain the sales and distribution suspension of all MON 863 hybrids potentially affected by the resistant pest populations or areas in which resistance is considered to be serious. This must be done within the affected region or if undetermined, the affected county(ies) and proximate surrounding counties. This sales suspension should remain in place until resistance has been determined to have subsided (within 5 to 10% or one standard deviation of baseline levels). In addition, Monsanto should develop, recommend, and implement alternative resistance management strategies for controlling the resistant pest(s) on corn with all necessary personnel (e.g. growers, extension agents, consultants, seed distributors, processors, university cooperators, and state/federal officials) in the affected region/county(ies) and surrounding counties of the resistance situation. All necessary personnel (e.g. growers, consultants, extension agents, seed distributors, processors, university cooperators, and state/federal authorities) in the affected region/county(ies) and surrounding counties of the resistance situation should be informed. Monitoring and surveillance in the affected area(s) for resistance and define the boundaries of the affected region should be intensified and studies on the rate of decline of resistance in the field should be conducted. Monsanto should continue to work with the Agency, states, grower groups, extension agents, consultants, university cooperators, or other expert personnel and other stakeholders to insure the implementation and development of appropriate mitigation measures for resistance in the affected areas.

Grower Education and Compliance

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. The 2000 SAP also suggested that a comprehensive education program may help increase IRM compliance (SAP 2001). Ideally, the educational messages presented to growers should be consistent (among different registrants if applicable for CRW) and reflect the most current resistance management guidelines. 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.

To avoid confusing or discouraging growers, new IRM programs should be kept simple and consistent with existing programs so that growers will not be discourage from properly implement IRM or will not grow transgenic crops. Growers should be required to sign a technology use agreement that outlines IRM requirements and acknowledges the growers responsibility to comply with them on an annual basis. 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. An annual industry-supported survey conducted by a third party should be submitted to the Agency as a tool to monitor grower compliance. Additional education efforts should target non-compliant growers and access to the technology will be limited for growers found to be non-compliant.

CONCLUSIONS

A non-Bt corn refuge should be planted as continuous blocks adjacent to the MON 863 fields, perimeter strips or non-transgenic strips planted within transgenic field. A 20% non-Bt corn refuge is necessary to produce an adequate number of CRW susceptible to the Cry3Bb1 protein. Considering the limited movement of CRW larvae, planting refuges close to transgenic fields in large blocks is preferred to narrow strips (Gray 1999, Meinke *et al.* 2001). If a 20% refuge is planted as row strips within a corn field, then at least 6 to 12 consecutive rows should be planted 0.5 meters apart and Bt corn strips should be 9 to 18 meters from refuge rows (Onstad *et al.* 2001).

Seed and granular insecticide treatments to control CRW larvae are acceptable on refuge acres. However, it is not acceptable to treat refuges for adult CRW control these treatments may diminish the effectiveness of the refuge. If growers spray their corn fields with insecticides to control pests other than CRW, then all acres (Bt and non-Bt) should be treated identically. Bt fields and the non-Bt refuge acres should be treated with identical agronomic practices such as irrigating all corn (Bt and non-Bt) at the same time. To ensure the production of similar numbers of CRW, Bt and non-Bt corn should be planted in fields with similar backgrounds. For example, if MON 863 hybrids are planted on continuous corn fields then the non-Bt refuge should be planted on continuous corn fields or both should be planted on first-year corn acres. Non-Bt refuges should not be planted on first year corn fields if the MON 863 hybrids are planted on rotated fields.

It has been suggested that corn fields should be scouted the previous growing season and MON

863 should only be planted when economic thresholds are reached. The Agency encourages scouting fields and the use of economic thresholds for all pest management programs including those utilizing PIPs. However, CRW resistance to MON 863 is not expected to occur during the three year interim registration requested by Monsanto. Therefore, mandatory scouting or prescriptive use of MON 863 is not necessary at this time. In addition, the potential for cross-resistance is not an issue for MON 863 since no microbial pesticides or PIPs are currently registered for corn with the same mode of action as Cry3Bb1 and CRW is not a listed pest on any current Bt microbial products.

Additional comments received by the Agency recommended limiting sales of MON 863 (e.g., maximum of 25% MON 863 grown per county). However, the 2002 SAP suggested limiting sales of MON 863 for IRM purposes to an on-farm level rather than by region, state or county. The Agency concludes that additional acreage limitations of MON 863 beyond the 20% refuge requirement are not necessary during the three interim period requested by Monsanto since market penetration is expected to be low relative to the potential market share at full maturity. According to Monsanto projections, MON 863 corn adoption will be similar to Roundup Ready® corn which had approximately 1% (790,000 acres), 2.5% (2 million acres) and 5% (4 million acres) market penetration during the first three years of commercial use. In addition, the Agency has predicted that acreage will be limited during the three year interim registration due to technology fees and grower practices. With any new technology, initial grower adoption rates are initially low and increase over time. Effectiveness of the technology, information dissemination, hybrid availability and grower characteristics influence the growers level of awareness, interest, evaluation, trial and eventual adoption of MON 863 corn. Growers will also probably utilize other CRW control techniques that will limit adoption of MON 863 over time particularly as seed treatments and other transgenic products become available. In addition, models have predicted that a $15 technology fee will result in 43% adoption of MON 863 corn in the year 2013; therefore, less than 43% adoption is expected during the initial three years of commercialization.

Additional research is needed to establish a long-term IRM strategy for MON 863 corn. The August 2002 SAP recognized areas of research recommended by the NCR 46 and identified ten additional areas needing further investigation for a basic scientific assessment. The NCR 46 identified the following topics requiring additional research in order to develop a long-term IRM plan.

- Characterize tissue expression, dose, and the mechanism by which corn rootworms survive on transgenic corn expressing Cry3Bb.
- Continue to quantify movement patterns of corn rootworm larvae when feeding on transgenic (expressing Cry3Bb) and nontransgenic corn.
- Quantify pre- and post-mating dispersal of corn rootworm, movement within and between fields, and its implications for IRM.
- Quantify the relative fitness of rootworm individuals that survive on transgenic corn vs. nontransgenic corn.

- Re-evaluate the host status of major grassy cornfield weeds and other grasses commonly found near corn; estimate the potential impact these alternate hosts may have on corn rootworm population dynamics.
- Continue to develop toxicological bioassays and resistance monitoring techniques.
- Determine the genetic nature of resistance to corn rootworm-active Cry compounds.
- Improve rearing techniques for certain corn rootworm species to facilitate laboratory and greenhouse bioassays, genetic studies, etc.
- Generate more complete data sets on transgenic efficacy, adult emergence from transgenic corn, etc. for all targeted corn rootworm species.
- Evaluate IRM options other than a refuge strategy, especially if an event is not classified as high-dose.
- Examine the impacts of refuge configuration, including seed mixtures, on development of resistance and likelihood of farmer adoption.
- Continue to develop and refine computer simulation models that build on current knowledge to guide development of IRM strategies.
- Reconcile corn rootworm and ECB IRM needs into an optimal IRM plan."

In the November 6, 2002 memorandum from Paul Lewis to Marcia Mulkey, the August 2002 SAP identified the following areas needing further investigation.

- "What is the selection intensity on corn rootworm larvae from MON 863 in different regions/soils/moistures and at different densities?"
- "What is the selection intensity on corn rootworm male and female adults from MON 863?"
- "What is the selection progeny through maternal effects?"
- "What is the impact of using whole fields versus rows within fields as refuges on population dynamics and on percent of refuge beetles mating with resistant beetles from the Bt fields?"
- "How would use of a seed mix impact selection intensity?"
- "Are some of the surviving larvae on MON 863 more genetically tolerant of the Bt toxin than the general population?"
- "What could we learn from a quantitative genetic model?"
- "Is male/female movement different in different areas?"
- "Can we develop appropriate monitoring strategies?"
- "Can we develop appropriate mitigation strategies?"

A refuge strategy that utilizes a seed mix would include 20% non-Bt corn randomly mixed with 80% MON 863 corn. The SAP recommended investigation of a "seed mix impact on selection intensity"; however, the Agency recognizes that seed mix refuges may not be practical. Since the Bt corn should not be treated with insecticides to control CRW larvae, the non-Bt corn plants that are randomly planted throughout the MON 863 field will not be protected from damage. This may lead to a destruction of non-Bt corn plants and a decrease in yield. In addition, CRW

feeding damage leading to the destruction of non-Bt corn plants may result in a reduction in the effective refuge size. A seed mix is also not preferred because the Agency believes that IRM compliance will be greater if one consistent message is communicated to growers. Planting a Bt/non-Bt corn seed mix is not an acceptable refuge strategy for Bt corn currently grown to control lepidopteran pests. Recommending a seed mix as a refuge strategy for coleopteran-active Bt corn may mislead growers to conclude that a seed mix is acceptable for all Bt corn.

In addition to the NCR 46 and SAP recommendations for additional research that are listed above, MON 863 affect on CRW fitness should be investigated. Additional information is also needed on CRW biology, monitoring for resistance and mitigation/remedial action. These areas are covered in detail above as well as in the July 23 2002 memorandum from Robyn Rose to Mike Mendelsohn that includes a preliminary review of Monsanto's three year interim IRM strategy.

REFERENCES

Andow, D. A. and D. N. Alstad. 2002. Preliminary draft: efficacy of between field refuges for resistance management of transgenic corn rootworm insecticidal corn: model results. MRID 455770-01

Andow, D. A. University of Minnesota. December 23, 2002. Personal communication with Robyn Rose.

Bourguet, D., J. Chaufaux, M. Seguin, C. Buisson, J.L. Hinton, T.J. Stodola, P. Porter, G. Cronholm, L.L. Buschman and D.A. Andow*. 2002. Frequency of alleles conferring resistance to *Bt* maize in French and US corn belt populations of the European corn borer, *Ostrinia nubilalis*. In print.

Caprio. M. 1998. Evaluating resistance management strategies for multiple toxins in the presence of external refuges. J. Econ. Entomol. 91:1021-1031.

Caprio, M. Mississippi State University. December 20, 2002. Personal communication with Robyn Rose.

Ferre', J. and J. Van Rie. 2002. Biochemistry and Genetics of insect resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 47: 501-33.

FIFRA Scientific Advisory Panel (SAP). October 18-20, 2000. Bt Plant-Pesticides Risk Assessment and Benefit Assessment. Report No. 00-02B. http://www.epa.gov/scipoly/sap/2000/october/brad4_irm.pdf

FIFRA Scientific Advisory Panel (SAP). November 6, 2002. Memorandum of Meeting Minutes from Paul Lewis to Marcia Mulkey.

http://www.epa.gov/scipoly/sap/2002/August/august2002final.pdf

Gray, M. E. 1999. Transgenic insecticidal cultivars for corn rootworms: resistance management considerations. *In* Proceedings of the Crop Protection Technology Conference. University of Illinois, Urbana-Champaign. pp 50-55.

Groeters, F. R. and b. E. Tabashnik. 2000. Roles of selection intensity, major genes, and minor genes in evolution of insecticide resistance. J. Econ. Entomol. 93(6): 1580-1587.

Meinke, L *et al.* 2001. Letter from the NCR 46 to Sharlene Matten dated May 29, 2001.

Onstad, D. W., C. A. Guse, J. L. Spencer, E. Levine and M. E. Gray. 2001. Modeling the dynamics of adaptation to transgenic corn by western corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 94(2): 529-540.

Ward, D.P. December 13, 2002. Response to FIFRA Scientific Advisory Panel Report dated November 6, 2002..

Weiss, M.J., K.P. Seevers and Z.B. Mayo. 1985. Influence of Western corn rootworm larval densities and damage on corn root worm survival, developmental time, size an sex ratio (Coleoptera: Chrysomelidae). J. Kansas Entomol. Soc. 58(3): 397-402.

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

December 23, 2002

MEMORANDUM

Subject: Preliminary summary of new information Monsanto Co. provided to EPA in response to the August 27 to 29, 2002 FIFRA Scientific Advisory Panel (SAP)

To: Mike Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division, 7511C

From: Robyn Rose, Entomologist Robyn D. Rose
Biopesticides and Pollution Prevention Division, 7511C

Brief Summary of the SAP Remarks:
The SAP indicated that the current models show that the time to resistance does not substantially differ when the refuge size ranges from 10-25%; however, the Panel suggested that a 50% refuge will delay resistance substantially longer so it would be a conservative approach. According to the Panel, the amount gene frequency increases during an interim period is of greater importance than years to resistance because this may impact future IRM. Since MON 863 is a non-high dose product, the Panel suggested that the potential for heritable quantitative variation and rapid evolution of resistance should be considered. The Panel went on to suggest that the models only consider monogenic (single locus) resistance, but they should consider the potential for polygenic resistance in a non-high dose product.

Additional comments were made by the Panel regarding initial resistance allele frequency. The models (Andow *et al.*, Onstad *et al.*, Monsanto's modified Caprio model) submitted in support of Monsanto's IRM plan each used .001 as the initial resistance allele frequency. However, the Panel suggested that the initial resistance allele frequency may be as low as 0.1 in a non-high dose product. The Panel also pointed out that studies could be conducted to determine if the initial resistance allele frequency is less than .01. In addition, the models should investigate the full range of dominance values.

Internet Address (URL) • http://www.epa.gov
Recycled/Recyclable • Printed with Vegetable Oil Based Inks on Recycled Paper (Minimum 20% Postconsumer)

Brief Summary of Monsanto's Response to the SAP:
Monsanto summarized results from four data sets from research they sponsored on the efficacy of MON 863. The first and third data sets consisted of field data collected from 1999 to 2002 by 22 scientists from 15 universities located in 15 states. The second data set included data collected by Dr. Bruce Hibbard* (University of Missouri) and the fourth data set is from research conducted by Dr. Blair Seigfried (University of Nebraska). Results of the four data sets demonstrate that the initial allele frequency is ≤.01.

The first data set looked at 7500 corn plants artificially infested with ≥1200 CRW eggs/plant from naturally occurring populations. If the initial resistance allele frequency is 0.01 and Hardy-Weinberg is assumed, then 24 CRW/plant ($24 = 1200*(1-(1-0.01)^2)$) would be resistant and the damage rating on the Iowa scale would be 3.1. Weiss *et al.* (1985) showed that <20 CRW = 3.1 on the Iowa scale. Since the average damage recorded in data set #1 was 1.6, it can be concluded that the initial resistance allele frequency is <0.01.

Data set #2 evaluated larval survival. In this study, ≥30 larvae were recovered per non-Bt corn plant at a wide range of egg infestation rates. If the initial resistance allele frequency is 0.01 and Hardy-Weinberg is assumed, then 0.6 resistant larvae would occur per MON 863 corn plant. Since an average of 0.7 larvae were recovered (but not feeding normally), a ≤.01 initial resistance allele frequency is assumed.

Data set #3 evaluated the number of surviving adult CRW. This data set includes several studies that infest corn plants with over 1200 eggs. The non-transgenic plants resulted in an average of 30 adults surviving. If the initial resistance allele frequency is 0.01 and Hardy-Weinberg is assumed, then 0.6 resistant adults would occur per MON 863 plant and the damage rating on the Iowa scale would equal 3. Since damage averages 1.6 on the Iowa scale, a ≤.01 initial resistance allele frequency can be assumed.

The final data set (#4) examined 11 CRW populations reared in the lab from field collected adults. If the initial resistance allele frequency is 0.01 and Hardy-Weinberg is assumed, then 2% (>20) of the larvae assayed would be resistant. Since there were no large larvae at high doses which suggests no larvae survived and there was low variation (lower than with lepidopterans), a .01 initial resistance allele frequency can be assumed.

Based on these results, Monsanto has concluded that the initial resistance allele frequency is ≤0.01. Therefore, Monsanto modified Caprio's model to include an initial resistance allele frequency of .01. Results of running this model showed that a 20% refuge would delay resistance for approximately 16 years (see Fig 2 on page 13 and Fig 3 on page 14) of Monsanto's submission). For this model, SS survival was set at 0.5 and RS survival set at 0.8 which is partial dominance. Based on data collected by Monsanto and cooperators, MON 863 has been shown to control an average of 50% of the homozygous susceptible (SS) CRW. Therefore, the SS survival was designated 0.5 in the modified Caprio model.

According to Monsanto RS survival (dominance) probably equals 0.7 (communication during a 12/18/02 meeting). Therefore, basing dominance on ≥0.8 would be considered a very conservative approach. Monsanto modeled RS survival to range from 0.5 to 1. If a RS survival of 1 (absolute worst case) were to occur and the initial resistance allele frequency is assumed to be .01, then resistance would be delayed for approximately 13 years with a 20% refuge (Fig 1 on page 8 of Monsanto's submission). If RS survival is designated 0.8, then resistance will occur in approximately 16 years. According to Dr. David Andow* (University of Minnesota), RS survival ranges between 0.3 and 0.8 (personal communication with Andow on 12/23/02). Therefore, a conservative approach would be to designate RS as 0.8 which suggests that 80% of the heterozygotes survive and a 1.0 designation is a "worst case scenario."

Monsanto also discussed why it does not matter if resistance is monogenic or polygenic in their submission. During the December 18, 2002 meeting, Monsanto pointed out that Mike Caprio has run models based on monogenic and polygenic resistance. Caprio has shown that applying monogenic or polygenic resistance to the models does not affect the outcome in the absence of refuge (personal communication with Caprio on 12/20/02). In the presence of refuge, polygenic traits lead to a longer time to resistance.

Additional Publications Supporting 0.01 Initial Resistance Allele Frequency:
Bourguet *et al.* 2002: This paper stated that products with a resistance allele frequency ≥.01 would not have enough efficacy to justify commercialization. Therefore, we can assume that products with high enough efficacy for commercialization have an initial resistance allele frequency of ≤.01 and probably ≤.001. Monsanto's efficacy data summarized above reinforces this conclusion.

Ferre' and Van Rie 2002: This paper considers fitness costs. Insects that show a reduced fitness and have resistance genes would be less competitive so the frequency of resistance genes would be low. This paper suggests that existing scientific information suggests that initial resistance allele frequencies is probably <.001. (I need more time to thoroughly read this paper).

Groeters and Tabashnik: This papers concludes "that the intensity of selection , rather than the number of loci conferring resistance, is central in determining rates of resistance evolution and effectiveness of refuges." Bottom line- it doesn't matter if polygenic rather than monogenic.

Reviewer's Recommendations:
If the initial resistance allele frequency were 0.1, then the efficacy of the MON 863 corn would be so poor that it would not be a marketable product (See Table 1 of Monsanto's submission). At a 0.1 initial resistance allele frequency, damage would equal >>4.6 on the Iowa root rating scale and 0.01 would result in a 3 Iowa rating. The economic threshold in corn is a 3 on the Iowa rating scale. Monsanto has demonstrated that the average damage rating is 1.6. Since MON 863 provides enough protection to result in much less than a 3 root rating, it can be concluded that the initial resistance allele frequency is ≤.01.

22

Monsanto has rerun the modified Caprio model with an initial resistance allele frequency of .01 and RS degree of dominance of 0.8. This represents a conservative scenario. Results of running this model demonstrated that a 20% refuge will delay resistance for ~16 years and probably longer since the model also assumes 100% adoption. However, Monsanto assumes that 50% of the susceptible homozygotes (SS) will be controlled. Efficacy data submitted thus far shows 17% to 62% larval survival on MON 863 corn. If the SS input parameter were changed to a lower level of efficacy (e.g., 0.3), then the years to resistance may decrease.

Based on the results presented in Monsanto's recent submission and an overwhelming majority of national expert's recommendations, a 20% refuge should be adequate to delay resistance for >15 years. In addition, a 20% will lead to increased compliance based on grower feasability and presenting a consistent message to growers. A 20% refuge should be planted adjacent to or within fields. Additional research should be conducted during registration to support the continued use of a 20% refuge.

References:

Bourguet, D., J. Chaufaux, M. Seguin, C. Buisson, J.L. Hinton, T.J. Stodola, P. Porter, G. Cronholm, L.L. Buschman and D.A. Andow*. 2002. Frequency of alleles conferring resistance to *Bt* maize in French and US corn belt populations of the European corn borer, *Ostrinia nubilalis*. In print.

Ferre', J. and J. Van Rie. 2002. Biochemistry and Genetics of insect resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 47: 501-33.

Groeters, F. R. and b. E. Tabshnik. 2000. Roles of selection intensity, major genes, and minor genes in evolution of insecticide resistance. J. Econ. Entomol. 93(6): 1580-1587.

Weiss, M.J*., K.P. Seevers and Z.B. Mayo. 1985. Influence of Western corn rootworm larval densities and damage on corn root worm survival, developmental time, size an sex ratio (Coleoptera: Chrysomelidae). J. Kansas Entomol. Soc. 58(3): 397-402.

* These authors served on the August 2002 FIFRA SAP Panel.

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

FEB 12 2003

OFFICE OF PREVENTION, PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Ecological hazard assessment for *Bacillus thuringiensis* Cry3Bb1 protein, EPA Reg. No. **524-LEI; Barcode Nos. D275903, D280086, D282664, D281017, D275898, D268480, D268481 & D262045** submitted by Monsanto Co. for corn containing *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material necessary for its production (vector ZMIR13L) in corn for control of corn root worm (*Diabrotica* spp.)

FROM: Zigfridas Vaituzis, Ph.D., Senior Scientist
Phil Hutton, Chief
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511C

Zigfridas Vaituzis 2/12/03

PEER REVIEW: Robyn Rose, Entomologist
Biopesticides and Pollution Prevention Division, 7511C

Robyn J. Rose 2-12-03

TO: Michael Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division 7511C

Pesticide Name: Modified *Bacillus thuringiensis* Cry3Bb1 insecticidal protein and genetic material necessary for its expression in corn developed with event MON 863 (vector ZMIR13L).

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

ENVIRONMENTAL ASSESSMENT

Background

The Monsanto Company has requested a registration for *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material (ZMIR13L) necessary for its production in all corn lines and varieties. The Cry3Bb1 protein is intended to control the corn rootworm (CRW, *Diabrotica* spp.), a coleopteran pest of corn. The CRW is a primary pest of corn in the U.S., it feeds on corn roots as

24

larvae leading to a reduction in the plant's ability to absorb water and nutrients from soil and lodging (i.e., loss of structural integrity of the corn stalk). In areas where the CRW is a pest (e.g., Corn Belt), significant financial losses are realized from a decrease in production and chemical insecticide usage. Significant acres of corn are treated annually to control CRW with organophosphate, carbamate and pyrethroid insecticides.

In addition to the review of the final submission of the field sampling data by Monsanto, this section addresses comments and conclusions from the August 27, 2002 Scientific Advisory Panel (SAP) meeting titled *Corn Rootworm Plant-Incorporated Protectant Insect Resistance Management and Non-Target Insect Issues*. A memorandum dated November 6, 2002 from Paul I. Lewis to Marcia E. Mulkey included a transmittal of the meeting minutes of the November 27, SAP. Based on the SAP and public comments, the Agency is providing supplemental conclusions to those found in the May 20, 2002 preliminary review of ecological non-target insect studies for *Bacillus thuringiensis* Cry3Bb1 protein.

The Agency has conducted an environmental hazard assessment of the Cry3Bb1 producing corn lines. The general topics covered include gene flow to related wild plants, development of weediness, effects on wildlife, and fate of Cry3Bb1 proteins in the environment. The assessment is based on data submitted to the Agency during the development of the corn lines, additional data submitted for registration, FIFRA Scientific Advisory Panel (SAP) recommendations, consultations with scientific experts, and public comments received.

I. Non Target Wildlife Hazard Assessment

A. The Hazard Assessment Process

The Agency assesses the toxicity of a Cry protein (*B.t.* endotoxin) to representatives of potentially exposed non-target organisms by a tiered testing system starting with Tier I single species maximum hazard dose laboratory data using mortality as the end point. This approach was developed for the Agency by the American Institute of Biological Sciences and approved in 1996 as an acceptable ecological hazard assessment method by a FIFRA Scientific Advisory Panel for microbial pesticides and microbial toxins, and by the December 9, 1999 SAP for protein Plant Incorporated Protectants (PIP). The methods were last published as the Harmonized OPPTS Testing Guidelines (EPA 712-C-96-280, February 1996). The guidelines include (but are not limited to) bacteria and their toxins as defined in 40 CFR 152.20. The guidelines apply to microbes and microbial toxins when used as pesticides, including both those that are naturally occurring, and those that are strain-improved, either by natural selection or by deliberate genetic manipulation.

The guidelines in Tier I reflect a maximum hazard approach to testing. Negative results from tests using this approach provide a high degree of confidence that no unreasonable adverse effects are likely to occur. The OPPTS Harmonized Testing Guidelines utilize the tier testing scheme to ensure, to the greatest extent possible, that only the minimum data sufficient

to make scientifically sound regulatory decisions will be required. Moreover, the Agency believes that the Tier 1 maximum hazard dose testing requirement represents a reasonable approach to evaluating hazard related to the use of biological pesticides, and is one in which negative results allow a high degree of confidence in the safety of the test agents. The Agency expects that most of the plant incorporated Bt Cry proteins require testing only in the first tier for short term hazard assessment. Long range adverse effects have to be ascertained by higher Tier long term field testing. A SAP convened in October 2000 and the National Academy of Sciences (NAS 2000) also recommended testing non-target organisms directly in the field. This approach, together with an emphasis on testing of invertebrates found in the corn fields was also recommended by the August, 2002 SAP, and was supported by several public comments.

The maximum hazard dose approach for Tier I testing is based on a safety factor times the maximum amount of active ingredient expected to be available to terrestrial and aquatic plants and animals in the environment (the expected environmental concentration, or EEC). Therefore, data that establishes an LC_{50}, ED_{50}, or LD_{50} that is greater than the maximum hazard dosage level (e.g. LD_{50} >10 x EEC) is sufficient to evaluate adverse effects. If there are no effects at the maximum hazard dose, lower dose testing is not necessary.

If, however, significant toxic effects are noted at the maximum hazard dose level, the Harmonized Guidelines call for testing of multiple groups at lower doses in order to quantify the hazard. Sufficient doses and test organisms are required to determine an LD_{50} value and, if necessary, the No Observed Effect Level (NOEL), or reproductive and behavioral effects such as feeding inhibition, weight loss, etc. Appropriate statistical methods are to be used to express trends, and to evaluate the significance of differences in data obtained from different test groups. The statistical methods used must reflect the current state-of-the-art with appropriate statistical power.

The Guidelines call for testing of a single group or groups of test animals at the maximum hazard dose, and if deleterious effects are observed, testing with sequentially lower doses to establish a definitive LD_{50} with confidence limits. When the active ingredient is a toxin the appropriate endpoint would be death of the test organism. Each treatment and control group shall contain at least 10 test animals. When there is only one treatment group, at least 30 animals must be tested at that treatment level. The guidelines provide that the duration of all Tier I tests be about 30 days long. Some test species, notably non-target insects, may be difficult to culture and the test duration has been adjusted accordingly. Control and treated insects should be observed for a duration of at least 30 days after dosing, or in cases where an insect species cannot be cultured for 30 days, until control mortality rises above 20 percent.

On December 9, 1999, the Agency presented the maximum hazard dosing approach to testing of protein PIP and for possible new data requirements to a FIFRA Scientific Advisory Panel for their recommendations. The December 1999 SAP report was generally supportive of the Agency's testing and hazard evaluation. The Panel also recommended more testing of non-target invertebrates more closely related to the target species and species more likely to be present in the field of the GM crops. In addition, the October 2000 SAP recommended appropriate field

26

testing be conducted for non-target organisms. The August, 2002 SAP and certain public comments also agreed with this approach with some additions. It was recommended that the choice of appropriate indicator organisms for testing be based on the potential field exposure as deduced from data on Cry protein activity and expression in the plant. The SAP thought that appropriately chosen single species Tier I laboratory tests showing no detrimental effects are sufficient to make a short term hazard assessment and that field studies be conducted when these tests show toxicity (as higher Tier testing described in the OPPTS Microbial Testing Guidelines) but that proper multi year commercial field studies with appropriate statistical power are needed to determine long term ecological effects. The December 9, 1999, SAP, the August, 2002 SAP, and several public comments noted that the maximum hazard approach to non-target species testing was not statistically appropriate for determination of No Observed Effect Levels (NOEL). This comment is in agreement with the Agency's OPPTS Testing Guideline discussed above.

Bt Cry endotoxins are proteins and, unlike inorganic chemicals, do not have the potential to bioaccumulate and thereby result in delayed effects. An accumulation through the food chain is therefore not expected to take place, and there are no data to support this possibility for protein substances. The basic biological properties of proteins also make Bt Cry proteins readily susceptible to metabolic, microbial, and abiotic degradation once they are ingested or excreted into the environment. Although there are reports of soil binding under certain circumstances, the bound Cry proteins are also reported to be rapidly degraded by microbes upon elution from soil. The same sources also report that Bt proteins in the soil of Bt corn fields have no detectable effect on soil invertebrates or culturable microbial flora. In addition, Bt Cry proteins do not have any characteristics in common with persistent, bioaccumulative chemicals that are transferred through the food chain. Therefore, chronic effects testing of protein substances is not routinely performed.

B. Cry3Bb1 Protein Environmental Hazard Assessment

Introduction

Based on the evaluation of the submitted maximum hazard dose testing data and information on the general biology of Bt Cry proteins, no unreasonable adverse effects on the invertebrate fauna of the corn field are expected from Cry3Bb1 protein producing corn. EPA concludes that it is appropriate for long term environmental effects to be assessed by appropriately designed field monitoring during the initial years of the Cry3Bb1 corn registration. EPA believes that the development and review of such information will also address one of the major concerns in several public comments.

Specific data are cited relating to aquatic and terrestrial wildlife, Cry protein fate in soils, potential effects on soil biota represented by the earthworm and field census data, effects on non-target soil Coleoptera species, foliar insects and endangered or threatened species, particularly Coleoptera. The results of these studies are presented here in both tabular (Table 1) and more detailed descriptive format. The complete review record of the submitted data can be found in the

individual Data Evaluation Reports (DERs).

1. Summary of Non-Target Organism Toxicity Testing

Two separate SAP reports (October, 2000 and August, 2002) recommended that non-target testing be focused primarily on species exposed to the crop being registered. However, in addition to testing species directly exposed to the CryBb1 protein in the field, the full battery of non-target wildlife species testing was conducted to comply with the published Agency non-target data requirements for microbial toxins. (In the absence of PIP-specific data requirements, EPA requires applicants for PIP registrations to meet the 40 CFR. Part 158 data requirements for microbial toxins.) The Agency has determined that the non-target organisms most likely to be exposed to the protein in transgenic corn fields were beneficial insects feeding on corn pollen and nectar, and soil invertebrates, particularly Coleoptera ssp. Initially, in lieu of extensive and difficult single species laboratory soil coleopteran toxicity testing followed by an extrapolation to community risk assessment, direct field census data, and data on coleopteran insect effects and abundance in the field were requested, received and evaluated. The August, 2002 SAP, however, found the field census data unsatisfactory because of low statistical power. Maximum hazard dose toxicity testing on representative beneficials from several taxa were also performed. The toxicity of the Cry3Bb1 protein has been evaluated following challenge of several species of invertebrates, including: adult and larval honey bees, a parasitic hymenopteran (*Nasonia*), green lacewings, lady beetles, collembola, monarch butterfly and earthworms. Reproductive and developmental observations were also made on collembola, honey bee and lady beetle larva maturation studies. The August, 2002 SAP (as well as several public comments) however, found the green lacewing and parasitic wasp studies lacking and recommended testing of alternative species. Based on worst-case soil concentration, soil degradation studies show that Cry Bb1 protein in corn tissue is no longer detectable in agricultural field soil after 22 to 28 days. The August, 2002 SAP (as well as several public comments), however, suggested that additional soil degradation testing is desirable in a larger variety of soils and climactic conditions.

The non-target organisms tested are chosen as representative indicators of the major groups of wildlife and on the potential for field exposure as deduced from data on Cry3Bb1 protein expression in the plant. Although Bt Cry proteins are very specific in their activity to only certain insect species, for Cry3Bb1 protein in corn the Agency has examined the toxicity to birds, fish, honeybees and certain other beneficial insects even though a recent SAP (March, 2001) recommended against testing of non-targets species not related to those susceptible to the specific activity of Bt Cry proteins. However, in order to comply with the published Agency data requirements (40CFR Part 158) for registration of microbial toxins, the Agency asked for avian and aquatic invertebrate toxicity data, as well as Collembola (springtail) and earthworm species to ascertain effects on beneficial soil invertebrates because prolonged exposure to Cry3Bb1 proteins in soil was a possibility. Earthworm studies were also conducted and voluntarily submitted to the Agency by the registrant to demonstrate a lack of MON 863 effects on beneficial decomposers. Honey bee effects on brood as well as adults were required as exposure to the Cry3Bb1 protein in pollen is expected.

The form of the test substances used in the studies for this assessment are plant material such as leaves, roots, pollen or purified bacterially-produced Cry3Bb1 protein incorporated into the test species diet. The October 2000 SAP provided guidance to the Agency that while actual plant material is the preferred test material, bacterially-derived protein is also a valid test substance, especially in testing where the test animals do not consume corn plant tissue and where large amounts of Cry protein are needed for maximum hazard dose testing. As per the OPPTS Harmonized Testing Guidelines, the adult insect studies were generally of 30 days duration or until the negative control mortality reached 20%. Larval studies were through pupation and adult emergence.

Table 1. Tabular results of non-target wildlife and soil fate studies

Guideline No	Study	Results	MRID No.
USEPA OPPTS 885.4150	Wild Mammal Testing, Tier 1	Mammalian wildlife exposure to Cry3Bb1 protein is considered likely; however, the Cry3Bb1 toxicity data for Human Health Assessment indicate that there is no significant toxicity to rodents from testing at the maximum hazard dose. Therefore no hazard to mammalian wildlife is anticipated.	Not assigned
885.4050	A Dietary Toxicity Study with the Northern Bobwhite	The dietary LC_{50} value for Cry3Bb1 corn grain to juvenile Northern Bobwhite was greater than 70,000 ppm (10% of the diet) in a ~~13~~-day study (eight day observation). No adverse effects on avian wildlife is expected from incidental field exposure to Cry3Bb1 corn. A higher corn concentration and longer duration broiler study with MON863 corn is recommended.	449043-15
885.4200	Freshwater Fish Testing	No treatment mortality or behavior change was observed among channel catfish in an 8 week sub-chronic study when fed diets containing 35% Cry3Bb1 corn lines MON 853 and MON 859.	449043-19
885.4200	Freshwater Fish Testing	The requirement for a fresh water fish static renewal rainbow trout toxicity study has been waived based on a lack of any substantial exposure of fish to the Cry3Bb1proteins produced in corn crops.	Not assigned
Series 72, Subdivision E	Acute Toxicity Test with the Cladoceran (*Daphnia magna*)	The 48-hour LC_{50} value for Cry3Bb1 corn pollen when administered to neonate daphnids was >120 mg pollen/L, a maximum hazard dose. No other adverse effects were noted. Therefore, no hazard to daphnia are expected from incidental exposure to Cry3Bb1-containing corn pollen.	449043-18
885.4280	Estuarine and Marine Animal testing, Tier I	The Estuarine and Marine animal studies are waived for this product because of very low to no potential for exposure to Cry3Bb1 protein from field corn.	Not assigned

29

Guideline No	Study	Results	MRID No.
885.4300	Nontarget Plant Studies, Tier I	Since the active ingredient in this product is an insect toxin (Bt endotoxin) that has never shown any toxicity to aquatic or terrestrial plants, these studies have been waived for this product. Outcrossing issues are addressed below.	Not assigned
885.4380	Honey Bee Larva Testing	The LC_{50} for honey bee larvae and maturation to adult bees was determined to be >1,790 ppm Cry3Bb1 protein, (100X the concentration in pollen)in a maximum hazard dose study. Therefore no hazard to honey bee larvae and adult bee emergence is anticipated.	449043-10
885.4380	Adult Honey Bee Testing	An adult honey bee maximum hazard dose feeding study showed the LC_{50} of the Cry3Bb1 protein to be >360µg/mL. (20X the concentration found in pollen). Therefore, no hazard from the Cry3Bb1 protein to honey bees is expected.	449043-11
885.4340	Parasitic Hymenoptera Larva Testing	The LC_{50} for parasitic Hymenoptera was determined to be >400 ppm Cry3Bb1 protein. Although 400 ppm Cry3Bb1 protein is only 1X field concentration in plants rather than 10X, parasitic Hymenoptera are not expected to feed directly on corn plant tissue. Therefore, minimal exposure and no hazard to parasitic Hymenoptera from Cry3Bb1 protein is expected. Testing of a species more common to corn fields is recommended.	449043-13
885.4340	A Dietary Toxicity Study with Green Lacewing Larvae	The LC_{50} for green lacewing larvae was determined to be >8,000 ppm Cry3Bb1 protein (20X field exposure). Based on these results it can be concluded that green lacewing will not be adversely affected when exposed to Cry3Bb1 in the field. Because of questionable ingestion of the test material another species (e.g, minute pirate bug, predatory carabid) more likely to be exposed to Cry3Bb1 should be tested.	449043-12
885.4340	Effects of Bt Protein 11231 on Adult Lady Beetles *(H. convergens)*	This maximum hazard dose study showed that the LC_{50} for Cry3Bb1when fed to adult *H. convergens* is >8,000 µg purified Bt protein/mL diet., equivalent to 20X the maximum Bt protein concentration in plant tissue. A follow-up pollen feeding study was requested.	449043-14
885.4340	Lady Beetle Larval Pollen Feeding Study (*C. maculata*)	The LC_{50} for Cry3Bb1 expressed in pollen is >93 µg/g fresh pollen weight. The larvae were observed through pupation to adult emergence. It can be concluded from this study that *Coleomegilla maculata* larvae will not be adversely affected by Cry3Bb1 field corn pollen.	455382-04
885.4340	Adult Lady Beetle Pollen Feeding Study *(C. maculata)*	No significant adverse effects were noted in a 30 day 50% pollen feeding study. Based on these results , no hazard to *Coleomegilla maculata* is expected when feeding on Cry3Bb1 corn pollen in the field.	453613-01

Guideline No	Study	Results	MRID No.
885.4340	Adult Lady Beetle Pollen Feeding Study (*H. convergens)*	No significant adverse effects were noted in a 15 day 50% pollen in honey water feeding study. Based on these results , no hazard to *Hippodamia convergens* is expected if feeding on Cry3Bb1corn pollen in the field.	453613-02
885.4340	Collembola Chronic Dietary Toxicity Study	The LC_{50} of the Cry3Bb1 protein for Collembola was found to be >872.5µg (50% corn leaf tissue in the diet). No adverse reproductive effects were noted. It can be concluded from this test that Cry3Bb1protein does not pose a hazard to Collembola, a representative of a beneficial decomposer soil inhabiting species.	449043-17
850.6200	Earthworm Toxicity Study	A maximum hazard dose 14-day LC_{50} for earthworms exposed to Cry3Bb1 protein in an artificial soil substrate was determined to be > 570 mg Cry3Bb1 protein/kg dry soil, or greater than 10 times the maximum EEC of the protein. The data show that no adverse effects to earthworms are expected from exposure to Cry3Bb1 protein producing corn plants.	449043-16
OECD Guideline 207	Earthworm Toxicity Study	There were no earthworm mortalities or other remarkable observations during the 14 day study. The LC_{50} value is greater than the highest maximum hazard concentration tested [166.6 mg Cry3Bb1 protein variant 11098 (Q349R)/kg dry soil].	457571-01
885.4340	Monarch Butterfly Larval Pollen Feeding Study	This study has demonstrated that corn pollen expressing the Cry3Bb1 protein will not result in acute toxic or developmental effects to monarch larvae. The SAP recommended testing *Tetraopes* (red milkweed) beetles as a more logical choice than the monarch butterfly.	455382-05
N/A	Insecticidal Activity Spectrum study	Bioassays of six Families of the Order Coleoptera and two Lepidoptera species detected activity only against beetle species of the family Chrysomelidae (corn rootworm and Colorado potato beetle).	455328-07
154-3500	Field evaluation of Cry3Bb1corn exposure on non-target organisms	Preliminary results from two year Tier IV field census studies. These studies are supplemental to Tier I maximum hazard dose testing. The data do not show any MON 863 corn related adverse effect on non-target and beneficial invertebrate abundance in the field.	455382-06
154-3500	Non-target organism field scale risk assessment	Final report for MRID 455382-06 two year field census study. MON863 showed no overall differences in the abundance of non-target invertebrates and had less impact on certain beneficial insects compared to traditional insecticides, especially soil and foliar applications. These studies are supplemental to Tier I maximum hazard dose testing and are of inadequate statistical power for long term effects determination.	457916-01

Guideline No	Study	Results	MRID No.
154-3500	Field and Laboratory invertebrate studies	Summary (without data) of preliminary findings from several one year supplemental higher Tier field and laboratory studies not triggered by Tier I maximum hazard dose testing data. Final report of studies to be submitted.	456530-03
885.5200	Aerobic Soil Degradation of the Cry3Bb1 Protein 11098	Finely ground corn leaf tissue in sandy loam field soil degradation data at worst-case field concentrations show that the Cry3Bb1 protein DT_{50} based on insect bioassays and ELISA were 2.37 and 2.76 days respectively. The DT_{90} estimates for the insect bioassays and ELISA were 7.87 and 9.16 days respectively. At ≤28 days the CryBb1 protein was below the detection level. These results verify that the Cry3Bb1 protein degrades rapidly and does not accumulate in the soil. Additional testing in different soil types is requested.	451568-04
885.5200	Aerobic Soil Degradation of Cry3Bb1 Produced by CRW Protected Corn Event MON 863	The DT_{50} values for Cry3Bb1in several dosing regimes and soil types ranged from 0.6 days to 2.3 days and the DT_{90} values ranged from 4.03 days to 50 days. Cry3Bb1 levels in soil sample extracts show that concentrations were near or below the ELISA LOQ (0.16 µg/g) after 2 months of incubation. Additional studies with whole plant tissue are requested.	457571-02
None	Endangered Species Impact Assessment	Monsanto conducted a hazard assessment, exposure assessment and risk characterization to demonstrate that Cry3Bb1 does not pose a risk to endangered Coleoptera. The Agency performed an independent assessment and determined that no adverse affects from Cry3Bb1event MON 863 are expected to endangered/threatened Coleoptera species listed by the USFWS. Non-coleopteran endangered species are not expected to be susceptible to MON 863.	455770-03

2. Non-target Wildlife Testing and Hazard Assessment

a. Mammalian Wildlife

Mammalian wildlife exposure to Cry3Bb1 protein is considered likely; however, the mammalian toxicology information gathered to date on Bt Cry proteins does not show a hazard to wild or domesticated mammals. The Cry3Bb1 toxicity data for Human Health Assessment indicate that there is no significant toxicity to rodents from acute oral testing at the maximum hazard dose. Therefore no hazard to mammalian wildlife is anticipated.

b. Avian hazard assessment

The methods used in conducting the study (MRID No. 449043-15) followed procedures specified in EPA's Registration Guidelines, Pesticide Assessment Guidelines, FIFRA Subdivision E,

Hazard Evaluation: Wildlife and Aquatic Organisms; OECD Guideline 205, Guideline for Testing of Chemicals, Avian Dietary Toxicity Test and on ASTM Standard E857-87, "Standard Practice of Conducting Subacute Dietary Toxicity Tests with Avian Species."

The dietary LC_{50} value for Cry3Bb1 corn grain (MON 853, MON 854 and MON 855) when fed to juvenile Northern Bobwhite for 5 days was reported to be greater than 70,000 ppm (10% of the diet), the only concentration tested. No adverse effects on bobwhite quail were seen in eight days. These data show that there will be no hazard to avian wildlife from incidental field exposure to Cry3Bb1 corn. These data are, however, not sufficient to make a hazard assessment from repeated exposure to higher doses of Cry3Bb1 corn. This study is classified as supplemental. The concentration tested (10% corn in the diet) is too low. A six week broiler study with 60% - 70% MON 863 corn in the diet is needed to assess hazard to non-target birds from continuous exposure to high levels of Cry3Bb1 protein.

c. Aquatic species testing

There is no evidence for sensitivity of aquatic (including endangered) species to Cry proteins. Toxicity studies with Cry proteins on aquatic organisms show no hazard for fish or invertebrates exposed to either corn pollen or to bacterially expressed Cry protein. In addition, aquatic exposure from Bt corn is extremely small. When a simple standard pond scenario (1 hectare pond, 2 meters deep draining a 10 hectare watershed planted with corn) was used to develop a worst case EEC for Cry3Bb1 protein on the basis of corn pollen loadings from airborne pollen deposition and agricultural runoff from corn plant tissue left in the field at the end of harvest (assuming that no degradation of the protein takes place), airborne and agricultural runoff is calculated to be 3.9 ng Cry3Bb1 protein/mL. Thus, total water concentration of less than 3.9 ng Cry3Bb1 protein/mL is projected under worst case conditions.

i. Freshwater fish

The Harmonized Testing Guidelines requirement for a static renewal freshwater fish toxicity study is usually waived based on low to nonexistent exposure to Cry protein produced in corn. Exposure from corn pollen, if it does take place, will be of a very short duration and quantity and is not expected to have any detectable effect on freshwater fish. Nevertheless a subchronic eight week farmed channel catfish feeding study was performed and submitted for review.

The procedures used in the catfish study (MRID No. 449043-19) follow those recommended by EPA Pesticide Testing Guidelines for Microbial and Biochemical Pest Control Agents, Subdivision M and OPPTS Series Guideline 885.4200 - Freshwater Fish Testing. The testing was done at the Thad Cochran National Warmwater Aquaculture Center (Testing Facility), Mississippi State University, Stoneville, MS . The study was conducted in compliance with the U.S. EPA FIFRA Good Laboratory Practic Regulations (40 CFR Part 160) with three minor deviations which had no impact on the integrity of the study.

The study is scientifically sound and no treatment mortality or behavior change was observed

among channel catfish fed diets containing finely ground corn grain from two insect-protected Cry3Bb1 corn lines (MON 853 and MON 859) for eight weeks. The results indicate that corn grain derived from the two transgenic lines producing Cry3Bb1 can be used as a feed ingredient in channel catfish diets at levels of up to 35% without adverse effect on fish growth, feed conversion efficiency, survival, behavior, or body composition. Significant differences were observed only as lower percentage fillet moisture among fish fed corn grain of the line MON 859; however, these are relatively unremarkable and are unlikely related to the different diets. There were no significant differences noted in feed consumption, weight gain, feed conversion ratio, survival, percentage visceral fat, or percentages fat, protein, or ash in fillets of channel catfish fed the different test diets. No abnormal fish behavior was observed in the study.

In view of the lack of demonstrated toxicity to channel catfish and minimal aquatic exposure, no fresh water fish hazard is expected from the uses of Cry3Bb1 protein in corn crops.

ii. Aquatic invertebrates

This study (MRID No. 449043-18) was conducted according to procedures specified in Series 72 of EPA's Registration Guidelines, Pesticide Assessment Guidelines, FIFRA Subdivision E, Hazard Evaluation for acute toxicity testing of pesticidal substances to freshwater aquatic invertebrates.

The study was performed on *Daphnia magna*, a freshwater invertebrate. The test material consisted of corn pollen from corn plants, line MON 858. The Cry3Bb1 content was estimated to be 18.8 µg/g fresh weight pollen. The study is procedurally sound and no treatment mortality or behavior change was reported between the dosed and control replicates for the 48-hr exposure period.

The October, 2000 and August, 2002 SAP reports recommended that non-target testing be focused on species exposed to the crop being registered. The Agency has determined that the non-target organisms most likely to be exposed to the protein in transgenic corn fields were beneficial insects feeding on corn pollen and nectar, and soil invertebrates, particularly Coleoptera spp. Therefore, testing of aquatic invertebrates was performed primarily to satisfy the testing requirements for microbial toxins published in 40 CFR Part 158. No substantial aquatic exposure to Cry3Bb1 protein contained within corn plant tissue is expected except for possibly small amounts of pollen. Several public comments have raised questions about using corn pollen in aquatic invertebrate testing with *Daphnia magna* because corn pollen is thought to be too large for ingestion by these filter feeders . However, there is some observational evidence that daphnids do ingest pollen. As indicated in some study reports reviewed by the Agency, daphnids were actually yellow in color, which can be indicative of ingestion of the yellow pollen test material. However, there is no clear evidence that *Daphnia magna* is capable of ingesting particles as large as pollen. Therefore only a statement of no effect from exposure to pollen, and no statement on lack of toxicity can be made from this study. However, since the Cry3Bb1 protein is confined to corn tissue, and the worst case aquatic EEC is calculated to be 3.9 ng Cry3Bb1 protein/mL, there is no substantial exposure to aquatic invertebrates, and therefore no

hazard from the registered used of Cry3Bb1 containing corn is anticipated. As a result, no further aquatic invertebrate testing is required at this time.

iii. Estuarine and Marine Animals

The Estuarine and Marine animal studies are waived for this product because of very low to no potential for exposure to CryBb1 protein from field corn.

iv. Terrestrial and Aquatic Plants

Since the active ingredient in this product is an insect toxin (Bt endotoxin) that has never shown any toxicity to plants, these studies have been waived for this product. Outcrossing issues are addressed below.

3. Terrestrial Invertebrate Testing and Hazard Assessment

Background:

The October 2000 SAP concluded that invertebrates such as earthworms and springtails (collembola) are appropriate indicator species for Cry protein testing because of the specific nature of the Cry protein toxicity to select target species. When it initially reviewed the applications for PIP products that were registered in 1995, EPA considered requiring studies evaluating effects upon the representative beneficial soil invertebrates Collembola and earthworms. The Agency was concerned (1) that such soil organisms may be subject to long-term exposure as a result of soil incorporation of crop residues or when crop residues are left on the soil surface and (2) that adverse effects on such soil organisms could result in an accumulation of plant detritus in fields. Recent reports of exudation of Cry proteins by corn roots throughout the growing season add to this concern. However, the Agency understands that routine agronomic practices have included the long term use of chemical insecticides, which have adverse effects on soil organisms, but there has not been an accumulation of significant amounts of plant detritus in soils . Thus, Cry3Bb1 corn, which is expected to have less impact on these species than chemical pesticides, should not result in any increased build up of plant detritus or Cry proteins at toxic levels. Supporting this conclusion are data received by EPA that indicate that such proteins are known to degrade rapidly in field soils. Cry proteins that are bound to soil particles have been shown to be rapidly degraded by soil microbes upon elution from the soil particles. Therefore the potential for significant soil buildup and adverse effects to non-target soil organisms are not anticipated. It has been confirmed in published literature that Bt Cry protein released from root exudates and biomass of Bt corn plants has no apparent effect on earthworms, nematodes, protozoa, algae, bacteria, actinomyces and fungi in soil in spite of the fact that enough detectable Cry protein is bound to soil particles to show toxicity to the target pest. These results suggest that despite its presence in soil, the Cry protein released in root exudates of Bt corn, or from the degradation of the biomass of Bt corn, is not toxic to a variety of organisms in the soil environment. It has also been reported that the same degree of Bt Cry protein persistence takes place in soils that have been exposed to repeated Bt microbial spray

applications. In addition, new plants grown in Bt containing soil do not take up the Bt protein. Nevertheless, data on insects closely related to the target pest, as well as other studies to address the published data requirements for registration of microbial toxins (40 CFR §158) have been received and reviewed.

a. Single Species Laboratory Testing

i. Effects on Honey Bee Larvae

An acceptable study (MRID No. 449043-10) was conducted based on OPPTS Series 885-4380, Honey bee testing Tier I. This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test.

Testing was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (purity 96%; 1.79 mg active protein/mL water; current nomenclature refers to this protein as Cry3Bb1) inoculated directly into larval brood cells prior to capping. Within 18 days after treatments were administered, all larvae emerged from capped brood cells. All of the larvae (100%) treated with Cry3Bb1 protein survived to pupation or "capping"; whereas, 97.5% (2.5% mortality) of the honey bee larvae in the control group survived to pupation. There was no statistical difference ($p=0.05$) in total percent mortality during the larval development or adult emergence stages between treated and control groups. Based on the results presented in the study, it can be concluded that honey bee development and survival are not affected by exposure to the Cry3Bb1 protein. There was 88.8% mortality of larvae treated with the reference substance potassium arsenate which indicated that bees were exposed to the treatments. The LC_{50} for honey bee larvae was determined to be >1,790 ppm Cry3Bb1 protein.

According to the OPPTS Harmonized Testing Guidelines, non-target insects should be tested at 10-100X the field dosage. This test was conducted at an acceptable level 100X the concentration in pollen or 1,790 ppm Cry3Bb1 protein. Since potential exposure of honey bees to Cry3Bb1 will be from pollen, this test was conducted at an appropriate maximum hazard dose. Therefore, no hazard to honey bee larvae and their development is expected from exposure to the Cry3Bb1 protein in corn pollen.

ii. Adult Honey Bee Testing

This study (MRID No. 449043-11) was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on OPPTS Series 885-4380, Honey bee testing Tier I.

The testing consisted of a control group fed 30% sucrose in deionized water, a reference group fed 100µg/mL potassium arsenate, and a test group fed 360 µg/mL of Cry3Bb1 protein and a

water only group. The study concluded that 360 µg/mL Cry3Bb1 protein did not affect survival or behavior of adult honey bees. The maximum hazard dose LC_{50} of >360 µg/mL is 20X the concentration found in pollen. Therefore, no hazard to adult honey bees is expected from exposure to the Cry3Bb1 protein in corn pollen.

iii. **Parasitic Hymenoptera Testing**

This study (MRID No. 449043-13) was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on OPPTS Series 885-4340 Non-target Insect Testing, Tier I.

A dietary toxicity study with the parasitic Hymenoptera (*Nasonia vitripennis*) was conducted with *Bacillus thuringiensis* Cry 3B2.11231 (Cry3Bb1) protein (purity 96%; 34.5 mg active protein/mL water). Wasps were tested at rates of 400 and 8,000 ppm Cry3Bb1 protein which is approximately equivalent to 1X and 20X the maximum protein concentration in plant tissue. The LC_{50} for parasitic Hymenoptera was determined to be >8,000 ppm Cry3Bb1 protein. When an adjustment for mortality in the control group is considered, mortality in the 8,000 ppm treatment group is 45%. Although differences in mortality between the control and treatment groups were not significantly different ($p>0.05$), a treatment effect at 20X EECcould not be precluded in this study. At test termination mortality for the 100 ppm potassium arsenate reference group was 33% (24 of 73) and 100% mortality (70 of 70) in the 1,000 ppm reference group.

Based on this test, the LC_{50} for adult parasitic Hymenoptera exposed to dietary Cry3Bb1 is >8,000 ppm. The hazard assessment is based on 4000 ppm Cry3Bb1 protein which is 10X the field concentration in plants. However, because parasitic Hymenoptera do not feed directly on corn plant tissues, minimal exposure of parasitic Hymenoptera to Cry3Bb1 protein is expected. As a result, no hazard to *Nasonia vitripennis* is expected from exposure to MON 863 Cry3Bb1 corn.

The preliminary review of the *Nasonia vitripennis* study was initially found acceptable by the Agency (memorandum from Robyn Rose to Mike Mendelsohn dated May 20, 2002). However the August 27, 2002 SAP concluded that the parasitic Hymenoptera (*Nasonia vitripennis*) testing was not appropriate. The SAP concluded that "[t]he levels of exposure of ... *Nasonia* to active protein were not, for example, determined throughout their respective tests. The test protein ... within a diet broth ... could have degraded considerably." Not only were the procedures in this study questioned by the SAP, the appropriateness of testing this organism is questionable. *N. vitripennis* is a dipteran parasitoid that does not occur in corn fields. A more appropriate parasitoid that occurs in corn fields (*e.g. Tricogramma* or *Macrocentrus grandii*) should be considered. Since *Tricogramma* and *Macrocentrus* are lepidopteran parasitoids, testing another beneficial organism rather than a parasitoid is appropriate. Therefore, the Agency recommends additional maximum hazard dose laboratory testing of a beneficial coleopteran such as a carabid (ground beetle).

iv. Green Lacewing Larva Testing

An acceptable study (MRID No. 449043-12) was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on OPPTS Series 885-4340 Non-target Insect Testing, Tier I.

Green lacewing larvae were fed the Cry 3Bb1 protein in a moth egg (*Sitotroga* sp.) and water meal diet at rates of 400 and 8,000 ppm which is approximately equivalent to 1X and 20X the maximum protein concentration in plant tissue. There was 20% mortality in the negative control group on Day 10. Compared to the negative control, at day 10, there was no significant increase in green lacewing larval mortality when fed 1X (400 ppm) and 20X (8,000 ppm) the maximum Cry3Bb1 protein concentration found in plant tissue. At test termination mortality for the 1,000 ppm reference group was 43% (13 of 30) and 100% mortality in the 10,000 ppm reference group (potassium arsenate). The data show that the LD_{50} for green lacewing larvae exposed to Cry3Bb1 in diet is >8,000 ppm . Based on these results it is not expected that the green lacewing will be adversely affected when exposed to Cry3Bb1 in the field.

The preliminary review of the green lacewing larva study was initially found acceptable by the Agency. However the August 27, 2002 SAP concluded that the green lacewing (*Chrysoperla carnea*) testing was not appropriate. Several public comments also addressed this issue. The SAP concluded that "[t]he levels of exposure of *Chrysoperla* to active protein were not, for example, determined throughout their respective tests. The test protein was held for a week within a diet broth in the *Chrysoperla* test chamber, and could have degraded considerably."

Additional problems were recognized with the *Chrysoperla* laboratory study. Green lacewing are difficult to test in the laboratory because of a high rate of mortality. In this instance (MRID No. 449043-12), the test was terminated after 10 days because there was >20% mortality in the positive control. In addition, it is questionable whether the green lacewings are ingesting the Cry3Bb1 protein that is coated around moth eggs in a diet. Since green lacewing have piercing-sucking mouthparts, they may not be exposed to the protein on the external surface of the egg diet. Therefore, Monsanto should conduct a laboratory insect toxicity test on an alternate organism. The minute pirate bug (*Orius insidiosus*) would be a more appropriate species to test than the green lacewing. *Orius* typically occur in corn fields as egg predators and they typically feed on pollen. Therefore, a laboratory study should be conducted feeding *O. insidiosus* both pollen and purified protein in diet. Feeding *O. insidiosus* Cry3Bb1 protein in diet will allow for a test at the maximum hazard dose; whereas, feeding *O. insidiosus* pollen expressing the Cry3Bb1 protein will provide an evaluation of potential effects from actual exposure scenarios.

v. Lady Beetle Testing

Since the Cry3Bb1 protein specifically targets coleopteran (beetle) insects, particular attention is warranted regarding potential effects of MON 863 on lady beetles. In a memorandum from

Robyn Rose to Mike Mendelsohn dated March 10, 2000, in addition to a dietary exposure to the purified Cry protein (44903-14), the Agency requested a test demonstrating the effect of lady beetles feeding on corn pollen containing Cry3Bb1. Monsanto conducted three additional laboratory studies (MRID Nos 453613-01, 453613-02 and 455382-04) on two different lady beetle species (*Coleomegilla maculata* and *Hippodamia convergens*) in response to this request.

Adult Lady Beetle Protein Dietary Study

A diet containing purified Cry protein and honey was fed to the adult lady beetle (*H. convergens)* at rates one and 20 times the maximum protein concentration found in corn leaf tissue (MRID No. 449043-14) . When the negative control group reached 20% mortality on day 10 , the results showed no significant differences in the mortality rate between lady beetles fed 400 and 8,000 µg Cry3Bb1/mL of diet. Results from this study showed that the LC_{50} for Cry3Bb1 when incorporated in diet and fed to *H. convergens* is >8,000 µg Cry3Bb1 protein/mL diet. Mortality for the 1,000 and 10,000 µg potassium arsenate/mL diet groups were 55% and 95% respectively at day 10. This demonstrates that toxicity can be measured by mixing a test substance in the lady beetle diet. Lady beetles do not feed on corn plant tissue. They do, however, feed on corn pollen and prey on pest insects that may feed on corn tissue and contain Cry3Bb1 in their gut, thus exposing lady beetles to the Bt protein. There is approximately 390 µg Cry3Bb1/g fresh weight corn tissue. Lady beetle exposure is expected to be significantly lower than this since the corn tissue would be metabolized, eliminated, or otherwise degraded within the prey species. Since the maximum hazard dose LC_{50} was found to be 8,000 µg Cry3Bb1/mL diet which is 20 times higher than maximum expected exposure levels, no hazard from Cry3Bb1 in corn plants to adult lady beetles is anticipated.

Larval Lady Beetle Pollen Feeding Study

At certain times corn pollen may comprise up to 50% of lady beetle larvae's diet. Therefore the effects of corn pollen containing event MON 863 Cry3Bb1 protein on lady beetle larvae (*Coleomegilla maculata*) was evaluated (MRID No. 455382-04). Pollen was fed to lady beetle larvae in a diet consisting of equal amounts of lyophilized tephritid fruit fly eggs and bee pollen. Diets contained 50% pollen (93 µg Cry3Bb1/g fresh pollen weight) since this is the potential level of field exposure and an equal amount of the tephritid fruit fly diet. First instar lady beetle larvae were individually placed in test arenas to avoid cannibalism. There was not a statistically significant difference between developmental time of larvae to pupae and/or adults; nor was there a difference in adult weight survival between larvae fed bee pollen or corn pollen nor was there a difference between larvae fed Bt and non-Bt pollen. There was a significant difference between the reference group (potassium arsenate) and other test groups since no larvae survived in the reference group. The 100% mortality observed in the reference group verified that the lady beetles were ingesting the diet. This test was conducted with pollen levels greater than or equal to levels lady beetle larvae are expected to be exposed to in the field. Therefore, the LD_{50} for Cry3Bb1 expressed in corn pollen is >93 µg/g fresh pollen weight. This study demonstrates that lady beetle larvae will not be adversely affected by Cry3Bb1 field corn.

Adult Coleomegilla maculata and *Hippodamia convergens Pollen Feeding Studies*

Coleomegilla maculata lady beetle adults were fed diets of transgenic corn pollen mixed with fruit fly eggs to determine the potential effects of transgenic pollen to beetles (MRID No. 453613-01). The corn (MON 863) test pollen (assayed at the time of testing) contained the Cry3Bb1 protein at a concentration of 37.4 µg/g pollen. After 30 days of diet exposure, 83.3 and 80.0% of adult *C. maculata* survived in the test and control pollen groups, respectively. While these survival rates were significantly less than that in the assay control group (bee pollen which exhibited 100% survival), there were no significant differences between the test and control pollen groups. All adults in the positive control (arsenate treated corn pollen) died in less than 8 days. Results indicated that transgenic Bt corn pollen expressing the variant Cry3Bb1 protein have no significant negative effects on the survival of *Coleomegilla maculata* adults.

Hippodamia convergens adults were fed diets of transgenic corn pollen in honey to determine the potential effects of transgenic pollen to non-target beetles (MRID No. 453613-02). The corn (MON 863) test pollen (assayed at the time of testing) contained the Cry3Bb1 protein at a concentration of 37.4 µg/g pollen. After 15 days of diet exposure, 84% and 81% of adult *Hippodamia convergens* survived in the test pollen and control pollen groups, respectively. There were no significant differences in survival among the test pollen, control pollen and the assay control (honey only) treatment groups. Only 5% of beetles exposed to the positive control (arsenate treated corn pollen) survived. Results demonstrate that transgenic Bt corn pollen expressing the variant Cry3Bb1 protein had no significant negative effects on the survival of *Hippodamia convergens* adults from dietary exposure.

No adverse effects were detected when *Coleomegilla maculata* and *Hippodamia convergens* were fed MON 863 pollen in diet in the laboratory. Pollen levels fed on by the lady beetles in this study exceeded concentrations that are expected to be encountered in the field. Therefore, it can be concluded the MON 863 will not pose a hazard to lady beetle adults in the field.

vi. Collembola Feeding study

This study (MRID No. 449043-17) was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on OPPTS Series 885-4340 Non-target Insect Testing, Tier I

Collembola (*Folsomia candida*) were fed diets consisting of transgenic corn leaf tissue containing Cry3Bb1 protein mixed with dry granulated Brewer's yeast. Diets contained a ratio of 0.50, 5.0 and 50% corn leaf tissue in Brewer's yeast which was equivalent to 8.73, 87.3 and 872.5 µg Cry protein/gram diet respectively. The corn leaf tissue contained 1,745 µg Cry3Bb1 protein/g dried leaf tissue.

These results show a LD_{50} > 872.5 µg/g diet of Cry3Bb1 protein. The study also noted that a diet containing 50% corn leaf tissue expressing the Cry3Bb1 Bt protein (a maximum hazard dose) did

not adversely affect reproduction of Collembola. This test was conducted at concentration levels much greater than Collembola are expected to be exposed to in the field. The primary route Collembola would be exposed to Cry3Bb1 in the field is from decaying root tissue (and possibly from pollen to a much lesser degree). MON 863 is expressed in corn roots in the range of 3-66 µg/g which is significantly lower than the levels used in this test.

This study adequately addresses potential concerns for Cry3Bb1 protein expressed in transgenic corn to Collembola (*Folsomia candida*) a representative of beneficial soil insect species. The results of this study demonstrate that Cry3Bb1 proteins found in transgenic corn pose no hazard to soil inhabiting Collembola species, and by inference to other beneficial non-coleopteran soil insects. It is notable that recent recommendations by the Scientific Advisory Panel (March, 2001) are that invertebrates of different orders than those known to be affected by the Cry protein in question not be tested.

vii. Earthworm Toxicity Testing

Background:

Earthworm feeding studies submitted to the Agency for all of the registered Cry proteins demonstrate that the Cry proteins are not toxic to earthworms at the worst case environmental concentration. Some public comments have voiced concerns as to whether the earthworms actually ingested the Bt Cry proteins when these are incorporated into the soil in the test systems used. Recently published data show that the earthworms do, however, ingest the Bt Cry proteins with the soil without harmful effects. The data also show that there were no significant differences in the percent mortality and weight of earthworms after 40 days in soil planted with Bt or non-Bt corn, in fallow fields, or after 45 days in soil amended with biomass of Bt or non-Bt corn or not amended. The Bt Cry protein was shown to be present in both the casts and guts of the worms.

Cry3Bb1 Earthworm testing:

MRID No. 449043-16

The study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Parts 160 and 792; Organization for Economic Development (OECD) Principles of Good Laboratory Practice; and Japan Ministries of Agricultural Forestry and Fisheries (MAFF), with certain exceptions that did not affect the integrity of the test. The testing was conducted based on OPPTS Series 850.6200 Earthworm Subchronic Toxicity Test and OECD Guideline 207. This study meets current testing requirements for assessing risks to earthworms from plant-incorporated protectants derived from *Bacillus thuringiensis*.

The 14-day LC_{50} for earthworms exposed to Cry3Bb1 protein 11231 in an artificial soil substrate was determined to be greater than 570 mg Cry3Bb1 protein/kg dry soil. However, the percent mortality reported was 38% . The mortality in the in the 57.0 mg/kg group was 8%. It was noted

in the study design that the levels of buffer salt in the test groups were higher than expected because of a miscalculation. The actual concentration of sodium bicarbonate salt in the 57.0 and 570 mg Cry3Bb1 protein/kg treatment groups was 70 and 699 mg/kg, respectively. The higher concentrations did not appear to have any influence on the overall conclusions of the study. However, another earthworm study (MRID No. 457571-01) was performed.

MRID No. 457571-01

The submitted study is classified as acceptable, it is scientifically sound, and is consistent with current testing requirements for earthworm hazard assessment. The 14-day LC_{50} for earthworms exposed to purified 11098 Cry3Bb1 (*E. coli* -produced) protein in an artificial soil substrate was determined to be greater than 166.6 mg /kg dry soil (the highest concentration tested), or greater than 20 times the worst case EEC in a corn field. There was no apparent effect of the phosphate buffer on the earthworms. There were no earthworm mortalities in the any of the controls or Cry-protein treated soils during the 14 day study. Changes in average body weights were not statistically different ($p>0.05$) among the controls and protein-amended soils. There were no other remarkable observations. At the end of the study, mortality in the 10 and 20 mg chloroacetamide/kg soil was 2.5% (1 of 40) and 85% (34 of 40), respectively. Percent mortality of earthworms in the reference substance (chloroacetamide) groups was consistent with historical results, and further confirmed the adequacy and consistency of the protocol used in the definitive test.

The reviewed data show that no adverse effects to earthworms are expected in fields growing Cry3Bb1 corn plants.

viii. Monarch Butterfly Larval Pollen Feeding Study

This study (MRID No. 455382-05) was not required nor requested for Cry3Bb1 because Cry3Bb1 it is a coleopteran active protein that is not expected to affect lepidopterans such as the monarch butterfly. In addition, extensive research conducted on the potential affects of monarch feeding on lepidopteran-active Bt corn pollen has shown a lack of concern for subchronic toxicity. However, due to recent public concern for possible adverse effects of Cry3Bb1 corn on monarchs, Monsanto sponsored this study and submitted it to the Agency for review. This study has demonstrated that corn pollen expressing the Cry3Bb1 protein will not result in subchronic toxic or developmental effects to monarch larvae. Neonate monarch survival was not affected after feeding on milkweed dusted with up to 3200 pollen grains/cm^2. expressing Cry3Bb1 for 2, 4 or 10 days of pollen exposure. Larval development, weight gain and milkweed leaf consumption were also not affected by feeding on Bt pollen 96 hours and 10 days after exposure. Pollen densities in the field are not expected to be as great as 3200 grains/cm^2. Pollen densities in the field average 150 grains/cm^2. Levels of 400 and 800 pollen grains/cm^2 would probably be rare. Therefore, results of this study indicate that young monarch larvae (at the most sensitive stage) will not be adversely affected by exposure to corn pollen expressing Cry3Bb1 in the field. However, since Cry3Bb1 expressed in MON 863 is a coleopteran-active protein, the August 27, 2002 SAP concluded that the monarch butterfly was not an appropriate indicator organism to be tested. The SAP recommended testing *Tetraopes* (red milkweed beetles) as a more logical

choice than the monarch butterfly.

ix. Insecticidal Activity Spectrum Study

Insecticidal spectrum of activity bioassays were conducted on six Coleoptera Families and two Lepidoptera species (MRID No. 455328-07). A series of six to eight concentrations of CryBb1 protein (1ppm - 8000ppm) in standard insect diet preparations were used to conduct seven day mortality testing. Significant insecticidal activity was seen only in the family Chrysomelidae (corn rootworm and Colorado potato beetle) of the Order Coleoptera. No activity was seen against the cowpea weevil (fam. Bruchidae), lady bird beetle (Coccinellidae), red flour beetle (Tenebrionidae), cotton boll weevil, pepper weevil and rice weevil (Curculionidae). The Lepidoptera corn earworm (Noctuidae) and European corn borer (Crambidae) were also not affected. This efficacy study was reviewed for environmental assessment purposes to expand the number of insect species examined for possible toxicity of the Cry3Bb1 protein. The results confirm the assertion that Bt Cry proteins have a very specific and narrow range of target species.

b. Field Evaluation of Cry3Bb1 Corn Exposure on non-target Invertebrates

i. Introduction

The Scientific Advisory Panels (October, 2000 and August, 2002) recommended that non-target testing be focused on species exposed to the crop being registered. The Agency has determined that the non-target organisms most likely to be exposed to the Cry3Bb1 protein in transgenic corn fields were beneficial insects feeding on corn pollen and nectar, and soil invertebrates, particularly Coleoptera spp. In addition to extensive and difficult maximum hazard dose single species soil coleopteran toxicity testing followed by an extrapolation from the results to a community risk assessment, and the fact that all of the species cannot be tested in the laboratory, direct field test and field census data on coleopteran insect effects and abundance were requested, received and evaluated. These studies are being conducted in several States by Monsanto Co. and several independent University scientists. The results of these studies are summarized below. Some of the submissions consists of preliminary results of studies in progress. The Agency is requesting that Monsanto submit the final reports of these studies as they become available.

These preliminary field and field census data with the study design methodologies have been presented to a Scientific Advisory Panels (August, 2002). The SAP commented that the study designs lack appropriate statistical power, but that methodology for conducting statistically valid field census studies at the scale necessary to determine ecosystem effects is not available. Such methodology is yet to be developed. As a result, the Agency is reviewing the available field studies as data supplemental to the maximum hazard dose single species laboratory testing but useful for short range assessment of non-target invertebrate abundance in Cry3Bb1 corn test plots. It is an accepted practice in the Office of Pesticide Programs to use the trends seen in several supplemental studies for hazard assessment when a perfect study is not available.

ii. Preliminary Invertebrate Field Census data (MRID No.455382-06)

These two year Tier IV field studies are intended to supplement the Tier 1 maximum hazard dose findings. Invertebrates in Cry3Bb1 corn field plots were sampled from the soil, soil surface and foliage. Soil-dwelling invertebrates were collected using a "pan trap" which utilized a modified Burlese extraction method. Surface-dwelling invertebrates were sampled in the field with pitfall traps. Foliage-dwelling invertebrates were monitored by yellow sticky traps (Pherocon AM™) set in the field at canopy level and adjusted as the season progressed. Sampling for lady beetles was also done using a drop cloth technique. Preliminary results do not show any MON 863 corn related adverse effect on non-target and beneficial invertebrate abundance in the field. The final report (MRID No. 457916-01) is reviewed below.

iii. **Final Invertebrate Field Census data** (MRID No. 457916-01)

Methods:

During the 2000 and 2001 growing seasons, event MON 863 CRW-protected corn and non-transgenic corn (hybrid RX670) were grown in Warren County near Monmouth, IL. Plots were planted in different locations located approximately 1.5 miles apart in Warren County each year. The field used in 2000 consisted of silt loam (3.6% organic matter; pH of 6.1) and in 2001 was a silty clay loam (4.0% organic matter; soil pH of 6.4). Corn was planted in both fields the previous year and soybeans were planted two years prior to conducting these field trials. All experimental plots were managed according to typical cultural practices of commercially grown corn in the region and included the application of the herbicides acetochlor and atrazine after planting and before emergence.

During the 2000 and 2001 growing seasons, Bt (MON 863) and control hybrids (RX670) were the main plots planted in a split-plot design with four replications planted 20 ft apart. Rows were planted 30 inches apart, seeded at a rate of approximately 1.7 seeds/ft and planted 1.5 - 1.75 inches deep. Plots (240 ft × 60 ft) were divided into 24 row subplots (60 ft × 60 ft) that served as replications receiving one of 4 insecticide regimes. Insecticide treatments of the Bt and non-Bt plots included: 1. No insecticide; 2. Seed treated with Gaucho® prior to planting; 3. The granular insecticide Force 3G® applied and incorporated in furrows at planting; and 4. A foliar insecticide, Pounce 3.2 EC®, applied at the V10 and R2 corn growth stages to control 1st and 2nd generation CRW adults. A 4-row buffer of non-transgenic corn was planted around each plot to minimize edge effects from adjacent subplots.

Data were collected on agronomic and phenotypic characteristics, pest (insect and disease) susceptibility, soil quality and fertility, microbial populations and non-target invertebrate abundance. Eight inch deep soil samples were taken to evaluate quality and fertility. Four samples were taken during the growing season and two samples post-harvest in 2000; two samples were taken during and after the growing season in 2001 for a total of ten samples. Microbial populations were evaluated from test and control plots that received no insecticide regime a total of 14 times during the growing season from the top six inches of soil within six inches of the rhizosphere. Samples were taken in 2000 and 2001 during the V2, V4, V8, R1 and R6 stages as well as after tillage and the following spring. Soil samples from the 2000 growing season were analyzed for bacteria, mold and yeast by a heterotrophic plate count method. Four of

the seven soil samples collected during the 2001 growing season were analyzed for bacteria, actinomycetes and fungi by the "viable plate count method".

Soil-dwelling invertebrates were collected from root balls including soil during the V6, V10 and R1 growth stages during the 2000 and 2001 growing seasons. Three eight inch root balls were collected during the V6, V10 and R1 corn growth stages from all control and test plots and processed through a Berlese funnel to extract invertebrates. Earthworms were also collected from soil samples by hand sorting. Ground surface-dwelling invertebrate were collected from all test and control plots under all insecticide regimes using pitfall traps. Four pitfall traps were placed in each subplot from the V6 to R4 corn growth stages for three-day periods. Key invertebrates from pitfall traps were counted and identified to family level. Flying and foliage-dwelling invertebrate were collected from each subplot using yellow sticky traps. Three traps per subplot were placed at canopy level from the V6 to R4 corn growth stages. Traps were left in the field for seven days and all key taxa were counted and identified to family or genus level. Data were analyzed using a mixed linear repeated measures model for each invertebrate collected by each sample method.

Results:

Among the three sample methods (soil, pitfall and sticky trap) there was a total of 156,572 organisms from 16 orders and 36 families identified during the 2000 and 2001 growing seasons. Collected invertebrates included pests, predators, parasitoids, detritivores and decomposers. The predominant nontarget invertebrates collected in each sample method are summarized in Table 2.

Table 2. Predominant non-target invertebrates collected in each sample method

Sample Method	Order (Family)
Soil and Root Samples (soil-dwelling invertebrate)	Diplura (Japygidae), Chilopoda, Aranea, Acari, Oligocaeta (earthworms), Coleoptera (Carabidae, Staphylinidae, Nitidulidae, Lanthridiidae), Hymenoptera (Formicidae)
Pitfall Trap Samples (ground surface-dwelling invertebrate)	Orthoptera (Gryllidae), Coleoptera (Carabidae, Staphylinidae, Nitidulidae, Scarabeidae, Chrysomelidae), Hymenoptera (Formicidae), Araneae, Chilopoda
Yellow Sticky Trap Samples (flying & foliage-dwelling invertebrate)	Coleoptera (Chrysomelidae, Nitidulidae, Coccinellidae), Hymenoptera, Homoptera (Aphidoidae, Cicadellidae), Hemiptera (Anthocoridae), Diptera (Syrphidae), Neuroptera (Chrysopidae, Hemerobiidae), Aranea

Overall, there was no statistical difference between MON 863 and non-Bt plots (RX670) in the abundance of the predominant invertebrates collected in soil samples. According to the soil sample data, the number of Japygidae (diplurans) did not differ between Bt and non-Bt plots; however, there were significantly less collected in the insecticide treated plots. Of the coleopteran insects collected, there were generally more carabids (ground beetles) and staphylinids (rove beetles) than nitidulids (sap beetles) and lanthridiids (minute brown scavenger beetles). There was no statistical difference in the number of coleopteran insects captured between the MON 863 and non-transgenic corn isolines. Statistical analysis showed that insecticide treatments significantly reduced the number of carabids in 2000 and the number of

staphylinids on the last sample date in 2001. Invertebrates from the Araneae (spider) and Acari (mite) families were also not significantly different between Bt and non-Bt plots; however the number of acarids (mites) were statistically greater in the plots treated with foliar insecticides in 2001. The number of chilopods (centipedes), the most abundant non-insect arthropod sampled, was not different between Bt and non-Bt isolines, but more were collected in plots treated with seed, soil and foliar insecticides in 2001 particularly the MON 863 treated plots. Although the number of earthworms collected did not differ between Bt and non-Bt plots, there were significantly less earthworms in the plots treated with foliar insecticides than the other insecticide regimes. The hand-sorting method also showed no differences in the number of earthworms collected in MON 863 and non-transgenic plots, nor was there a difference found between insecticide regimes.

Overall, there was no statistical difference between MON 863 and non-Bt plots (RX670) in the abundance of the predominant invertebrates collected in pitfall trap samples. Of the coleopterans captured in pitfall traps, there were more nitidulids (sap beetles), carabids (ground beetles) and staphylinids (rove beetles) captured than chrysomelids and scarabids collected. There was no statistical difference between MON 863 and RX670 field-plots in the overall number of coleopterans collected in pitfall traps. The different insecticide regimes tested resulted in varied and inconsistent effects on the abundance of the predominant Coleoptera sampled. The number of gryllids (crickets) and formicids (ants) collected in pitfall traps was not statically different between Bt and non-Bt plots or different insecticide regimes. Chilopod (centipede) and Araneae (spider) abundance was not different between MON 863 and RX670 plots nor was there an affect from insecticide regimes in most cases. Statistical analysis showed that there were significantly fewer Araneae (spider) in the plots with soil and foliar insecticide treatments in 2000.

Based on yellow sticky trap counts, there were consistently less corn rootworm (CRW; *Diabrotica virgifera* and *Diabrotica barberi*) in the MON 863 plots than the non-Bt plots in all insecticide regimes. However, the difference in the number of CRW captured was not statistically different. In both 2000 and 2001, there were no significant differences between MON 863 and RX670 plots across all insecticide regimes in the number of nitidulids (sap beetles), coccinellids (lady beetles), aphidids (grass hoppers), cicadellids (potato leaf hoppers), braconids (*Macrocentrus grandii* - a parasitoid), syrphids (syrphid or hover flies), hemerobiids (brown lacewing), chrysopids (green lacewing) and Araneae (spiders). There was a reduction in the abundance of *Coleomegilla maculata* (lady beetles) and *M grandii* and an increase in *Empoasca fabae* (potato leaf hopper) in plots receiving a foliar spray. Syrphid fly abundance was reduced in 2000 by soil insecticide treatments.

Field Census Summary:

Data collected during the 2000 and 2001 growing seasons indicate that MON 863 and RX670 corn are agronomically and phenotypically equivalent and there are not differences in their susceptibility to pathogens. Soil quality and fertility were also found to be consistent among MON 863 and RX670 field plots. Sampling data collected in 2000 and 2001 also showed that there are no overall differences in the abundance of non-target invertebrate collected in MON 863 and RX670 plots. However, corn pests such as *D. virgifera*, *C. pulicaria* and *R. maidis* as well as predators (e.g., *O. insidiosus* and *C. maculata* [lady beetle], parasitoids (e.g., *M. grandii*)

and decomposers (e.g., earthworms and diphrans) were significantly impacted by insecticide regimes. Foliar sprays and soil treatments resulted in the greatest impact on non-target organisms such as carabids, spiders, *O. insidiosus* (a generalist predator), *C. maculata* (lady beetle) and *M. grandii* (parasitic wasp). Therefore, the report concluded: "MON 863 had less impact on certain beneficial insects compared to traditional insecticide control programs, especially soil and foliar applications. Thus, the use of MON 863 for corn rootworm control can lead to reduced use of insecticides and increased compatibility with Integrated Pest Management programs in corn."

Field Census Conclusions:

According to the data submitted to the Agency by Monsanto, MON 863 corn does not adversely impact the abundance of non-target invertebrate found in corn fields. However, plot size (240 ft × 60 ft plots divided into 24 row 60 ft × 60 ft subplots) was small and only replicated four times. In addition, each plot only included three root and sticky trap samples and four pitfall trap samples. The August, 2002 SAP concluded that field experiments must be appropriately designed to provide a measure of ecological impacts. In addition, the SAP opinion was that a two year field study would not be sufficient to determine if MON 863 corn will have long term impact on non-target invertebrates. Several public comments also expressed this concern. Short-term field studies are not adequate to draw conclusions on the variations in non-target invertebrate populations. Large field-scale studies conducted for at least three to four years would be needed to draw a conclusion on non-target impacts. The Panel generally concluded that "the state-of-the science" needed for long-term studies must improve for the research to be appropriately conducted to provide meaningful results. The statistical power (avoiding Type II experimental error) needed to gain useful results from field studies would require very large fields, more replications and more samples per plot (e.g., 10 soil and pitfall samples) plus the addition of visual plant samples (e.g., >50/plot). Since the endpoint for field census studies has not been determined, it is difficult to determine how large the fields should be, how many replications are needed and how many samples per plot are needed to achieve appropriate statistical power. Therefore, additional field census studies should not be conducted until the endpoints and logistics of the study have been determined. If Tier I maximum hazard dose single species laboratory studies show a hazard, intermediate field or semi-field studies between laboratory and full-scale field studies should be conducted. Additional full scale field or semi-field studies with appropriate end points and statistical power should also be considered based on recommendations of the August 27, 2002 SAP.

However, the submitted field census data demonstrating an abundant presence and diversity of invertebrates in the corn CryBb1 corn field are useful for short term hazard assessment as supplementary information which shows the same no-hazard trend seen in the maximum hazard dose single species laboratory testing.

Year 2001: Field and Laboratory invertebrate studies (MRID No. 456530-03)

A summary of preliminary findings from several one year supplemental higher Tier field and laboratory studies was submitted (MRID No. 456530-03). These studies were not triggered by Tier I maximum hazard dose testing data, however they appear to have value for assessing

possible for long term invertebrate population shift effects. These studies are being conducted in Kansas, Nebraska, Illinois, Virginia, South Dakota and New York to evaluate the ecological impact of MON 863 Bt corn grown under different insecticide regimes on abundance of non-target organisms relative to non-transgenic corn. The submitted summary of preliminary findings shows some possible effects of MON 863 on corn field insects. Preliminary data are being analyzed which suggest an increase in Coccinellid (ladybeetle) larvae, an increase in abundance of mites on MON 863 residues, a laboratory study showing significantly reduced root population and egg production of the plant pathogenic nematode *M. incognita*, a reduction in the population of the non-pathogenic *C. elegans* in root extracts (no effects in soil leachate were seen), and no effects were noted on the entomo-pathogenic nematode *S. carpocapsae*. It was also reported that of 14 species of Collembola, a decrease in one species was noted, although no effect was seen on total Collembola abundance. One inconclusive study found a decrease in Carabids, however these data are suspect because of limitations in movement of ground dwelling insects due to problems with plot layout designs. The studies are being repeated in more adequately rearranged and randomized plots. A final report of these studies is to be submitted to the Agency for review.

4. Soil Degradation Studies (Environmental Fate)

Soil organisms may be exposed to Cry3Bb1 protein by exposure to roots, incorporation of above ground plant tissues into soil after harvest, or by pollen deposited on the soil. Root exposure may occur by feeding on living or dead roots or, theoretically, by ingestion or absorption after secretion of the Cry protein into the soil. In addition, some evidence suggests that Cry proteins while bound to some soil components, e.g. clays and humic acids, are recalcitrant to degradation by soil microorganisms, but without eliminating their insect toxicity. Several factors influence either the affinity of binding or the rate of degradation. In particular, pH near neutrality generally substantially increases degradation. Corn does not grow well below ~pH 5.6 and therefore most corn growing soils are expected to be at a higher pH. Therefore, under most production conditions, corn would not be grown on soils that would inhibit the rate of degradation compared to what is seen at near neutral pH. Nevertheless, these issues are being evaluated on a case-by case basis by environmental fate studies designed to determine the rate of Cry protein degradation over sufficiently long periods to assure an accurate assessment of degradation in agricultural soils

MRID No. 451568-04

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

Methods:

An insect bioassay and an ELISA was conducted to measure the level of functional and non-functional Cry3Bb1 protein present in field soil samples. The amount of lyophilized corn tissue added to the field soil in this study was based on the amount of plant tissue that could potentially be incorporated into the top six inches of soil under field conditions. Since field incorporation of plant tissue usually will not take place until the Fall, this amount of Cry3Bb1 protein represents

the worst-case scenario during the growing season, including possible exudation of Cry protein through the roots into soil. Based on this calculation 0.03 g (rounded up from 0.028) dry weight plant tissue was added to each gram of dry sandy loam field soil (from Fayette County of Lexington, KY.); therefore, 3% of the dry weight of soil was dry weight of lyophilized plant tissue. An additional test was conducted with 10% of the soil containing lyophilized dry weight plant tissue (0.10 g leaf tissue/g soil). Insect bioassays included a mixture of test and control substances with an agar-based insect diet added to wells of bioassay trays. Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) larvae were added one larva/well and each treatment bioassay was replicated twice for a total of 16 CPB/replicate. CPB are more sensitive to the Cry3Bb1 protein then CRW and were, therefore, expected to result in a more measurable response than CRW, the target species. In addition to an insect bioassay, an ELISA was conducted to measure the level of Cry3Bb1 protein present in samples. However, the ELISA test will only show extractable protein and does not distinguish between functional and non-functional proteins.

Results:

Results from this study show the DT_{50} and DT_{90} (degradation time) for Cry3Bb1 in leaf tissues protein in sandy loam soil based on ELISA test to be 2.76 and 9.16 days. The 21 day ELISA sample was the last to show traces of Cry protein. At 28 days the Cry3Bb1 protein was below the detection level. However, the value of these results need to be considered with regard to biological activity because it is unknown if the extractable protein in the ELISA test was functional or non-functional. Therefore the insect bioassays were performed with CPB and determined the DT_{50} and DT_{90} to be 2.37 and 7.87 days respectively. The no-detection level was in the range of the results obtained by ELISA.

Conclusion:

Based on these results Cry3Bb1 protein does degrade rapidly and does not accumulate in sandy loam soil. However, corn is not necessarily grown in sandy loam soil in all regions. Corn is grown in other soil types such as clay loam and silt loam soils in various regions of the U.S. Testing clay soils would, therefore, be considered a "worst case scenario." In addition, this test does not account for all plant tissue such as roots, or root exudation of the Cry protein in the field. It is possible that root tissue is degrading slower then leaf tissue in the soil which may result in a longer duration of degradation time of the Cry3Bb1 protein. Therefore, it was recommended that field testing should be continued in a variety of soil types including clay and humic acid soils, over a three year period of time, to determine the long-term degradation rate and accumulation/persistence of Cry3Bb1 protein in soil.

Since the submission and review of the above study (MRID No. 451568-04), the Agency convened a Scientific Advisory Panel (SAP) on August 27, 2002 to address corn rootworm plant incorporated protectant (PIP) non-target issues including the degradation of the Bt protein in soil. The following is a review of a second aerobic soil degradation study (MRID No. 457571-02) designed to address some of the deficiencies identified in the first study above, and a review of comments made by the August SAP as well as by several public comments.

MRID No. 457571-02

This study is intended to address the issues raised in the study (MRID No. 451568-04) described above. That study was classified as "[s]upplemental to conducting the study in the field with all plant tissue incorporated into the soil in fields that have had MON 863 corn grown for one to three consecutive years. Studies should also be conducted in a variety of soil types particularly soil high in clay and humic acids" as well as other tissue, particularly roots. In addition, the previously submitted aerobic soil degradation study (MRID No. 451568-04) utilized plant tissue from Cry3Bb1 transformation event MON 859 rather than MON 863. The second submission (MRID No. 457571-02) utilized MON 863 corn root and shoot tissue.

Methods:

The test substance consisted of finely ground and lyophilized root and shoot tissue of the corn event MON 863 containing the Cry3Bb1 insecticidal protein. The concentration of Cry3Bb1 protein in the lyophilized corn tissue, determined by ELISA, was 487 µg/g in the root and 468 µg/g in the shoot. A purified Cry3Bb1 protein obtained from a genetically modified *E. coli* strain containing the identical sequence as the MON 863 corn was also included in the study. MON 863 corn shoot and root tissue were collected from field grown plants in Richland, IA. The test matrix consisted of three soils collected from the top six inches, Horizon A, of corn fields in Carlyle, IL, Monmouth, IL and Richland, IA. Soil properties for these three soils (Table 3) and a microbial analysis were characterized. Soil viability was confirmed at test initiation and at approximately four and eight weeks of incubation. Soils were shown to remain active and viable throughout the study (144-228 microbial biomass carbon/50 g soil). The highest concentration of Cry3Bb1 protein in roots 35-days after planting is 66 µg/g. On this basis the maximum field loading of Cry3Bb1 in corn shoots is 3.93 µg/g soil (equivalent to 8 mg shoots/g soil) and for corn root tissue the loading is 2.79 µg/g soil (equivalent to 6 mg of roots/g soil).
For additional conservatism, the maximum values were exaggerated 3X for dosing of soils. Therefore, 24 mg of shoot tissue and 18 mg of root tissue was added to soils. Also, the purified Cry3Bb1 control dosing concentration was exaggerated ~25X to model the unlikely scenario that large amounts of protein would be exuded by roots into the soil throughout the growing season. Approximately 48 µg of purified protein was added to 0.5 g of soil. Soils from the three test locations were air dried and dosed with the Cry3Bb1 test substance at these rates. All vials were mixed thoroughly and soil moisture adjusted with deionized water to obtain soil moisture of 75% field capacity at 0.33 bar.

Table 3. Physicochemical characteristics of soils collected from Horizon A (top 6 inches).

Parameter		*Soil Source*		
		Carlyle, IL	**Monmouth, IL**	**Richland, IA**
USDA Textural Class		Silt Loam	Silt Loam	Silt Loam
Particle Size Distribution (%)	Sand Silt Clay	21 58 21	18 56 26	13 62 25
Bulk Density (g/cm^3)		1.00	1.03	1.07

Parameter		Soil Source		
		Carlyle, IL	Monmouth, IL	Richland, IA
% Organic Matter		2.5	4.6	4.0
CEC (meq/100g)		16.6	23.9	20.7
Field Moisture Capacity	@ 1/3 Bar	28.2	30.7	30.3
	@ 15 bar	15.7	17.9	17.2

Table copied from page 29 of 80 of MRID No. 457571-02

Results:

Cry3Bb1 protein levels in soil sample extracts determined by ELISA show that Cry3Bb1 protein concentrations were near or below the ELISA detection limit (LOQ) of 0.16 µg/g after 2 months of incubation. After two months of soil incubation, Cry3Bb1 concentrations were at least an order of magnitude below the initial concentration of Cry3Bb1 in the dosed samples. The DT_{50} values for all dosing regimes and soil types ranged from 0.6 days to 2.3 days and the DT_{90} values ranged from 4.03 days to 50 days (Table 4 reproduced from page 36 of the study submission). Purified Cry3Bb1 protein degraded faster than when corn shoot or root tissue was applied. Visual observation verified that root tissues are slower to degrade in soil than shoot tissue. These results also indicate, as expected, that the longer DT_{50} and DT_{90} values for corn tissues are due to the time required for the tissue to decay and for the Cry3Bb1 protein to move from tissue to soil. In addition, the rapid degradation (DT90 4.0 to 5.2 days) of the purified Cry3Bb1 protein suggest that any Cry3Bb1 protein reaching the soil by root exudation or release from slowly decaying plant tissue would be >90% degraded in less than six days.

Table 4. DT50 and DT90 estimates for the dissipation of Cry3Bb1 protein in soils

Protein Source	Soil Source	DT50[a] (days)	DT90[b] (days)
Purified Protein	Carlyle, IL	0.63 (0.49, 0.78)[c]	4.03 (3.67, 4.42)
	Monmouth, IL	0.64 (0.50,0.80)	4.18 (3.77, 4.62)
	Richland, IA	0.73 (0.50, 1.00)	5.23 (4.48, 6.07
MON 863 Corn Root Tissue	Carlyle, IL	1.74 (0.78, 3.20)	27.29 (14.52, 50.58)
	Monmouth, IL	1.19 (0.86, 1.57)	12.48 (9.90,15.67)
	Richland, IA	2.27 (1.68, 2.98)	50.02 (34.57, 72.18)
MON 863 Corn Shoot Tissue	Carlyle, IL	1.45 (0.82, 2.30)	18.68 (12.61, 27.46)
	Monmouth, IL	0.90 (0.61, 1.24)	7.43 (6.01, 9.12)
	Richland, IA	1.77 (1.40, 2.21)	28.59 (23.16, 35.23)

[a]DT50=Time to 50% dissipation of original protein concentration
[b]DT90=Time to 90% dissipation of original protein concentration
[c]Lower and upper 95% confidence interval on the DT value

Conclusions

It is difficult to determine a DT_{50} or DT_{90} for Cry3Bb1 expressed in corn tissue in the field from this study because corn shoot and root tissue were analyzed separately and not all plant material was included. Therefore, methods utilized in this study do not represent actual field conditions. It is unknown whether these laboratory results can be adequately correlated to the field. Additional field studies should be conducted that include the incorporation of all non-harvested plant tissue in a variety of soil types particularly areas high in clay (>26% tested here) and humic acids. These studies should be conducted for at least one growing season after harvest and continue until no Cry3Bb1 protein is detected. In addition the persistence of the Cry3Bb1 protein under less than optimum conditions (e.g., high or low temperatures; high or low soil moisture content) should be examined. Additional studies conducted to address the degradation of Cry3Bb1 protein in the soil should include an insect bioassay utilizing a known sensitive species such as the Colorado potato beetle.

These conclusions are based in part on the August 27, 2002 SAP and several public comments. The Panel concluded that several different soils should be examined and monitored for a minimum of one growing season after harvest and continued until the Cry3Bb1 protein can no longer be detected. The Panel also recommended that an additional sample or two should be examined to verify that an analytical error was not the cause for the lack of detection. According to the Panel, at least two additional soil types should be evaluated for Cry3Bb1 persistence. Soils that are high in organic matter and clay should be concentrated on since there is the highest potential of persistence in these soil types. However, additional soils should also be considered. The Panel also recommended that the soil degradation studies be conducted under less than optimum conditions such as high or low temperatures or high or low moisture content. Since corn roots grow deep into the soil to areas with reduced microbial activity, degradation rates may be reduced. Therefore, degradation of Cry3Bb1 from deep sites should also be examined. The Panel also addressed the protein source that is appropriate for the soil degradation studies. Future studies should utilize plant material that is representative of actual field conditions. For example, whole plant tissue should be incorporated. Plant tissue should not be ground prior to incorporation because it artificially increases the surface area exposed to microorganisms which may lead to an increase in the rate of degradation of the protein. Since more protein may be present than is detected by an ELISA, an insect bioassay using a sensitive species such as the Colorado potato beetle should be conducted. The SAP concluded that "[r]eal life or true persistence is likely to be equal to or less than that measured with ELISA." If an ELISA is conducted, the results should be compared to results from a beetle bioassay.

This study, although rated as supplemental, provides further evidence that the Cry3Bb1 protein in corn event MON 863 produces no short term risk of unreasonable adverse effects for the environment. Because the Agency believes that additional studies would be useful in completing the database for long term effects assessment, it is requesting additional supplementary studies regarding Cry3Bb1 protein degradation in soil.

5. Effects on Soil Microorganisms

Published studies on the impact of transgenic Cry producing plants indicates that adverse effects

on soil microorganisms are unlikely. No effects have been seen due to the protein itself, and only a minimal, transient increase observed in soil microbes attributed to the transgenic cotton plant tissue rather than the Cry protein expressed in that tissue. No adverse effects have been observed in a similar season long field study with Cry3A potato.

6. Horizontal Transfer of Transgenes from Bt Crops to Soil Organisms

The Agency has evaluated the potential for horizontal gene transfer (hgt) from Bt crops and has considered possible risk implications if it occurred. Several experiments published in the scientific literature have been conducted to assess the likelihood of hgt, and have been unable to detect gene transfer under typical conditions. Hgt has only been detected under conditions designed to favor transfer. In addition, the genes that have been engineered into the Bt crops are mostly found in, or have their origin in, soil inhabiting bacteria. Soil is also the habitat of anthrax, tetanus and botulinum toxin producing bacteria. No transfer of these genes or toxin to other microorganisms or plants has been detected. Therefore, the Agency concluded that hgt is at most an artificial event, and the traits engineered into the Bt crops are already present in soil bacteria or are unlikely to have selective value for soil microorganisms. In considering these data the Agency further concludes that there is no significant risk from hgt from the transgenes found in the Cry3Bb1 producing corn.

7. Gene Flow and Weediness Potential

The movement of transgenes from the host plant into weeds has been a significant concern for the Agency due to the possibility of novel exposures to the pesticidal substance. The Agency has determined that there is no significant risk of gene capture and expression of Cry3Bb1 protein by wild or weedy relatives of corn in the U.S., its possessions or territories. In addition, the USDA/APHIS has made this same determination under its statutory authority under the Plant Pest Act.

Under FIFRA, the Agency has reviewed the potential for gene capture and expression of the *B.t.* endotoxins by wild or weedy relatives of corn, cotton and potatoes in the U.S., its possessions or territories. *B.t.* plant-incorporated protectants that have been registered to date have been expressed in agronomic plant species that, for the most part, do not have a reasonable possibility of passing their traits to wild native plants. Feral species related to these crops, as found within the United States, cannot be pollinated by these crops (corn, potato and cotton) due to differences in chromosome number, phenology (*i.e.*, periodicity or timing of events within an organism's life cycle as related to climate, *e.g.*, flowering time) and habitat. The only exception, however, is the possibility of gene transfer from *B.t.* cotton to wild or feral cotton relatives in Hawaii, Florida and the Caribbean.

The Scientific Advisory Panel meeting held on October 18-20, 2000 further discussed the matter of gene flow and offered some issues for consideration in this matter. The panel agreed that the potential for gene transfer between corn (maize) and any receptive plants within the U.S., its possessions and territories was of limited probability and nearly risk free.

Concern over the potential for species related to maize (*Zea mays* ssp. *mays*), such as *Tripsacum*

species and the teosintes, as potential recipients of gene flow from genetically modified *Zea mays* indicated a need for review of what is known related to gene flow potential of *Zea mays*. Some *Zea* spp., such as the teosintes, are known to be interfertile with maize and are discussed as potential recipients of pollen directed gene flow from maize. This issue is of particular concern based upon the increased planting of genetically modified maize. Therefore, the Agency conducted a reevaluation in early 2000, the results of which are reported here.

a. *Zea mays* ssp. *mays* - Maize - General Biology

Zea mays is a wind-pollinated, monoecious, annual species with imperfect flowers. This means that spatially separate tassels (male flowers) and silks (female flowers) are found on the same plant, a feature that limits inbreeding. A large variety of types are known to exist (*e.g.*, dent, flint, flour, pop, sweet) and have been selected for specific seed characteristics through standard breeding techniques. Maize cultivars and landraces are known to be diploid (2n = 20) and interfertile to a large degree. However, some evidence for genetic incompatibility exists within the species (*e.g.*, popcorn x dent crosses; Mexican maize landraces x Chalco teosinte). *Zea mays* has been domesticated for its current use by selection of key agronomic characters, such as a non-shattering rachis, grain yield and resistance to pests. The origin of corn is thought to be in Mexico or Central America, based largely on archaeological evidence of early cob-like maize in indigenous cultures approximately 7200 years ago.

A recent study has indicated that cross-pollination of commercial maize cultivars at 100 ft downwind from the source of genetically modified maize was 1 % and this proportion declined exponentially to 0.1 % at 130 ft and further declined to 0.03 % at 160 ft. At 1000 ft, the farthest distance measured, no cross-pollination was detected (Jemison and Vayda, 2000). For production of Foundation Seed, a distance of 660 ft has been generally required to mitigate outcrossing between different genotypes. The relatively large size of corn pollen and its short viability period under most conditions reduce long distance transfer for purposes of outcrossing (Schoper, personal communication,1999). Under conditions of high temperature or low humidity, corn pollen may only survive for a matter of minutes. Under more favorable conditions in the field or with controlled handling in the laboratory, pollen life may be extended to several hours.

b. *Tripsacum* species - Gama Grass - General Biology

Close relatives of corn or maize are found in the genus *Tripsacum*. Sixteen species of *Tripsacum* are known worldwide and generally recognized by taxonomists and agrostologists; most of the 16 different *Tripsacum* species recognized are native to Mexico, Central and South America, but three occur within the U.S. In the Manual of Grasses of the United States, A. S. Hitchcock (revisions by Agnes Chase; 1971) reports the presence of three species of *Tripsacum* in the continental United States: *T. dactyloides*, *T. floridanum* and *T. lanceolatum*. Of these, *T. dactyloides*, Eastern Gama Grass, is the only species of widespread occurrence and of any agricultural importance. It is commonly grown as a forage grass and has been the subject of some agronomic improvement (*i.e.*, selection and classical breeding). *T. floridanum* is known from southern Florida and *T. lanceolatum* is present in the Mule Mountains of Arizona and possibly southern New Mexico.

For the species occurring in the United States, *T. floridanum* has a diploid chromosome number of 2n = 36 and is native to Southern Florida; *T. dactyloides* includes 2n = 36 forms which are native to the central and western U.S., and 2n = 72 forms which extend along the Eastern seaboard and along the Gulf Coast from Florida to Texas, but which have also been found in IL and KS; these latter forms may represent tetraploids (x = 9 or 18; Lambert, personal communication, 1999); and *T. lanceolatum* (2n = 72) which occurs in the Southwestern U.S. *Tripsacum* differs from corn in many respects, including chromosome number (*T. dactyloides* n = 18; *Zea mays* n = 10). Many species of *Tripsacum* can cross with *Zea*, or at least some accessions of each species can cross, but only with difficulty and the resulting hybrids are primarily male and female sterile (Duvick, personal communication, 1999; Galinat, 1988; Wilkes, 1967). *Tripsacum*/maize hybrids have not been observed in the field, but have been accomplished in the laboratory using special techniques under highly controlled conditions.

Eastern Gama Grass is considered by some to be an ancestor of *Zea mays* or cultivated maize (Mangelsdorf, 1947), while others dispute this (Galinat, 1983; Iltis, 1983; Beadle 1980), based largely on the disparity in chromosome number between the two species (maize n = 10; Gama Grass x = 9 or 18, with diploid, triploid and tetraploid races existing; 2n = 36 or 72), as well as radically different phenotypic appearance. Albeit with some difficulty, hybrids between the two species have been made (Mangelsdorf and Reeves, 1939; Chet DeWald, personal communication;1999). In most cases these progeny have been sterile or viable only by culturing with *in vitro* 'embryo rescue' techniques.

Even though some *Tripsacum* species occur in areas where maize is cultivated, gene introgression from maize under natural conditions is highly unlikely, if not impossible (Beadle, 1980). Hybrids of *Tripsacum* species with *Zea mays* are difficult to obtain outside of the controlled conditions of laboratory and greenhouse. Seed obtained from such crosses are often sterile or progeny have greatly reduced fertility. Approximately 10 - 20% of maize-*Tripsacum* hybrids will set seed when backcrossed to maize, and none are able to withstand even the mildest winters. The only known case of a naturally occurring *Zea* - *Tripsacum* hybrid is a species native to Guatemala known as *Tripsacum andersonii*. It is 100% male and nearly 99% female sterile and is thought to have arisen from gene flow to teosinte, but the lineage is uncertain (Doebley, personal communication, 2000). *Zea mays* is not known to harbor properties that indicate it has weedy potential and, other than occasional volunteer plants in the previous season's corn field, maize is not considered as a weed in the U.S.

In a telephone conversation with Dr. Chester 'Chet' DeWald, USDA-ARS, Woodward, OK, a geneticist working on improvement of grasses, he stated that relatively few accessions of *T. dactyloides* will cross with maize and the majority of progeny are not fertile or viable even in those that do. In controlled crosses, if the female parent is maize, there is a greater likelihood of obtaining viable seed. When these hybrids have been backcrossed to maize in attempts to introgress *Tripsacum* genes for quality enhancement or disease resistance, the *Tripsacum* chromosomes are typically lost in successive generations. In many instances where hybridization has been directed between these two species, the resultant genome is lacking in most or all of the chromosomal complements of one of the parent species in subsequent generations.

Only recently has Dr. DeWald (or anyone else) succeeded in obtaining a true *Tripsacum* cytoplasm with a maize nuclear background. This was done by using gama grass as the female parent and maize as the male or pollen donor. Numerous accessions were tested and crosses made before this came to fruition. The *Tripsacum* derived mitochondrial chondrome and chloroplast plastome in these hybrids contribute to the seed qualities of the plants, but the nuclear genome appears to be totally maize in origin (DeWald *et al.*, 1999).

Dr. DeWald concluded that the possibility of maize contributing genetic material to Eastern Gama Grass through random pollen flow in agricultural or natural situations is extremely remote based upon his experience trying to create hybrids under the best of conditions. He also felt that no other known grass species present in the continental U.S. would interbreed with commercial maize populations (*i.e.*, be recipients of pollen-directed gene flow). This is in agreement with Holm *et al.* (1979) who determined that none of the sexually compatible relatives of corn in the U.S. are considered to be serious, principal, or common weeds in the U.S.

c. *Zea* species - **Teosintes - General Biology**

Teosintes, specifically *Z. mays* ssp. *mexicana* (Schrader) Iltis, *Z. mays* ssp. *parviglumis* Iltis and Doebley, *Z. mays* ssp. *huehuetenangensis* (Iltis and Doebley) Doebley, *Z. luxurians* (Durieu and Ascherson) Bird, *Z. perennis* (Hitchc.) Reeves and Mangelsdorf and *Z. diploperennis* Iltis, Doebley and Guzman, have co-existed and co-evolved in close proximity to maize in the Americas over thousands of years; however, maize and teosinte maintain distinct genetic constitutions despite sporadic introgression (Doebley, 1990).

The teosintes retain a reduced cob-like fruit / inflorescence that shatters more than cultivated maize, but still restricts the movement of seeds as compared to more widely dispersed weedy species. Hence, the dispersal of large numbers of seeds, as is typical of weeds, is not characteristic of teosintes or maize. In their native habitat, some teosintes have been observed to be spread by animals feeding on the plants. Teosintes and teosinte-maize hybrids do not survive even mild winters and could not propagate in the U.S. corn belt. Additionally, some types have strict day length requirements that preclude flowering within a normal season (*i.e.*, they would be induced to flower in November or December) and, hence, seed production under our temperate climate (Beadle, 1980; Iltis, personal communication; 2000; Wilkes, personal communication; 2000; Wilkes, 1967).

Since both teosinte and *Tripsacum* are included in botanical gardens in the U.S., the possibility exists (although unlikely) that exchange of genes could occur between corn and its wild relatives. The Agency is not aware, however, of any such case being reported in the United States. Gene exchange between cultivated corn and transformed corn would be similar to what naturally occurs at the present time within cultivated corn hybrids and landraces. Plant architecture and reproductive capacity of the intercrossed plants will be similar to normal corn, and the chance that a weedy type of corn will result from gene flow with cultivated corn is extremely remote.

Like corn, *Zea mays* ssp. *mexicana* (annual teosinte) and *Zea diploperennis* (diploid perennial teosinte) have 10 pairs of chromosomes, are wind pollinated, and tend to outcross, but are highly variable species that are often genetically compatible and interfertile with corn, especially when

maize acts as the female parent. *Zea perennis* (perennial teosinte) has 20 pairs of chromosomes and forms less stable hybrids with maize (Edwards *et al.*, 1996; Magoja and Pischedda, 1994). Corn and compatible species of teosinte are capable of hybridization when in proximity to each other. In Mexico and Guatemala, teosintes exist as weeds around the margins of corn fields. The F_1 hybrids have been found to vary in their fertility and vigor. Those that are fertile are capable of backcrossing to corn. A few isolated populations of annual and perennial teosinte were said to exist in Florida and Texas, respectively (USDA-APHIS, 1997). The Florida populations were presumably an escape from previous use of *Z. mays* ssp. *mexicana* as a forage grass, but local botanists have not documented any natural populations of this species for approximately twenty-five years (Keith Bradley, personal communication, 2000; David Hall, personal communication, 2000; Richard Wunderlin, personal communication, 2000).

Consultation with botanists and agronomists familiar with Texas flora suggested that no teosinte populations exist in the state (Benz, personal communication, 2000; Read, personal communication, 2000; Orzell, personal communication, 2000; Wilson, personal communication, 2000). Further, given the day length characteristics of *Z. diploperennis*, it is highly unlikely a sustaining population would result from introduction of this species. *Z. mays* ssp. *mexicana, Z. mays* ssp. *parviglumis, Z. luxurians* and *Z. diploperennis* may cross with maize to produce fertile hybrids in many instances (Wilkes, 1967). None of these teosinte species have, however, been shown to be aggressive weeds in their native or introduced habitats (John Schoper, personal communication, 1999). Except for special plantings as noted above, teosinte is not present in the U.S. or its territories. Its natural distribution is limited to Mexico, Honduras, Nicaragua, El Salvador and Guatemala.

Given the cultural and biological relationships of various teosinte species and cultivated maize over the previous two millennia, it would appear that significant gene exchange has occurred (based upon morphological characters) between these two groups of plants and that no weedy types have successfully evolved as a result. More recent cytogenetic, biochemical and molecular analyses have indicated that the degree of gene exchange is far less than previously thought (Doebley, 1984; Doebley *et al.*, 1987; Kato, 1997a, 1997b; Smith *et al.*, 1985). Partial and complete gametophytic incompatibility has been documented among cultivated maize, landraces and teosinte (Kermicle, 1997). The former is demonstrated by differential pollen growth and a skewed recovery of alleles linked to incompatibility genes. Complete incompatibility mechanisms serve to isolate a species or subspecies and are evidenced as pollen exclusion or non-functioning of pollen types on certain genotypes. Attempts to cross six collections of *Zea mays* ssp. *mexicana* with U.S. maize cultivars (W22, W23) yielded no or few seeds in five of the six groups (Kermicle and Allen, 1990).

Based on the ability of maize to hybridize with some teosintes, the suggestion of previous genetic exchange amongst these species over centuries, and their general growth habits, any introgression of genes into wild teosinte from *Zea mays* is not considered to be a significant agricultural or environmental risk. The growth habits of teosintes are such that the potential for serious weedy propagation and development is not biologically plausible in the United States.

d. Conclusions

The potential for pollen-directed gene flow from maize to Eastern Gama Grass is extremely remote. This is evidenced by the difficulty with which *Tripsacum dactyloides* x *Zea mays* hybrids are produced in structured breeding programs. Additionally, the genus does not represent any species considered as serious or pernicious weeds in the United States or its territories. Any introgression of genes into this species as a result of cross fertilization with genetically-modified maize is not expected to result in a species that is weedy or difficult to control. In many instances where hybridization has been directed between these two species, the resultant genome is lacking in most or all of the maize chromosomal complement in subsequent generations.

Many of the *Zea* species loosely referred to as "teosintes" will produce viable offspring when crossed with *Zea mays* ssp. *mays*. None of these plants are known to harbor weedy characteristics and none of the native teosinte species, subspecies or races are considered to be aggressive weeds in their native or introduced habitats. In fact, many are on the brink of extinction where they are indigenous and will be lost without human intervention (*i.e.*, conservation measures). Further, none of the landraces or cultivated lines of *Zea mays* are considered to have weedy potential and are generally considered to be incapable of survival in the wild as a result of breeding practices (*i.e.*, selection) during domestication of the crop.

C. Endangered Species Considerations

Cry3 proteins including Cry3Bb1 are known to be highly specific against coleopteran insects and are not hazardous to vertebrate animals. It has been generally demonstrated that Cry3 proteins do not pose a hazard to non-target animals or invertebrates. The Cry3Bb1 protein appears to be specifically toxic to Chrysomelid beetles including corn rootworms (*Diabrotica* spp.) and Colorado potato beetles (*Leptinotarsa decemlineata*) (MRID No. 455328-07). Currently, there are no Chrysomelid species listed on the endangered species list and no other species are known to be sensitive to Cry3Bb1. Therefore, no adverse affects from Cry3Bb1 event MON 863 are expected against endangered species. Nevertheless, all endangered/threatened beetle species habitats found in the counties where corn is grown were examined to determine possible exposure to corn pollen. Their habitat (and breeding grounds) were found not to overlap with corn fields. Therefore no endangered beetles will be exposed to potentially harmful levels of corn tissue or pollen containing Cry3Bb1 protein.

Terrestrial and aquatic exposure were considered in this assessment since non-target coleopterans may be exposed to the Cry3Bb1 protein within corn fields or in surrounding areas from plant tissue (e.g., pollen) movement offsite. However, the distance pollen moves outside of the corn field should be considered. Published data show that less than 25 grains of pollen per cm^2 are expected 4-5 meters from the corn field edge. A relative comparison of surface ratio of milkweed to other substrates (e.g., other host plants, arthropod prey, animal carrion) can be used as a basis for estimating the amount of pollen that may leave the field. The maximum concentration of Cry3Bb1 protein has been determined to be 93 µg/g fresh weight pollen. Based on this concentration, <0.03 µg Cry3Bb1 protein/g of diet would be expected to be deposited 4-5 meters from the field edge. The potential of aquatic organisms to be exposed to the Cry3Bb1 protein is minimal. Such exposure would occur from runoff of the protein (either free or sequestered in plant debris) into adjacent water bodies or pollen drift. Since movement of Cry3Bb1 in soil into water bodies is expected to be negligible, pollen drift was considered the

primary source of potential hazard to endangered aquatic Coleoptera. According to estimates based on published studies, if 100% of the pollen grains leaving the field were deposited in a 1 ha pond with 2 m depth and located ≥1 m from the edge of the corn field, <0.0001 µg Cry3Bb1/mL of water would be expected.. This is a few orders below the toxic level to any insect.

Many of the endangered and threatened beetles occur in cave or aquatic habitats. None of these endangered beetles are expected to occur in or near corn fields. The American burying beetle may occur in old fields or cropland hedge rows. However, based upon the feeding habits of the American burying beetle, the beetle is not expected to occur within corn fields nor will it be exposed to Cry3Bb1 protein. Adult American burying beetles are classified as opportunistic scavengers that feed on anything dead and bury vertebrate carcasses on which their larvae feed. Carrion regurgitated by adults is fed to larvae until they are able to feed directly on a carcass.

In addition, Monsanto conducted a hazard assessment, exposure assessment and risk characterization to demonstrate that Cry3Bb1 does not pose a risk to endangered Coleoptera (MRID No. 455770-03). This endangered species assessment was based on the Hazard Evaluation Division, Standard Evaluation Procedure, Ecological Risk Assessment (U.S. EPA, 1986). The Agency reviewed this assessment and found it acceptable.

An examination of the endangered bird and bat species shows that their breeding habitats are mostly non-agricultural. Insectivorous bats do not prey on larvae. They rely on flying insects. Taking these, and other pertinent issues into consideration, it becomes apparent that reduction in the target pests of corn would not have an effect on the food source of endangered birds and bats. Of those that do encroach on agricultural fields in the rare instances where these species may feed on the target pests, the reduction in the pest species will merely cause them to rely on other plentiful insects as a source of food. Submitted and published field data reviewed in this document show that a wide variety of insects remain abundant in Cry3Bb1 corn fields as opposed to non-Bt fields when conventional insect pest control practices are used. Therefore the data show that Bt crops should actually be beneficial to bird and bat populations.

The reviewed non-target data confirm the expectation that Cry3Bb1 corn will have no adverse effect on endangered and/or threatened species listed by the US Fish and Wildlife Service, including mammals, birds or terrestrial and aquatic plants and invertebrate species. Therefore, no consultation with the USFWS is required under the Endangered Species Act.

D. Environmental Assessment Summary:

The Agency is using a Maximum Hazard Dose Tiered system for Biopesticide non-target wildlife hazard assessment. When no adverse effect at the maximum hazard dose are observed, the Agency concludes that there are no unreasonable adverse effects from the use of the pesticide. From all of the required and voluntarily developed indicator and host range species test data on Cry3Bb1 corn, including the supplementary two year field data, the Agency concludes that the levels of Cry3Bb1 protein in corn will not pose unreasonable adverse effects to corn field flora and fauna. Available data also indicate that there should be minimal short term accumulation of Cry3Bb1 protein in agricultural soil. In addition, no adverse effect on listed endangered and

threatened species listed by the US Fish and Wildlife Service is expected from the proposed Cry3Bb1 CRW resistant corn registration.

At present, the Agency is aware of no identified significant adverse effects of Cry3Bb1 proteins on the abundance of non-target beneficial organisms in any population in the field, whether they are pest parasites, pest predators, or pollinators. Field testing and field census data submitted to the Agency show minimal to undetectable changes in the beneficial insect abundance or diversity. In corn fields densities of predatory and non-target insects are generally higher on Cry3Bb1 corn than non-Bt corn primarily because the Cry3Bb1 corn is not subjected to the same number of applications of nonspecific pesticides. Two year invertebrate abundance studies do not show a shift in the biodiversity in Cry3Bb1 corn, except in cases where the predators are dependent on the pest insect as prey. In contrast, treatment with chemical pesticides, when studied, had significant effects on the total numbers of insects and on the numbers within the specific groups. To date the available field test data show that compared to crops treated with conventional chemical pesticides, the transgenic crops have no detrimental effect on the abundance of non-target insect populations. However, annual insect monitoring of representative commercial fields will continue for long term biodiversity effects assessment.

The Agency believes that cultivation of Cry3Bb1 corn may result in fewer adverse impacts to non-target organisms than result from the use of chemical pesticides. Under normal circumstances, Cry3Bb1 corn requires substantially fewer applications of chemical pesticides. This should result in fewer adverse impacts to non-target organisms because application of nonspecific conventional chemical pesticides is known to have an adverse effect on non-target beneficial organisms found living in the complex environment of an agricultural field. Many of these beneficial organisms are important integrated pest management controls (IPM) for secondary pests such as aphids and leafhoppers. The overall result of cultivation of corn expressing Cry3Bb1 proteins is that the number of chemical insecticide applications for non-target pest control is reduced for management of multiple pest problems.

The movement of transgenes from Cry3Bb1 host plant into weeds and other crops has also been considered. The Agency has determined that there is no significant risk of gene capture and expression of Cry3Bb1 protein by wild or weedy relatives of corn in the U.S., its possessions or territories. The fate of Cry3Bb1 protein in soils and indirect effects on soil biota have also been evaluated. Test data show that most of the Cry protein deposited into soil is quickly degraded, although a residual amount may persist in biologically active form for a much longer period of time. It is also reported that the same degree of Bt Cry protein persistence takes place in soils that have been exposed to repeat Bt spray applications when compared to soil exposed to growing Bt crop. Limited data do not indicate that Cry proteins have any measurable effect on microbial populations in the soil. Horizontal transfer from transgenic plants to soil bacteria has not been demonstrated. Published studies of Bt Cry protein in soil show no effect on bacteria, actinomyces, fungi, protozoa, algae, nematodes, springtails or earthworms. In addition, new plants planted in Bt Cry protein containing soil do not take up the Bt protein.

Conclusions:

This reassessment finds no hazard to the environment at the present time from cultivation of

Cry3Bb1 protein expressing corn for a time-limited registration.

E. Public Comments

Public comments expressing specific environmental concerns received in response to the FR Notice announcing the receipt of Monsanto application are being addressed in a separate document.

F. Supplemental Studies Needed for Long Term Cry3Bb1 non-target Hazard Assessment

The Agency has sufficient information to believe that there is no risk from the proposed uses of Cry3Bb1 corn to non-target wildlife, aquatic and soil organisms. However, after consultation with the FIFRA Scientific Advisory Panel in August, 2002 and from several public comments, the Agency is requesting additional data. The supplementary studies would provide additional weight to support the Agency's conclusions. Therefore, the Agency is requesting the following data (Table 5) to ascertain any possible adverse environmental effects from long term use of this product, as well as testing on more appropriate non-target invertebrates found in corn fields. The Agency does not believe that this data requirement was reasonably foreseeable by the applicant at the time of application.

Table 5. Supplemental data:

Testing Category	Type of Data
Avian chronic exposure testing	The submitted avian dietary toxicity data are not sufficient to make a final avian hazard assessment from repeated exposure(s) to higher doses of Cry3Bb1 corn. A six week broiler dietary study with 60% - 70% MON 863 corn in the diet is needed to assess hazard to wild and domesticated fowl from chronic exposure to high levels of Cry3Bb1 protein.
Non-target insect more appropriate for corn fields	Conduct a maximum hazard dose laboratory toxicity test with *Orius insidiosus* (minute pirate bug).
Non-target insect more appropriate for corn fields	Conduct a maximum hazard dose laboratory toxicity test with a carabid (ground beetle).
Non-target insect more appropriate for corn fields	Maximum hazard testing with *Tetraopes* (red milkweed beetle) should be performed because they are a more logical choice than the monarch butterfly
Field community effects	Submit final results to field studies previously summarized in MRID No.456530-03. The carabid and nematode data are of particular interest.
Ecosystem effects	Additional long range field studies should also be conducted based on recommendations of the August, 2002 SAP found in the conclusion section of the review of MRID No. 457916-01 above.
Soil fate studies	Additional long range soil degradation field studies should also be conducted including the parameters outlined by the August 2002 SAP found in the conclusion section of the review of MRID No. 45757t-02 above

Gene Flow References:

b1

Beadle, G. 1980, The ancestry of corn. Sci. American 242:112-119.

Benz, Bruce, personal communication, Botanist, Professor, Department of Biology, Texas Wesleyan University, Fort Worth, TX, February, 2000

Bradley, Keith, personal communication, Botanist, Institute for Regional Conservation, Miami, FL, January, 2000.

DeWald, Chester 'Chet', personal communication, Plant Breeder and Geneticist, USDA-ARS, Woodward, OK, December, 1999; (580-256-7449).

DeWald, C.L., P. Sims, Y. Li, and V.A. Sokolov. 1999, A novel cytoplasm for maize. Maize Genetics Conference Abstracts, 41:P114.

Doebley, J. F., personal communication, Geneticist, visiting professor, Department of Genetics, University of Wisconsin, Madison, WI. January, 2000. (608-265-5803).

Doebley, J. 1990, Molecular evidence for gene flow among *Zea* species. BioScience 40:443-448.

Doebley, J. F., 1984, Maize introgression into teosinte - A reappraisal. Ann. Missouri Bot. Gard. 71:1100-1113.

Duvick, Sue, personal communication, Geneticist, Department of Plant Genetics, Iowa State University, Ames, Iowa, December, 1999. (515-294-9375).

Edwards, J.W., J.O. Allen, and J.G. Coors, 1996, Teosinte cytoplasmic genomes: 1. Performance of maize inbreds with teosinte cytoplasms. Crop Sci. 36:1088-1091.

Galinat, W.C., 1983, The origin of maize as shown by key morphological traits of its ancestor teosinte. Maydica 28:121-138.

Galinat, W. C. 1988, The Origin of Corn, pp. 1-31. *In*: Corn and Corn Improvement, Third Edition. Sprague, G. F., Dudley, J. W. (Eds.). American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, Madison, WI.

Hall, David, personal communication, Forensic Botanist and Environmental Consultant, Gainesville, FL, January, 2000. (352-375-1370).

Holm, L., Pancho, J. V., Herberger, J. P., and Plucknett, D. L. 1979, A Geographical Atlas of World Weeds, p. 391, John Wiley and Sons, New York.

Iltis, H.H., 1983, From teosinte to maize: The catastrophic sexual transmutation. Science 222: 886-894.

Iltis, Hugh, personal communication, Professor Emeritus of Botany, University of Wisconsin, Madison, WI, January, 2000. (608-262-7247).

Jemison, J. and M. Vayda, University of Maine at Orono, Pollen transport from genetically engineered corn to forage corn hybrids: A case study, Abstract presented to the Maine Agricultural Trade Show, January, 2000.

Kato Y., T.A., 1997a, Review of Introgression between maize and teosinte. *In*: Gene Flow Among Maize Landraces, Improved Maize Varieties, and Teosinte: Implications for Transgenic Maize, pp.44-53, Serratos, J.A., Wilcox, M.C., and Castillo-Gonzalez, F. (Eds.), Mexico, D.F., CIMMYT.

Kato Y., T.A., 1997b, Plenary session: Analysis of workshop reports and discussions. Group I report. *In*: Gene Flow Among Maize Landraces, Improved Maize Varieties, and Teosinte: Implications for Transgenic Maize, pp.94-103, Serratos, J.A., Wilcox, M.C., and Castillo-Gonzalez, F. (Eds.), Mexico, D.F., CIMMYT.

Kermicle, J.L., 1997, Cross incompatibility within the genus *Zea*. *In*: Gene Flow Among Maize Landraces, Improved Maize Varieties, and Teosinte: Implications for Transgenic Maize, pp.40-43, Serratos, J.A., Wilcox, M.C., and Castillo-Gonzalez, F. (Eds.), Mexico, D.F., CIMMYT

Kermicle, J.L. and J.O. Allen, 1990, Cross-incompatibility between maize and teosinte. Maydica 35:399-408.

Lambert, John, personal communication, Plant Breeder and Geneticist, Department of Crop Sciences, University of Illinois, Champaign-Urbana, IL, December, 1999. (217-333-9642).

Magoja, J.L. and G. Pischedda, 1994, Maize x Teosinte hybridization. Biotechnology in Agriculture and Forestry 25:84-101, *In*, Maize, (Ed.) Y.P.S. Bajaj, Springer-Verlag, Berlin, Heidelberg.

Mangelsdorf, P.C., 1947, The origin and evolution of maize. *In*, Advances in Genetics, (Ed.) M. Demerec, 1:161-207, Academic Press, NY.

Mangelsdorf, P.C. and R.G. Reeves, 1939, The origin of Indian corn and its relatives, Texas Agricultural Experiment Station Bulletin 574 (monograph):80-81, 89-109.

Orzell, Steve, personal communication, Botanist / Ecologist, United States Air Force, Avon Park Air Force Range, Florida, 2000.

Read, James, personal communication, Professor, Texas Agricultural Experiment Station, Dallas, TX, January, 2000. (972-231-5362).

Schoper, John, personal communication, Geneticist, Pioneer Hi-Bred International, Johnston, IA, December, 1999. (515-270-3544).

Smith, J.S.C., M.M. Goodman, and C.W. Stuber, 1985, Relationships between maize and teosinte of Mexico and Guatemala: Numerical analysis of allozyme data. Econ. Bot. 39:12-24.

USDA, APHIS. 1997. USDA/APHIS Petition 97-265-01 for Determination of Nonregulated Status for *Bt* Cry9C Insect Resistant and Glufosinate Tolerant Corn Transformation Event CBH-351: Environmental Assessment. USDA, APHIS, Riverdale, MD.

Wilkes, G.H., 1967, Teosinte: The closest relative of maize. Bussey Inst., Harvard University, Cambridge, MA

Wilkes, H. Garrison, personal communication, Professor of Plant Genetics, University of Massachusetts, Amherst, MA, January, 2000. (617-287-6662).

Wilson, Hugh, personal communication, Professor of Biology, Texas A&M University, College Station, TX, January, 2000. (409-845-3354).

Wunderlin, Richard, personal communication, Professor of Botany, Institute for Systematic Botany, University of South Florida, Tampa, FL, January, 2000. (813-974-2359).

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

JAN 14 2003

MEMORANDUM

SUBJECT: Supplemental review of ecological assessment of non-target organisms for *Bacillus thuringiensis* Cry3Bb1 Field Com (EPA Reg. No. 524-LEI; Barcode No. D285418; Case No. 069017; Submission No. S621756; MRID No. 457916-01).

TO: Mike Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division, 7511C

FROM: Robyn Rose, Entomologist Robyn Rose 1/13/03
Biopesticides and Pollution Prevention Division, 7511C

PEER REVIEW: Zigfridas Vaituzis, Ph. D., Microbiologist
Phil Hutton, Chief Zigfridas Vaituzis 1/14/03
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511C

CLASSIFICATION: **Supplemental to:**

1. Submit final results to field studies previously summarized in MRID No.456530-03. Additional field studies should also be conducted based on recommendations of the August 27, 2002 FIFRA SAP.

2. Conduct a laboratory toxicity/pathogenicity test with *Orius insidiosus*.

3. Conduct a laboratory toxicity/pathogenicity test with a carabid.

4. Tests should be conducted with *Tetraopes* beetles because they are a more logical choice than the monarch butterfly.

65

BACKGROUND:

Monsanto has requested a registration for the *Bacillus thuringiensis* (Bt) Cry3Bb1 protein and the genetic material necessary for its production in field corn event MON 863. The Cry3Bb1 protein is expressed in corn is designated event MON 863 and provides protection against the corn rootworm (CRW), *Diabrotica* spp. (Coleoptera: Chrysomelidae). EPA has already reviewed several ecological effects and environmental fate studies submitted in support of Monsanto's request to register MON 863 corn. These studies, including MRID Nos. 449043-10, 449043-11, 449043-12, 449043-13, 449043-17, 455382-04, 455382-05, 455382-06 and 455770-03 were reviewed in a memorandum from Robyn Rose to Mike Mendelsohn dated May 20, 2002. Monsanto has recently submitted a two year field study that evaluates the potential ecological impact of MON 863 corn on non-target organism populations. This information is intended to be supplemental data that provides additional evidence that MON 863 corn will not pose an unreasonable adverse effect in the environment.

The first years results of this field census study were summarized in MRID 455382-06 and review by the Agency in a memorandum from Robyn Rose to Mike Mendelsohn dated May 20, 2002. Conclusions of this review stated: A final report will be submitted to the Agency after the 2001 data is collected and analyzed. The final report should include the location of the 2000 and 2001 test sites as well as the additional data on soil fertility, microbial populations and soil Bt protein collected during this study but not reported at this time. This study should also be conducted at additional test sites in different geographic locations to account for regional differences in insect populations.

In addition to the final submission of the field sampling study submitted by Monsanto, this memorandum will address comments and conclusions from the August 27, 2002 Scientific Advisory Panel (SAP) meeting titled Corn Rootworm Plant-Incorporated Protectant Insect Resistance Management and Non-Target Insect Issues. A memorandum dated November 6, 2002 from Paul I. Lewis to Marcia E. Mulkey included a transmittal of the meeting minutes of the November 27, SAP. Based on the SAP comments, the Agency is providing supplemental conclusions to those found in the May 20, 2002 preliminary review of ecological non-target insect studies for *Bacillus thuringiensis* Cry3Bb1 protein.

CONCLUSIONS:

Field Studies:

The August 27, 2002 FIFRA Scientific Advisory Panel (SAP) concluded that field experiments must be appropriately designed to provide a measure of ecological impacts. The SAP opinion was that a two year field study would not be sufficient to determine if MON 863 corn will have a negative impact on non-target invertebrates. Short-term field studies are not adequate to draw conclusions on the variations in non-target invertebrate populations. Large field-scale studies conducted for at least three to four years are needed to draw a conclusion on non-target impacts. The Panel generally concluded that "the state-of-the science" needed for long-term studies must

66

improve for the research to be appropriately conducted to provide meaningful results. The statistical power (avoiding Type II experimental error) needed to gain useful results from field census studies would require very large fields, more replications and more samples per plot (e.g., 10 soil and pitfall samples) plus the addition of visual plant samples (e.g., >50/plot).

Since the endpoint for field census studies has not been determined, it is difficult to determine how large the fields should be, how many replications are needed and how many samples per plot are needed to achieve appropriate statistical power. Therefore, additional field census studies should not be conducted until the endpoints and logistics of the study have been determined. Instead, small-scale field studies between laboratory and full-scale field studies should be conducted such as those found in MRID No. 456530-03 and summaries of these smaller scale field studies should be submitted to the Agency upon their completion. Additional full scale field or semi-field studies with appropriate end points and statistical power should also be considered based on recommendations of the August 27, 2002 FIFRA SAP.

Laboratory Studies:
The preliminary review of the insect toxicity/pathogenicity studies conducted in the laboratory (MRID Nos. 449043-10, 449043-11, 449043-12, 449043-13, 449043-17, 455382-04, 455382-05 and 44903-14) were initially found acceptable by the Agency (see memorandum from Robyn Rose to Mike Mendelsohn dated May 20, 2002). However the August 27, 2002 FIFRA SAP concluded that the green lacewing (*Chrysoperla carnea*) and parasitic Hymenoptera (*Nasonia vitripennis*) were not appropriate. The SAP concluded that "[t]he levels of exposure of *Chrysoperla* and *Nasonia* to active protein were not, for example, determined throughout their respective tests. The test protein was held for a week within a diet broth in the *Chrysoperla* test chamber, and could have degraded considerably."

The Agency also recognizes additional problems with the *Chrysoperla* and *Nasonia* laboratory test. Green lacewing are difficult to test in the laboratory because of a high rate of mortality. In this instance (MRID No. 449043-12), the test was terminated after 10 day because there was >20% mortality in the positive control. In addition, it is questionable whether the green lacewings are ingesting the Cry3Bb1 protein that is coated around moth eggs in a diet. Since green lacewing have piercing-sucking mouthparts, they may not be exposed to the protein on the external surface of the egg diet. Therefore, Monsanto should conduct a laboratory insect toxicity/pathogenicity test on an alternate organism. The minute pirate bug (*Orius insidiosus*) would be a more appropriate species to test than the green lacewing. *Orius* typically occur in corn fields as egg predators and they typically feed on pollen. Therefore, a laboratory study should be conducted feeding *O. insidiosus* both pollen and purified protein in diet. Feeding *O. insidiosus* Cry3Bb1 protein in diet will allow for a test at the maximum hazard dose; whereas, feeding *O. insidiosus* pollen expressing the Cry3Bb1 protein will provide an evaluation of potential effects from actual exposure scenarios.

A *Nasonia vitripennis* laboratory test was summarized in MRID No. 449043-13. Not only were the procedures in this study questioned by the SAP, the appropriateness of testing this organism

67

is questionable. *N. vitripennis* is a dipteran parasitoid that does not occur in corn fields. A more appropriate parasitoid that occurs in corn fields (*e.g. Tricogramma* or *Macrocentrus grandii*) should be considered. Since *Tricogramma* and *Macrocentrus* are lepidopteran parasitoids, testing another beneficial organism rather than a parasitoid is appropriate. Therefore, the Agency recommends testing a beneficial coleopteran such as a carabid (ground beetle) in the laboratory.

A monarch butterfly larval pollen feeding study (MRID No 455382-05) was also conducted in support of the MON 863 registration. However, Cry3Bb1 expressed in MON 863 is a coleopteran-active protein. Therefore, the monarch butterfly was not an appropriate indicator organism to be tested. The SAP recommended testing *Tetraopes* beetles as a more logical choice than the monarch butterfly.

68

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Biopesticides and Pollution Prevention Division
PEER REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target organism field scale risk assessment
MRID NO.:	457916-01
STUDY NO.:	MSL-17531
SPONSOR:	Monsanto Company, Biotechnology Regulatory Sciences, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LABS:	Monsanto Agronomy Center, Monmouth, IL 61462, Monsanto Company, 800 N Lindbergh Blvd., St Louis, MO 63141 and AGVISE Laboratories, 604 Highway 15, Box 510 Northwood, ND 58267, and Key Agricultural Services, Inc., Macomb, IL 61455
TEST MATERIAL:	*Bacillus thuringiensis* Cry3Bb1 Protein and the Genetic Material (ZMIR13L) necessary for its Production in Corn Event MON 863
STUDY COORDINATOR:	Muhammad A. Bhatti
REPORT DATE:	August 14, 2002
CLASSIFICATION:	Supplemental to submitting data from additional field studies currently underway

---

Study Summary

Title: "Ecological Assessment for Non-target Organisms in the Plots of Corn Rootworm Insect-Protected Corn Hybrid Containing MON 863 Event: 2000-2001 Field Trials"

Authors: Muhammad A Bhatti, Jian Duan, Carol L. Pilcher, Clinton D. Pilcher, Michael J. McKee, Thomas E. Nickson, Graham P. Head and Changjian Jiang

Objective: This study was conducted to generate field data on the potential effect of MON 863 corn on non-target organisms compared to conventionally grown corn. Phenotypic characteristics, soil quality and fertility, soil microbial populations and relative abundance of invertebrate non-target organisms were evaluated.

Methods:
During the 2000 and 2001 growing seasons, event MON 863 CRW-protected corn and non-transgenic corn (hybrid RX670) were grown in Warren County near Monmouth, IL. Seed were characterized at the molecular level by PCR at planting and harvest to verify that Cry3Bb1 was present in MON 863 corn and absent in the control line (RX670). Plots were planted in different locations located approximately 1.5 miles apart in Warren County each year. The field used in 2000 consisted of silt loam (3.6% organic matter; pH of 6.1) and in 2001 was a silty clay loam (4.0% organic matter; soil pH of 6.4). Corn was planted in both fields the previous year and

soybeans were planted two years prior to conducting these field trials. All experimental plots were managed according to typical cultural practices of commercially grown corn in the region and included the application of the herbicides acetochlor and atrazine after planting and before emergence.

During the 2000 and 2001 growing seasons, Bt (MON 863) and control hybrids (RX670) were the main plots planted in a split-plot design with four replications planted 20 ft apart. Rows were planted 30 inches apart, seeded at a rate of approximately 1.7 seeds/ft and planted 1.5 - 1.75 inches deep. Plots (240 ft × 60 ft) were divided into 24 row subplots (60 ft × 60 ft) that served as replications receiving one of 4 insecticide regimes. Insecticide treatments of the Bt and non-Bt plots included: 1. No insecticide; 2. Seed treated with Gaucho® prior to planting; 3. The granular insecticide Force 3G® applied and incorporated in furrows at planting; and 4. A foliar insecticide, Pounce 3.2 EC®, applied at the V10 and R2 corn growth stages to control 1st and 2nd generation CRW adults. A 4-row buffer of non-transgenic corn was planted around each plot to minimize edge effects from adjacent subplots.

Data were collected on agronomic and phenotypic characteristics, pest (insect and disease) susceptibility, soil quality and fertility, microbial populations and non-target invertebrate abundance. Agronomic and phenotypic observations were made on emergence and seedling vigor, early plant stand count, days to 50% silking and pollen shed, plant height, plant ability to stay green and yield. Insect and disease damage was evaluated from the middle two rows of test plots three times during the growing season; during the V4-V6, Vt-V16 and R3-R4 growth stages. Plants were also evaluated for staik rot at the R6 growth stage which represents physiological maturity.

Eight inch deep soil samples were taken to evaluate quality and fertility parameters such as organic matter, phosphorus, potassium, magnesium, calcium, pH, cation exchange capacity and texture. Four samples were taken during the growing season and two samples post-harvest in 2000; two samples were taken during and after the growing season in 2001 for a total of ten samples. Microbial populations were evaluated from test and control plots that received no insecticide regime a total of 14 times during the growing season from the top six inches of soil within six inches of the rhizosphere. Samples were taken in 2000 and 2001 during the V2, V4, V8, R1 and R6 stages as well as after tillage and the following spring. Soil samples from the 2000 growing season were sent to Microbial Inotech Labs Inc, 12133 Bridgeton Sq. Dr., St. Louis, MO to determine bacteria, mold and yeast by a heterotrophic plate count method. AGVISE Laboratories, 604 Highway 15, Box 510 Northwood, ND 58267 analyzed four of seven soil samples collected during the 2001 growing season. The "viable plate count method" was used to analyze bacteria, actinomycetes and fungi.

In this study, non-target invertebrate were also sampled. Soil-dwelling invertebrate were collected from root balls including soil during the V6, V10 and R1 growth stages during the 2000 and 2001 growing seasons. Three eight inch root balls were collected during the V6, V10 and R1 corn growth stages from all control and test plots and processed through a Berlese funnel to

extract invertebrate. Earthworms were also collected from soil samples by hand sorting. Ground surface-dwelling invertebrate were collected from all test and control plots under all insecticide regimes using pitfall traps. Four pitfall traps were placed in each subplot from the V6 to R4 corn growth stages for three-day periods. Key invertebrate from pitfall traps were counted and identified to family level. Flying and foliage-dwelling invertebrate were collected from each subplot using yellow sticky traps (Pherocon AM™, no-bait sticky traps distributed by Great Lakes IPM Inc., Vestburg, MI). Three traps per subplot were placed at canopy level from the V6 to R4 corn growth stages. Traps were left in the field for seven days and all key taxa were counted and identified to family or genus level.

Data were analyzed using a mixed linear repeated measures model for each invertebrate collected by each sample method. The repeated measure was the sampling date within each insecticide regime. A least significant difference (LSD) test was used to determine mean separations when main effects of insecticide regimes were significant. A mixed model procedure (PROC MIXED) was conducted in SAS for each invertebrate taxonomic group that was considered abundant enough for statistical analysis.

Results:

The agronomic and phenotypic evaluation of the MON 863 and non-transgenic isoline showed no statistical difference ($P>0.05$) between the hybrids in emergence, seedling vigor, 50% silking, pollen shed, plant height, stay green and yield. There was a significant difference in stand count in 2000 ($P=0.0270$, MON 863 = 28 plants, RX670 = 29 plants), but no difference occurred in 2001. Bt and non-Bt plots receiving foliar applied insecticides yielded significantly less than other insecticide regimes in 2000, but were not different from other treatment methods in 2001. Otherwise, insecticide regime did not result in a significant effect on agronomic and phenotypic characteristics. There were also no significant differences between Bt and non-Bt plots or insecticide regimes in insect pests or pathogens observed during the V4, VT and R3 corn plant growth stages.

Soil texture including the proportion of sand, silt and clay did not differ between Bt and non-Bt plots during or after the growing season. There was also no difference in soil fertility and quality indicators between MON 863 and RX670 plots. In general, there was no difference in maintaining soil health and quality between the MON 863 and RX670 cropping systems. Microbial populations were not significantly different ($P>0.05$) in any of soil samples analyzed from the MON 863 and RX670 plots. In general, the microbial populations increased during the growing season and after harvest.

Among the three sample methods (soil, pitfall and sticky trap) there was a total of 156,572 organisms from 16 orders and 36 families identified during the 2000 and 2001 growing seasons. Collected invertebrate included pests, predators, parasitoids, detritivores and decomposers. The predominant non-target invertebrate collected in each sample method are summarized in Table 1.

71

Table 1. Predominant non-target invertebrate collected in each sample method

Sample Method	Order (Family)
Soil and Root Samples (soil-dwelling invertebrate)	Diplura (Japygidae), Chilopoda, Aranea, Acari, Oligocaeta (earthworms), Coleoptera (Carabidae, Staphylinidae, Nitidulidae, Lanthridiidae), Hymenoptera (Formicidae)
Pitfall Trap Samples (ground surface-dwelling invertebrate)	Orthoptera (Gryllidae), Coleoptera (Carabidae, Staphylinidae, Nitidulidae, Scarabeidae, Chrysomelidae), Hymenoptera (Formicidae), Araneae, Chilopoda
Yellow Sticky Trap Samples (flying & foliage-dwelling invertebrate)	Coleoptera (Chrysomelidae, Nitidulidae, Coccinellidae), Hymenoptera, Homoptera (Aphididae, Cicadellidae), Hemiptera (Anthocoridae), Diptera (Syrphidae), Neuroptera (Chrysopidae, Hemerobiidae), Aranea

Overall, there was no statistical difference between MON 863 and non-Bt plots (RX670) in the abundance of the predominant invertebrate collected in soil samples. According to the soil sample data, the number of Japygidae did not differ between Bt and non-Bt plots; however, there were significantly less collected in the insecticide treated plots. Of the coleopteran insects collected, there were generally more carabids (ground beetles) and staphylinids (rove beetles) than nitidulids (sap beetles) and lanthridiids (minute brown scavenger beetles). There was no statistical difference in the number of coleopteran insects captured between the MON 863 and non-transgenic corn isolines. Statistical analysis showed that insecticide treatments significantly reduced the number of carabids in 2000 and the number of staphylinids on the last sample date in 2001. Invertebrate from the Araneae and Acari families were also not significantly different between Bt and non-Bt plots; however the number of acarids were statistically greater in the plots treated with foliar insecticides in 2001. The number of chilopods, the most abundant non-insect arthropod sampled, was not different between Bt and non-Bt isolines, but more were collected in plots treated with seed, soil and foliar insecticides in 2001 particularly the MON 863 treated plots. Although the number of earthworms collected did not differ between Bt and non-Bt plots, there were significantly less earthworms in the plots treated with foliar insecticides than the other insecticide regimes. The hand-sorting method also showed no differences in the number of earthworms collected in MON 863 and non-transgenic plots, nor was there a difference found between insecticide regimes.

Overall, there was no statistical difference between MON 863 and non-Bt plots (RX670) in the abundance of the predominant invertebrate collected in pitfall trap samples. Of the coleopterans captured in pitfall traps, there were more nitidulids, carabids and staphylinids captured than chrysomelids and scarabids collected. There was no statistical difference between MON 863 and RX670 field-plots in the overall number of coleopterans collected in pitfall traps. The different insecticide regimes tested resulted in varied and inconsistent effects on the abundance of the predominant Coleoptera sampled. The number of gryllids (crickets) and formicids (ants) collected in pitfall traps was not statically different between Bt and non-Bt plots or different

92

insecticide regimes. Chilopod and Araneae abundance was not different between MON 863 and RX670 plots nor was there an affect from insecticide regimes in most cases. Statistical analysis showed that there were significantly fewer Araneae in the plots with soil and foliar insecticide treatments in 2000.

Based on yellow sticky trap counts, there were consistently less corn rootworm (CRW; *Diabrotica virgifera* and *Diabrotica barberi*) in the MON 863 plots than the non-Bt plots in all insecticide regimes. However, the difference in the number of CRW captured was not statistically different. In both 2000 and 2001, there were no significant differences between MON 863 and RX670 plots across all insecticide regimes in the number of nitidulids (sap beetles), coccinellids (lady beetles), aphidids (grass hoppers), cicadellids (potato leaf hoppers), braconids (*Macrocentrus grandii*), syrphids (syrphid or hover flies), hemerobiids (brown lacewing), chrysopids (green lacewing) and Araneae (spiders). There was a reduction in the abundance of *Coleomegilla maculata* and *M grandii* and an increase in *Empoasca fabae* (potato leaf hopper) in plots receiving a foliar spray. Syrphid fly abundance was reduced in 2000 by soil insecticide treatments.

Study Author's Conclusions:
Data collected during the 2000 and 2001 growing seasons indicate that MON 863 and RX670 corn are agronomically and phenotypically equivalent and there are not differences in their susceptibility to pathogens. Soil quality and fertility were also found to be consistent among MON 863 and RX670 field plots. Sampling data collected in 2000 and 2001 also showed that there are no overall differences in the abundance of non-target invertebrate collected in MON 863 and RX670 plots. However, corn pests such as *D. virgifera*, *C. pulicaria* and *R. maidis* as well as predators (e.g., *O. insidiosus* and *C. maculata*, parasitoids (e.g., *M. grandii*) and decomposers (e.g., earthworms and diplurans) were significantly impacted by insecticide regimes. Foliar sprays and soil treatments resulted in the greatest impact on non-target organisms such as carabids, spiders, *O. insidiosus*, *C. maculata* and *M. grandii*. Therefore, the study author concluded: "MON 863 had less impact on certain beneficial insects compared to traditional insecticide control programs, especially soil and foliar applications. Thus, the use of MON 863 for corn rootworm control can lead to reduced use of insecticides and increased compatibility with Integrated Pest Management programs in corn."

Reviewer's Comments:
According to the data submitted to the Agency by Monsanto, MON 863 corn does not adversely impact the abundance of non-target invertebrate found in corn fields. However, plot size (240 ft × 60 ft plots divided into 24 row 60 ft × 60 ft subplots) was small and only replicated four times. In addition, each plot only included three root and sticky trap samples and four pitfall trap samples.

The August 27, 2002 FIFRA Scientific Advisory Panel (SAP) concluded that field experiments must be appropriately designed to provide a measure of ecological impacts. In addition, the SAP opinion was that a two year field study would not be sufficient to determine if MON 863 corn

Reviewer: Gail Tominatsu, Ph.D. Date: 1/10/2003
Microbial Pesticides Branch
Secondary Reviewer: Zigfridas Vaituzis, Ph.D. Date: 1/14/03
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Phil Hutton, Chief
Biopesticides and Pollution Prevention Division (7511C)

DATA EVALUATION REPORT

STUDY TYPE: Acute Toxicity Study- Aquatic Invertebrate (FIFRA Sub. E, Section 72-2; OECD Guideline 202

CITATION: Drottar, K. R. and H. O. Krueger. *Bacillus thuringiensis* Protein 11098 in Corn Pollen: A 48-Hour Static-Renewal Acute Toxicity Test with the Cladoceran (*Daphnia magna*). Wildlife International Ltd., 8598 Commerce Drive, Easton, MD 21601. Proj. No. 139A-236. Monsanto Study No. WL-98-295, August 13, 1999 (Amended). MRID 449043-18.

DP BARCODE: D262045 **CASE**:066221
REG./FILE#:000524-LRA **CHEMICAL/BIOL#**: 006482 Cry3Bb (Vector ZMIR14L)

COMPANY/SPONSOR: Monsanto Co., Regulatory Sciences, 700 Chesterfield Pkwy North, St. Louis, MO 63198

TEST MATERIALS: The test substance was pollen from MON 858 corn plants (Lot # 5827). The control substance was pollen from MON 847 plants (Lot No. 85827).

REVIEW CONCLUSION: The 48-hour EC_{50} value for Cry3Bb corn pollen when administered to neonate daphnids was greater than120 mg pollen/L, the only concentration tested. No adverse effects on daphnia were seen in 2 days. These data show that there will be no adverse effects on daphnia from incidental exposure to Cry3Bb-containing corn pollen.

ADEQUACY OF STUDY: Validation categories: Acceptable. This study meets EPA Guideline requirements for assessing acute pesticidal risks to *Daphnia*.

MATERIALS & METHODS: The methods used in conducting the study followed procedures specified in Series 72 of EPA's Registration Guidelines, Pesticide Assessment Guidelines, FIFRA Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms; OECD Guideline 202, Guideline for Testing of Chemicals: *Daphnia* sp. Acute Immobilization Test and Reproduction Test,, and on ASTM Standard E729-88a "Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians.

The test material, consisted of corn pollen from corn plants, line MON858; the Cry3Bb1 content was estimated to be 18.8 μg/g fresh weight pollen. It was described as a yellow powder and had a purity of 98.5%. The test material was assigned Wildlife International Ltd. identification

74

number 4780 and stored at -80°C. Cry3Bb1 also has been previously referred to as CryIIIB2 (Cry3B2) and Cry3Bb or CryIIIC.

Corn pollen from MON 847 plants, genetically similar to MON 858, was used as the control substance. It was also described as a yellow powder, with a purity of 98.7% and assigned an identification number of 4781 and stored at -80°C.

A qualitative ELISA analysis of the pollen from MON 858 was found to contain the B.t. protein; and the "B.t. protein of interest" was reported as "confirmed to be present in the pollen extract" as used in the daphnia test.

The test organisms were neonate daphnids obtained for testing from 6 individual adult daphnids which were observed to have no neonates present less than 24 hrs prior to test initiation. Adult daphnids in the cultures were held for at least 28 days prior to collection of the juveniles and fed a mixture of yeast, Cerophyll®, and trout chow and a suspension of *Selenastrum capricornutum,* a freshwater green alga. The adults showed no signs of disease or stress during the holding period. The neonates were not fed during the test. At test initiation, (30 min after the start of aeration), neonate daphnids were transferred indiscriminately to 10-mL glass beakers and subsequently from the beakers to the test compartments. Ten neonate daphnids were transferred in each chamber, and three replicate chambers (30 neonate daphnids/treatment) were maintained for each test and control groups. A wide-bore pipet was used to make all transfers from below the air/water interface.

The test chambers consisted of 2-L glass beakers which contained 500 mL of the test solution (depth was about 4.5 cm). These chambers were placed in a temperature-controlled water bath at about 20 ± 1°C, and aerated gently throughout the exposure period of 48 hours.

The test solutions were prepared by adding 0.06 **g** of the test or control substances to 500 mL of dilution water in the test chambers (The nominal test concentration was 120 mg pollen/L). Test solutions were not adjusted for percent pollen of the test (98.5%) nor the control substance (98.7%). The dilution water was twice-filtered freshwater from a 40-ft deep well located on Wildlife International Ltd. site, and characterized as moderately-hard.

Yellow particles were noted **on** the surface of the water and on the bottom of the test chambers containing test and control pollen solutions after test initiation and throughout the test. New test solutions were prepared and the daphnids were transferred from the old to the new solutions after about 24 hours.

Environmental conditions such as temperature, pH, and dissolved oxygen were measured at various intervals of test duration. Temperature was measured with a liquid-in-glass thermometer in each test chamber at the beginning and end of the test, and prior to and after the renewal of solutions. For the negative control, temperature was measured continuously using a Fulscope ER/C Recorder. The target test temperature during the study was 20 ± 1°C. The pH of all replicates was measured with a Fisher Accumet Model 915 pH meter at test initiation, at approximately 24 hours (prior to and after the renewal) and at the end of the test. Dissolved oxygen was measured with a Yellow Springs Instrument Model 51B dissolved oxygen meter at the same intervals. Specific conductance was measured with a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Measurements of hardness and alkalinity were made by titration based on procedures in *Standard Methods for the Examination of Water and Wastewater.* Lighting was

provided by fluorescent tubes (Colortone® 50) with a photoperiod of 16 hrs of light and 8 hrs of darkness, controlled by an automatic timer. To avoid sudden changes, a 30-min transition period of low light intensity was provided when lights went on and off..

Statistical analyses were not performed since this was a limit dose test and the EC50 values were estimated from the biological response data. Numbers of mortalities and immobility were visually assessed after 3, 24 and 48 hours after test initiation. Fisher's exact test was used to determine statistical differences in immobility/mortality in the limit concentration (120 mg test pollen/L) and the control groups.

REPORTED RESULTS:

A. Observations and Environmental Measurements. Temperatures in all the test chambers were reported within the 20 ± 1 °C range (19.9 °C to 20.1 °C). Dissolved oxygen levels were at least 81% saturated in all test chambers for the test duration; and values ranged from 7.4 mg/L to 8.9 mg/L. Values for pH were measured in the range of 8.2 to 8.5 throughout the test.

B. Mortalities and Clinical Observations. There were no mortalities or immobile *Daphnia* observed over the 48-hour study. The EC50 was therefore estimated to be >120 mg corn pollen/L.

C. Body Weight and Feed Consumption. There were no apparent treatment related effects on body weight or feed consumption in the commercial corn line reference groups or in any of the three genetically modified corn lines tested when compared to the non-genetically modified corn line control group (Tables 2 and 3, Appendices V and VI).

DISCUSSION: This study was conducted according to approved EPA guideline procedures for acute toxicity testing of pesticidal substances to freshwater aquatic invertebrates. There were no observations of mortality or behavioral effects in any of the treatments for 48-hr exposure period.

Consequently, the 48-hr EC_{50} for *Daphnia magna* exposed to Bt Cry3Bb protein in corn pollen was >120 mg corn pollen/L. .. Confirmations of the Cry3Bb1 protein present in only the test substance was validated by an ELISA technique.

REVIEWER'S COMMENTS:

A. **Test Procedures:** The procedures used follow those recommended by OPPTS Harmonized Series 885 Microbial Pesticide Test Guidelines.

B. **Statistical Analysis:** The LC_{50} value could not be subjected to statistical analysis because only one concentration was tested.

C. **Discussion/Hazard Assessment:** The study was procedurally sound and no treatment mortality or behavior change was reported between the dosed and control replicates.

D. **Adequacy of the Study:**

1. Validation Category: Acceptable.

76

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

Reviewer: Gail Tominatsu, Ph.D. Date: Jan 13 2003
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Secondary Reviewer: Zigfridas Vaituzis, Ph.D. Date: 1/14/03
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Phil Hutton, Chief
Biopesticides and Pollution Prevention Division (7511C)

STUDY TYPE: Nontarget Fish Testing (OPPTS Series 885.4200)

CITATION: Meng, H.L., E.H. Robinson (1999). Evaluation of Insect Protected Corn Lines MON 853 and MON 859 as a Feed Ingredient for Catfish. Thad Cochran National Warmwater Aquaculture Center (Testing Facility), Mississippi State University, P.O. Box 197, Stoneville, MS . MRID # 449043-19.

DP BARCODE: D262045 **CASE**: 066221
REG./FILE#:000524-LRA **CHEMICAL/BIOL#**: 006482 *Bacillus thuringiensis* CRY3Bb protein (Vector ZMIR14L)

COMPANY/SPONSOR:Monsanto Co., Regulatory Sciences, 700 Chesterfield Pkwy. North, St. Louis, MO 63198

TEST SUBSTANCES: Corn grain containing B.t. protein 11231 (also referred to as Cry3Bb1 or Cry3B2 protein)

REVIEW SUMMARY & CONCLUSION: The study was scientifically sound and there were no treatment mortalities or behavior changes among channel catfish fed the different diets. The results indicate that corn grain derived from the two transgenic lines engineered to produce the insecticidal protein, Cry3Bb1 can be used as a feed ingredient in channel catfish diets at levels of up to 35 % without adverse effect on fish growth, feed conversion efficiency, survival, behavior or body composition.

ADEQUACY OF STUDY: Acceptable. The study meets EPA Guideline requirements for maximum hazard dose (and a sub-chronic) toxicity testing of microbial toxins to freshwater fish.

METHODOLOGY: The methods used in conducting the study followed procedures specified in OPPTS Guideline 885.4200.

The test **materials** were finely ground corn grain from two insect-protected corn lines MON 853 and MON 859 prepared by the sponsor. Respective lot numbers of these test substances were MON 85328 and MON 85928. Both corn lines were genetically modified to contain the Cry3Bb1 protein. Cry3Bb1 protein has also been referred to as CryIIIB2 (or Cry3B2), as well as Cry3Bb or CryIIIC. The Cry3Bb1 designation from the most recent and accepted of nomenclature (Crickmore, et.al. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Bio. Rev. 62:807-13) is used in the present submission.

TS

Control materials consisted of finely ground corn grain of two non-genetically modified corn lines with similar genetics to the test substances. The non-genetically modified corn line MON 846 was the control for transgenic corn line MON 859; and MON 847 was the control corn line for transgenic MON 853. The control corn lines were assigned lot numbers of MON 84628 (for MON 846) and MON 84728 (for MON 847).

The reference substance consisted of finely ground corn grain from a commercial corn hybrid 2390 and was included to assess how commercial corn hybrids performed in the toxicity assay.

All test materials, control and reference substances were prepared by the Sponsor and were noted received on December 4, 1998.

Test diets were based on typical formulations of channel catfish feeds with approximately 32% crude protein using ground corn, soybean meal, menhaden fish meal, meat and bone/blood meal, and wheat middlings. Each diet consisted of 35% ground corn with vitamin and mineral supplements and was formulated using MIXIT-2 Version 3.3 computer software of Agricultural Consultants, Inc. (Kingsville, TX). Known nutrient requirements of channel catfish were met in accordance with published recommendations of the National Research Council (NRC. 1993. Nutritional requirements of fish. National Academy Press, Washington, D.C.)

Diets were prepared at the Thad Cochran National Warmwater Aquaculture Center (NWAC) in accordance with the NWAC Catfish Nutrition Laboratory Standard Operating Procedure SOP005-98-1; and based on procedures described in the open literature (Li, et al. 1993. Aquaculture 117:303-312). A 4-kg batch was prepared for each diet. Ingredients were weighed with a calibrated electronic balance and mixed in a V-mixer for 20 min before placing in a dough mixer to add catfish oil (1.5%) and distilled water (30%) and mixed for 10 min, respectively. After passing the feed mixture through a die in a meat grinder, the resulting feed strands were dried in a forced air oven at a temperature of 29 to 33° C for 2.5 to 3 hrs. A food blender was used to break up the dried feed into smaller particles, screened free of feed dust and mixed before storage at approximately -20 °C in sealed plastic bags.

The test organism (system) was the channel catfish, *Ictalurus punctatus*, Kansas strain. Fish were raised from eggs on site at the NWAC in aquaria containing 20 channel catfish apiece. In May 1998, they were hatched on site and raised indoors to an average weight of approximately 2.8 g/fish, then they were moved into a holding tank located in NWAC Catfish Nutrition Laboratory. During this holding period, they were fed a commercial catfish feed (36% protein floating fingerling feed) at approximately 3% body weight daily. The fish were 6 mos old at the time of study initiation.

The experimental design was based on aquaria as part of a flowing water system in the wet laboratory. Local well water was used as the water source for the study. Each aquarium was supplied with well water at a flow rate of approximately 1 liter per minute and continuous aeration. During this period the fish were fed a 32% protein diet twice daily to approximate satiation. Thirty fish were transferred into each of 25 aquaria located inside the wet laboratory for a 2-wk acclimation period.

After acclimation, all fish were pooled into one aquarium and graded for uniformity. Twenty fish of similar size were restocked into each of the 25 aquaria. The average initial weight of fish was 4.6 g. The diet of each corn line was distributed evenly to 100 catfish among five randomized

aquaria (80-L of water) each for the five dietary treatments. There were 20 fish/aquarium with 100 fish per dietary treatment, for a total of 500 fish used in the study. A one-way analysis of variance was conducted on total weight of fish by aquarium to assure that there was no significant variation in fish weight at study initiation. The fish were allowed to eat for 20 minutes and all fish were fed twice daily (0800 to 0900 and 1600 to 1700 hours) to approximate satiation, based on percentage of fish body weight. Feeding activities were recorded also for the duration of the 8-wk test period.

Recorded observations include daily mortality and behavioral assessments; and collective body weights noted at study initiation, and at weeks 4 and 8. Target water temperature was 30± 2°C and dissolved oxygen at 5 mg/L and above. These parameters were monitored daily using an oxygen/temperature meter.

Analyses of Diet Samples

Prior to initiation of the test, a 10-g sample was collected from freshly prepared diets, ground through a #40 mesh screen and assessed in accordance with NWAC Catfish Nutrition Laboratory Standard Operating Procedures SOP001-98-1, SOP002-98-1, SOP003-98-1 and SOP004-98-1. Proximate dietary analyses refer to the determination of dry matter/moisture, crude protein, crude fat, and ash. All standard operating procedures were based on standard AOAC (Association of Official Analytical Chemists International) methods (AOAC, 1995).

Dry matter was determined by oven drying at 135°C for 2 hours. Ash was determined by wet ashing on a hot plate using nitric acid and then dry ashing at 600°C for 12-16 hrs in a muffle furnace. The automated Kjeldahl method was used to determine crude protein: Sulfuric acid and hydrogen peroxide were used to digest samples on a digestion block for 1 hour under a fume hood. Sodium hydroxide solution was then added to release ammonia which was distilled by steam and titrated with standard hydrochloric acid solution. Samples were extracted in ether for 45 minutes to determine crude fat; the fat in each sample was collected into an aluminum cup which was dried in an oven at 100 to 102°C for 30 minutes before and after the extraction process.

Diet samples were also collected at the start and end of the study, stored on dry ice and returned to the Sponsor for confirmation of the Cry3Bb1 protein, using an ELISA assay in accordance with Monsanto SOP #BRME-0059-03.

Analyses of Fish Fillet Samples

After 8 weeks (end of the feeding period), fillets were cut from each of five fish from each aquarium and pooled by aquarium for proximate analyses of the cut tissue. Fish were sacrificed by overdosing with tricaine methanesulfonate (MS-222). The proximate analyses were performed on fish fillet samples in accordance to NWAC Catfish Nutrition Laboratory Standard Operating Procedures SOP001-98-1, SOP002-98-1, SOP003-98-1, and SOP004-98-1.

Crude protein and crude fat were determined from freeze-dried samples of ground fish fillet. These were prepared by grinding a 40-g fillet sample from each tank into a paste; 25 grams were removed, and freeze-dried for at least 12 hours. The remaining "wet" samples were used to determine moisture and ash. The procedures for proximate analyses of fillet samples were the same as described for diet samples except that fillet moisture was determined by oven drying at 100-102°C for 16 to 18 hours.

80

Data Collection, Calculations and Statistical Analysis

During this study, the following data were collected: feed consumption (total feed consumed/number of fish, g/fish); weight gain (final weight of fish - initial weight of fish, g/fish); feed conversion ratio (feed consumption/weight gain); visceral fat (visceral fat/whole fish weight, %); proximate composition of diets and fish fillets (% moisture, % crude protein, % crude fat, % ash); and observations concerning adverse effects, mortality or behavioral changes.

One-way analyses of variance (ANOVA) were conducted on several parameters: initial weight of fish, feed consumption, weight gain, feed conversion ratio, percentage visceral fat, and percentages moisture, ash, protein and fat of fish fillets (Steel and Torrie, 1980) using the Statistical Analysis System (SAS) for Windows, Release 6.12 (The SAS Institute, Cary, North Carolina). The least significant difference (LSD) test was conducted if the ANOVA indicated $p \leq 0.05$. For the mortality/survival data, the Fisher's exact test of equal proportions was performed using SAS.

REPORTED RESULTS

The mean and standard deviation for duplicate analyses of dry matter, crude protein, crude fat, and ash concentrations were calculated and reported. The average percent dry matter ranged from 85.80 (H2390) to 88.52 (MON846); average percent crude protein from 30.76 (H2390) to 32.37 (MON859); average percent crude fat ranged from 3.76 (H2390) to 3.92 (MON 853) and average percent ash ranged from 6.10 (H 2390) to 6.39 (MON846). There were no notable differences among the diets for the different corn lines. The Cry3Bb1 protein was verified as present in test substance diets and absent in control and reference substance diets.

The average weights for 20 fish per aquarium ranged from 12.6 to 13.8 g at week 4 (middle of the study) and after 8 weeks (test termination), average weights (of 20 fish/4 aquaria) were in the range of 30.8 to 34.9 g. There were no appreciable differences in the weights of channel catfish fed any of the diets containing the test substance, control or reference substances. Feed consumption, weight gain, feed conversion ratio and survival of channel catfish fed diets containing corn grain with the test substance were not different from those fed diets containing corn grain with the control or reference substances, following 8 weeks of feeding (Table 1).

Table 1. Mean feed consumption, weight gain, feed conversion ratio and survival of five channel catfish within four aquaria for each of five diets.

Corn Line Number	Feed Consumption (g/fish)	Weight Gain (g/fish)	Feed Conversion Ratio[1]	Survival (%)
H 2390	32.9	27.6	1.19	99.0
MON 846	32.6	27.3	1.20	100.0
MON 847	32.7	27.8	1.18	100.0
MON 853	32.7	27.8	1.17	100.0
MON 859	32.2	27.5	1.17	99.0

[1]Feed Conversion Ratio = weight of feed consumption (based on 90% dry matter)/weight gain of live fish.

81

Suvival of channel catfish ranged from 99 to 100% in all treatments. Abnormal fish behavior was not noticed in any tank. Also, no differences in percentage visceral fat or percentages fillet fat, protein, or ash were noted among channel catfish fed any of the experimental diets (Table 2).

Table 2. Mean visceral fat, fillet moisture, fat, protein, and ash (wet tissue basis) of channel catfish fed test diets.

Corn Line Number	Visceral Fat (%)	Fillet Moisture (%)	Fillet Fat (%)	Fillet Protein (%)	Fillet Ash (%)
H 2390	1.80	78.4bc	2.19	18.3	1.15
MON 846	1.50	78.8a	1.94	17.5	1.07
MON 847	1.83	78.7ab	2.16	17.8	1.17
MON 853	1.54	78.4bc	2.30	18.3	1.13
MON 859	1.73	78.2c[1]	2.27	18.1	1.13

[1]Least Significant Difference (LSD) = 0.38

Statistically significant differences were noted only in the percent fillet moisture of fish fed different test diets. A lower fillet moisture was noted in fish fed the diet containing corn grain of the line MON 859 that fish fed diet containing corn grain of line MON 846 (non-transgenic parent line of MON 859); whereas fillet moisture of fish fed diet containing grain of corn line MON 853 was not significantly different from those fed the diet containing MON 847 (non-transgenic parent line of MON 853). The statistical differences were relatively unremarkable, and determined unlikely to be related to test diets. Fish fed diets containing transgenic corn grain (MON 853 and MON 859) had a percentage fillet moisture similar to fish fed diets containing corn from a commercial hybrid (H 2390).

It was concluded that grain derived from the two transgenic corn lines with insect-protection could be used as a feed ingredient in channel catfish diet at levels up to 35% without adverse effects on fish growth, feed conversion efficiency, survival, behavior or body (fillet) composition.

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 and OPPTS Series Guideline 885.4200 - Freshwater Fish Testing. The study was conducted in compliance with the U.S. EPA FIFRA Good Laboratory Practic Regulations (40 CFR Part 160) except:

* The Dose Analysis summary was signed by the Sponsor rather than the Principal Investigator.

* For the Dose Analysis summary, the method used for qualitative analysis was a double sandwich ELISA based on polyclonal antisera that was generated against Cry3Bb1 protein.

82

* SDS-PAGE analysis was performed to reevaluate the molecular weights of the test substance after the initiation of the study. The resulting values were a more accurate assessment of the molecular weights than reported previously. There was no impact on the integrity of the study.

B. **Reported Results and Statistical Analyses:** Significant differences were observed only in percentage fillet moisture among fish fed different diets; however, these are relatively unremarkable and are unlikely related to the different diets. There were no significant differences in feed consumption, weight gain, feed conversion ratio, survival, percentage visceral fat, or percentages fat, protein, or ash in fillets of channel catfish fed the different test diets. No abnormal fish behavior was observed in the study.

C. **Discussion/Risk Assessment:** The study is scientifically sound and no treatment mortality or behavior change was observed among channel catfish fed the different diets. Under conditions of the study, the results indicate that corn grain derived from the two transgenic lines engineered to produce the insecticidal protein, Cry3Bb1 can be used as a feed ingredient in channel catfish diets at levels of up to 35% without adverse effect on fish growth, feed conversion efficiency, survival, behavior, or body composition

D. **Adequacy of the Study:**

1. Validation Category: Acceptable.
2. Rationale: This study meets EPA Guideline requirements for acute toxicity testing of pesticidal substances to nontarget fish.

83

Reviewer: Gail Tomimatsu, Ph.D. Date: January 14, 2003
Microbial Pesticides Branch
Secondary Reviewer: Zigfridas Vaituzis, Ph.D. Date: 1/14/03
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Phil Hutton, Chief
Biopesticides and Pollution Prevention Division (7511C)

DATA EVALUATION REPORT

STUDY TYPE: Acute Avian Dietary Toxicity Study (OPPTS Series 885.4050)

CITATION: S. P. Gallagher, J. Grimes, J. Beavers. *Bacillus thuringiensis* Protein 11231 in Corn Grain: A Dietary Toxicity Study with the Northern Bobwhite. Wildlife International Ltd., 8598 Commerce Drive, Easton, MD 21601. Proj. No.139-444. Monsanto Study No. WL-99-014, August 17, 1999 (Amended). MRID 449043-15.

DP BARCODE: D262045 **CASE**:066221
REG./FILE#:000524-LRA **CHEMICAL/BIOL#**: 006482 Cry3Bb (Vector ZMIR14L)

COMPANY/SPONSOR: Monsanto Co., Regulatory Sciences, 700 Chesterfield Pkwy North, St. Louis, MO 63198

TEST MATERIALS: Grain from three genetically modified corn lines containing Cry3Bb1 Protein - MON 853, MON 854 and MON 855. Cry3Bb1 is also referred to as Cry3Bb and B.t. Protein 11231.

REVIEW CONCLUSION: The acute dietary LC_{50} value for Cry3Bb corn grain when fed to juvenile Northern Bobwhite for 5 days was reported to be greater than 70,000 ppm (10% of the diet), the only concentration tested. No adverse effects on bobwhite quail were seen in eight days. These data show that there will be no adverse effects on avian wildlife from incidental field exposure to Cry3Bb corn. These data are, however, not sufficient to make a hazard assessment from repeated exposure to higher doses of Bt corn.

RECOMMENDATIONS: This study is classified as supplemental. The concentration tested (10% corn in the diet) is too low. A six week study with 60% - 70% corn in the diet is needed to assess hazard to non-target birds from continuous exposure to high levels of Cry3Bb protein.

ADEQUACY OF STUDY:

Validation category: Supplemental.
Rationale: The study is insufficient to assess hazards to non-target avian species which may be exposed continuously to high levels of Cry3Bb corn.

MATERIALS & METHODS: The methods used in conducting the study followed procedures specified in EPA's Registration Guidelines, Pesticide Assessment Guidelines, FIFRA Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms; OECD Guideline 205, Guideline for

Testing of Chemicals, Avian Dietary Toxicity Test and on ASTM Standard E857-87, "Standard Practice of Conducting Subacute Dietary Toxicity Tests with Avian Species."

The test materials, grain of corn plants from three corn lines (MON 853, MON 854, MON 855) genetically modified to contain the Cry3Bb1 protein to provide protection against certain insect pests. Cry3Bb1 has been previously referred to as CryIIIB2 (or Cry3B2), and CryIIIC (or Cry3Bb). The control material, (MON 847) was ground corn grain from a non-genetically modified corn line with genetics similar to the test substances. Ground corn grain from two commercial corn hybrids (Processed Corn Grain 3335 and Processed Corn Grain 3394) served as reference substances. All test, control and reference substances were stored in a freezer (-20° C) until used.

The test birds were 10 days old and all appeared to be in good health at initiation of the test. The birds (*Colinus virginianus*) were obtained from Wildlife International Ltd. Production Flock, Easton, MD and were hatched on January 15, 1999. Birds ranged in weight from 16 to 24 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 (about 10-day acclimation period). Each of the six experimental groups contained twenty chicks; housed in brooding pens containing five birds per pen.

The test chambers were indoor, thermostatically controlled pens manufactured by Beacon Steel Products Co. (Model No. B735Q). Each pen had floor space that measured approximately 72 x 90 cm. Ceiling height was approximately 23 cm. External walls, ceilings and floors were constructed of galvanized sheeting. During the test, the average temperature in the brooding compartment was 38° C ± 2° C. The average ambient room temperature was 26.3° C ± 0.5° C with an average relative humidity of 16% ± 9%. The photoperiod (maintained by a time clock) was 16 hr per day; the light source was fluorescent lights which closely approximate noon-day sunlight. The birds were exposed to an average of ~ 227 lux of illumination.

The test diets were prepared by incorporating the appropriate test, control and reference substances directly into a basal ration (formulated to Wildlife International Ltd.'s specifications-reported in separate Appendix) using a Hobart (Model No. AS200T) mixer. Diets were apportioned by mixing 900.0 g of the appropriate test, control, or reference substance with about 8100.0 g of the basal ration. Samples of diets for the test, control and reference substances were collected at Day 0 and Day 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. Samples were stored frozen until transferred to the sponsor.

Duration of test for the feeding of diets consisting of 10% test, control or reference substances plus 90% basal diet to assigned groups was 5 days. On days 6 - 8, all birds in the study were provided basal diets. Food and water were provided *ad libitum*. All birds were observed daily during acclimation. Birds exhibiting abnormal behavior or physical injury were not used. Signs of toxicity, abnormal behavior and mortality were noted for all birds on a twice-daily basis from the start of the test until the end of the test.

Statistical analyses were not performed since this was a single dose test and no end points were observed.

Quality assurance statements were submitted with the report. The study was conducted in compliance with EPA's Good Laboratory Practice Standards, OECD Principles of Good Laboratory Practice, and Japan MAFF. Several minor deviations with regard to the test substance analysis are listed. These have no impact on the scientific validity of the study.

REPORTED RESULTS:

A. Diet Analysis. Diets prepared with grain from genetically modified lines (MON 853, MON 854, and MON 855) were verified to contain the Cry3Bb1 protein by ELISA procedures. Diets from the non-transgenic corn lines (MON 847 and commercial corn lines 3335 and 3394) did not contain the protein. The raw data for the analysis was not reported in the present submission; and reportedly is located in the Monsanto Study File XX-98-297.

B. Mortalities and Clinical Observations. No mortalities were seen in the control groups which were fed non-genetically modified corn. One incidental mortality was reported in one of the commercial corn hybrid reference groups on Day 5 of the test; a bird had become trapped under a feeder. All other birds in the commercial corn hybrid groups were normal in appearance and behavior for the test duration. There were no mortalities or overt signs of toxicity in any of the birds exposed to the three genetically modified corn line test groups. Wing droop on Days 5, 6, and 7 was reported on one bird in the group feeding on the MON 855 grain; this abnormality was attributed to rapid growth of the bird's primaries and was not considered related to the treatment. All other birds in the MON 853, MON 854 and MON 855 treatment groups were normal in appearance and behavior for the duration of the test.

C. Body Weight and Feed Consumption. There were no apparent treatment related effects on body weight or feed consumption in the commercial corn line reference groups or in any of the three genetically modified corn lines tested when compared to the non-genetically modified corn line control group (Tables 2 and 3, Appendices V and VI).

DISCUSSION:

The study was procedurally sound and no treatment mortality or behavior change was reported between the dosed and control replicates. The estimated LC_{50} of 100,000 μg/g fresh wt grain would appear to indicate that Cry3Bb protein as expressed in corn grain is practically nontoxic to Northern Bobwhite at the concentrations tested (10% Bt corn in the diet).

REVIEWER'S COMMENTS:

A. **Test Procedures:** This study was conducted according to prescribed procedures and determined that the dietary LC_{50} for northern bobwhite exposed to corn grain containing Cry3Bb protein was greater than 100,000 µg Cry3Bb protein/g diet. There were no mortalities at 100,000 µg Cry3Bb protein/g diet. However, the amount of test material (10% Bt corn in the diet) was too low to make an avian hazard assessment from repeated exposure to corn containing Cry3Bb protein.

B. **Statistical Analysis:** The LC_{50} value could not be subjected to statistical analysis because only one concentration was tested.

86

C. **Discussion/Hazard Assessment:** The study was procedurally sound and no treatment mortality or behavior change was reported between the dosed and control replicates. The estimated LC_{50} of > 100,000 μg/g grain would appear to indicate that Cry3Bb protein as expressed in corn grain is practically nontoxic to Northern Bobwhite when exposed to 10% Bt corn in their diet for short time periods. The 8-day time period, and a 10% corn concentration represent a realistic exposure scenario for avian wildlife. These data do not, however address prolonged and repeated exposure to Bt corn, especially as would be the case in domesticated fowl rearing applications. Therefore a non-target avian hazard assessment from prolonged exposure to high levels of Cry3Bb corn cannot be made from the submitted data. A longer study of significant duration, e.g., in the range of 3 to 6 weeks with 60% - 70% corn in the diet is needed to assess hazard to non-target birds from long-term exposure to diets containing elevated levels of Cry3Bb protein.

D. **Adequacy of the Study:**

1. Validation Category: Supplemental.

2. Rationale: This study meets EPA Guideline requirements for acute toxicity testing of nontarget 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 nontarget avian species from continuous exposure to high levels of Cry3Bb. A 3 to 6 week study with 70% corn in the diet is necessary to assess hazards from chronic exposure of exposure of wild and domesticated fowl.

87

MEMORANDUM

SUBJECT: Review of earthworm toxicity study for *Bacillus thuringiensis* Cry3Bb1 protein variant 11098 (Q349R) (PC Code No. 006484; 000524-LEI; MRID No. 457571-01) Monsanto Company

FROM: Gail Tomimatsu, , Plant Pathologist 1/14/2003
Biopesticides and Pollution Prevention Division, 7511C

PEER REVIEW: Zig Vaituzis, Microbiologist 1/14/03
Biopesticides and Pollution Prevention Division, 7511C

Phil Hutton, Chief
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511C

TO: Mike Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division, 7511C

CLASSIFICATION: The submitted study is classified as **Acceptable**, it is scientifically sound, and is consistent with current testing requirements for earthworm hazard assessment of plant-incorporated *Bt* - Cry proteins. The study was prompted at the recommendation of the FIFRA Scientific Panel (October 2000), which expressed concerns of testing the appropriate Cry 3 protein for registration. In a cover letter (Ward to Hutton, 9/10/2002), the applicant states that Cry3Bb1 protein variant 11098 (Q349R) differs from the MON 863 corn-produced 11231 protein of Cry3Bb1 (MRID 449043-16) by 3 amino acids.

STUDY RESULTS: The 14-day LC_{50} for earthworms exposed to Cry3Bb1 protein variant (*E. coli*-produced) in an artificial soil substrate was determined to be greater than 166.6 mg Cry3Bb1 protein variant/kg dry soil, or greater than 20 times the maximum EEC of the protein. There was no apparent effect of the phosphate buffer on the earthworms. Percent mortality of earthworms in the reference substance (chloroacetamide) groups was consistent with historical results, and further confirmed the adequacy and consistency of the protocol used in the definitive test.

BPPD CONCLUSIONS: The results of the study indicate that no adverse effects to earthworms are expected from the planting of maize (corn, USA) containing the Cry3Bb1 protein variant 11098 (Q349R).

88

Reviewer: Gail Tomimatsu, Ph.D. _____ Date: Jan. 14, 2003
Microbial Pesticides Branch
Secondary Reviewer: Zigfridas Vaituzis, Ph.D. _____ Date: _____
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Phil Hutton, Chief
Biopesticides and Pollution Prevention Division (7511C)

DATA EVALUATION REPORT

STUDY TYPE: Acute Toxicity Study- Earthworm (OECD Guideline 207)

CITATION: Sindermann, A. B.; J. R. Porch, H.O. Kreuger. Evaluation of a Cry3Bb1 Protein Variant in an Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate. Wildlife International Ltd., 8598 Commerce Drive, Easton, MD 21601. Proj. No.139-457. Monsanto Study No. WL-2001-129. July 24, 2002. MRID #: 457571-01

DP BARCODE: D285418 **CASE**:069017
REG./FILE#:000524-LEI **CHEMICAL/BIOL#**: 006484 *Bacillus thuringiensis* Cry3Bb protein and the genetic material (Vector ZMIR13L) Necessary for its Production in Corn Event MON 863

COMPANY/SPONSOR: Monsanto Co., Regulatory Sciences, 700 Chesterfield Pkwy North, St. Louis, MO 63198

TEST MATERIALS: The test substance was Cry3Bb1 protein variant (produced by *E. coli*). The negative control was an aqueous phosphate buffer salt; the positive controls were two different concentrations of chloroacetamide.

REVIEW CONCLUSION: The study was procedurally sound and the data show that no adverse effects to earthworms are expected at twenty times the maximum environmental concentration (i.e., 166.6 mg Cry3Bb1 variant per kg of soil) of Cry3Bb1 protein variant.

ADEQUACY OF STUDY:

1. Validation Category: Acceptable .
2. Rationale: This study meets EPA approved requirements for assessing acute pesticidal risks to earthworms.

MATERIALS & METHODS: The methods used in conducting the study followed procedures

89

specified in the Organization for Economic Cooperation and Development (OECD) Guideline 207.

The test material, consisted of purified Cry3Bb1 protein (*E. coli* -produced) in purified water suspension as prepared by the Sponsor. The test substance was described as a pale yellow solid, and identified as Cry3Bb1 11098 (Q349R); Cry3Bb1 Protein Variant; Lot 6554026; Batch: 6705604 with a purity of 75.5% Cry3Bb1 Protein Variant. There was no expiration date and the protein was frozen at -80 °C .

The control substance was a 10 mM solution of Phosphate Buffer, pH 7.0; Lot number 6974939-A, and received frozen from the Sponsor with an expiration date of October 1, 2002.

The reference substance was chloroacetamide, received from Chem Service and described as a crystalline solid, identified as 2-chloroacetamide 0-258; Lot no. 212-63B, with a reported purity of 98%, expiration date: July, 2004. The reference substance was stored under ambient conditions.

The test organisms were adult earthworms with clitellum (*Eisenia fetida*) supplied by Worm Man's Worm Farm, Monroe Township, New Jersey. The worms were placed in a container of artificial "soil" mixture (70% quartz sand, 20% kaolin clay, 10% sphagnum peat carbonate, adjusted to ~ 33% moisture by weight for the acclimation period (24 hrs). The pH of the artificial soil mixture was adjusted to 6.0 with calcium carbonate. On the day of test initiation, worms were rinsed with deionized water, and impartially distributed by pairs into groups of 10 worms. Each group of worms was weighed and placed on the soil mixture in the test chamber. Worms were not fed during the test.

The test chambers consisted of 1-L glass beakers covered with plastic wrap and perforated for air exchange. There were four chambers, with 10 adult worms for each of 7 test groups (a total of 40 worms per treatment).

The test soils. To prepare the test soils, the appropriate test, control or reference substance was mixed with deionized water and then mixed into the dry artificial soil and adjusted to a moisture content of 33 %. To ensure homogeneity, the soil was mixed for 20 min with the appropriate test, reference or control substances. The tests were performed in 2 separate experiments (both of 14-day duration), but utilized worms from the same lot number. The first experiment included 2 levels of the Cry3Bb protein variant (10X and 20X maximum EC), a control substance (phosphate buffer) and an assay control (water only); the second experiment included two levels of chloroacetamide, 10 mg/kg soil, and 20 mg/kg soil, and an assay control. Seven hundred fifty grams of the test soils were added to each test chamber.

The maximum estimated environmental concentration of Cry3Bb1 protein variant in soil was calculated as 8.33 mg per kg dry weight soil, based on the assumption of a maximum plant tissue concentration of 93.0 μg Cry3Bb1/g fresh weight, and a planting of 25,000 maize plants /acre.

Environmental conditions for the test chambers were maintained in an environmental

90

chamber set to maintain a temperature of 20 ± 2°C. Air temperature was measured at least once daily in the chamber; the soil temperature was measured in one replicate of each treatment and control group on Days 0 and 14 of the test. The photoperiod during the test was 24 hrs of continuous light each day provided by overhead fluorescent bulbs. The intensity was approximately 400 to 800 lux.

Soil samples and analyses were collected for measurements of pH, moisture content and test initiation and termination. Measurements of pH were made with a waterproof hand-held pH meter. At the start and end of the test, soil samples were collected from each test, control and reference substance group and were used to measure the initial and final pH and moisture content of the soils. Moisture content was determined by measuring the initial weight of the soil sample, and weighing the soil sample after drying for 18 hrs at 105°C. Percent moisture was calculated using the formula:

%moisture = [(net wet weight - net dry weight) ÷ net wet weight] x100

Samples of treated soils were also collected to verify test concentrations and to confirm the homogeneity and stability of the test substances in the soil. On Day 0, samples for homogeneity (or verification) were collected from the top, middle and bottom of the mixing vessel. Larvae of the Colorado Potato Beetle were used in "[d]ose confirmation bioassays"(via agar diet overlays) conducted by the Sponsor based on Monsanto Company procedures SOP No.BR-ME-0044-02, entitled "Diet incorporation insect bioassay for the biological activity measurement of *Bacillus thuringiensis* and other insecticidal proteins."

Other observations and statistical analyses were recorded on Days 0, 7 and 14. Worms were placed on the surface of the soil in each test chamber and were observed for burrowing behavior on Days 0 and 7. The number of surviving worms were counted and observed for pathological or behavioral abnormalities on Days 7 and 14 of the test after the contents of each test chamber were emptied onto clean paper. Mortalities, signs of toxicity or abnormal behavior were noted. On Day 14, all worms were euthanized after observations; and all carcasses were disposed of by incineration. Body weights by group for each replicate were determined before placement in the test chambers on Day 0. Surviving worms were removed from each replicate test chamber, rinsed with de-ionized water and weighed as a group at test termination, Day 14. Individual body weights were calculated on the basis of the group average.

Body weights and changes in body weights were compared statistically with the Dunnett's test, after testing the data for homogeneity of variance and normal distribution.

REPORTED RESULTS:

A. Dose Confirmation: The Cry3Bb1 protein variant was found in soil aliquots collected from the top, middle and lower portions of the test chamber, indicating that the test material was distributed throughout the soil profile in the chamber at test initiation. Little bioactivity was observed in the Cry 3Bb1 amended soils (low and high doses) ; the low bioactivity was attributed

92

to rapid degradation of Cry3Bb1 protein variant and other research (Martin et al. 2000. Aerobic soil degradation of the B.t. protein 11098 as a component of insected protected corn. Monsanto Company MSL-16440). Soils amended with the phosphate buffer controls were not bioactive towards CPB.

B. Environmental Conditions: For the 14-day study, air temperatures in the chamber ranged from 21 to 22 °C; and under continuous lighting at an average intensity of 499 ±74.8 lux. Worms in the reference substance test were maintained under continuous lighting at an average intensity of 531 ± 65.6 lux (range of surface of test chambers: 443 to 631 lux).

There were little to no noticeable changes in soil pH, temperature and moisture content of test soils for the protein experiment and the reference substance tests. In the protein experiment, soil pH ranged from 8.1 to 8.3 soil temperature ranged from 21-22°C, and soil moisture ranged from 32 .4% to 31.0% at test initiation and test termination, respectively.

C. Mortalities and Clinical Observations.

Protein variant toxicity test. There were no earthworm mortalities in the any of the controls or protein variant-treated soils during the 14 day study. Changes in average body weights were not statistically different (p>0.05) among the controls and protein-amended soils. Other than a slight loss in body weight (expected), there were no other remarkable observations. An LC50 could not be statistically defined, therefore the LC50 value is greater than the highest concentration tested (166.6 mg Cry3Bb1 protein variant/kg dry soil).

Reference substance toxicity test. Although not run concurrently with the protein variant toxicity test, results were as expected, and average individual body weights, and changes in body weights from test initiation to test termination were reported consistent with those observed in other studies with chloroacetamide. At the end of the study, mortality in the 10 and 20 mg chloroacetamide/kg soil was 2.5%(1 of 40) and 85% (34 of 40), respectively.

DISCUSSION: The 14-day LC_{50} for earthworms exposed to Cry3Bb1 protein in an artificial soil substrate was determined to be greater than 166.6 mg Cry3Bb1 protein variant/kg dry soil, or greater than 20 times the maximum EEC of the protein. There was no apparent effect of the phosphate buffer on the earthworms. Percent mortality of earthworms in the reference substance (chloroacetamide) groups was consistent with historical results, and further confirmed the adequacy and consistency of the protocol used in the definitive test.

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.

Regulatory Compliance: This study was conducted according to OECD Guideline 207 and is in regulatory compliance with GLP Standards of 40 CFR Parts 160 and 792 (USEPA, 1989); OECD Principles of Good Laboratory Practice, ENV/MC/CHEM (98) 17 (OECD, 1998) and Japan MAFF, Notification No. 3850, Agricultural Production Bureau (1999) except that verification of the protein test concentrations, stability and homogeneity of chloroacetamide in the soil were not determined, and the purity and concentration of the protein test substance were not determined. These exceptions did not affect the overall integrity of the study.

B. **Discussion/Hazard Assessment:** The study was procedurally sound and the data show that no adverse effects to earthworms are expected from exposure to twenty times the maximum environmental concentration of Cry3Bb1 protein variant 11098 (Q349R).

C. **Adequacy of the Study:**

1. Validation Category: Acceptable .
2. Rationale: This study meets EPA Guideline requirements for assessing acute pesticidal risks to earthworms.

MEMORANDUM

SUBJECT: Review of earthworm toxicity study for *Bacillus thuringiensis* Cry3Bb1 protein **variant** 11098 (Q349R) (PC Code No. 006484; 000524-LEI; MRID No. 457571-01) Monsanto Company

FROM: Gail Tomimatsu, , Plant Pathologist 1/14/2003
Biopesticides and Pollution Prevention Division, 7511C

PEER REVIEW: Zig Vaituzis, Microbiologist 1/14/03
Biopesticides and Pollution Prevention Division, 7511C

Phil Hutton, Chief
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511C

TO: Mike Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division, 7511C

CLASSIFICATION: The submitted study is classified as **Acceptable**, it is scientifically sound, and is consistent with current testing requirements for earthworm hazard assessment of plant-incorporated *Bt* - Cry proteins. The study was prompted at the recommendation of the FIFRA Scientific Panel (October 2000), which expressed concerns of testing the appropriate Cry 3 protein for registration. In a cover letter (Ward to Hutton, 9/10/2002), the applicant states that Cry3Bb1 protein variant 11098 (Q349R) differs from the MON 863 corn-produced 11231 protein of Cry3Bb1 (MRID 449043-16) by 3 amino acids.

STUDY RESULTS: The 14-day LC_{50} for earthworms exposed to Cry3Bb1 protein variant (*E. coli*-produced) in an artificial soil substrate was determined to be greater than 166.6 mg Cry3Bb1 protein variant/kg dry soil, or greater than 20 times the maximum EEC of the protein. There was no apparent effect of the phosphate buffer on the earthworms. Percent mortality of earthworms in the reference substance (chloroacetamide) groups was consistent with historical results, and further confirmed the adequacy and consistency of the protocol used in the definitive test.

BPPD CONCLUSIONS: The results of the study indicate that no adverse effects to earthworms are expected from the planting of maize (corn, USA) containing the Cry3Bb1 protein **variant** 11098 (Q349R).

94

Reviewer: Gail Tomimatsu, Ph.D. _____ Date: Jan. 14, 2003
Microbial Pesticides Branch
Secondary Reviewer: Zigfridas Vaituzis, Ph.D. _____ Date: _____
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Phil Hutton, Chief
Biopesticides and Pollution Prevention Division (7511C)

DATA EVALUATION REPORT

STUDY TYPE: Acute Toxicity Study- Earthworm (OECD Guideline 207)

CITATION: Sindermann, A. B., J. R. Porch, H.O. Kreuger. Evaluation of a Cry3Bb1 Protein Variant in an Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate. Wildlife International Ltd., 8598 Commerce Drive, Easton, MD 21601. Proj. No.139-457. Monsanto Study No. WL-2001-129. July 24, 2002. MRID #: 457571-01

DP BARCODE: D285418 **CASE**:069017
REG./FILE#:000524-LEI **CHEMICAL/BIOL#**: 006484 *Bacillus thuringiensis* Cry3Bb protein and the genetic material (Vector ZMIR13L) Necessary for its Production in Corn Event MON 863

COMPANY/SPONSOR: Monsanto Co., Regulatory Sciences, 700 Chesterfield Pkwy North, St. Louis, MO 63198

TEST MATERIALS: The test substance was Cry3Bb1 protein **variant** (produced by *E. coli*). The negative control was an aqueous phosphate buffer salt; the positive controls were two different concentrations of chloroacetamide.

REVIEW CONCLUSION: The study was procedurally sound and the data show that no adverse effects to earthworms are expected at twenty times the maximum environmental concentration (i.e., 166.6 mg Cry3Bb1 variant per kg of soil) of Cry3Bb1 protein variant.

ADEQUACY OF STUDY:

1. Validation Category: Acceptable .
2. Rationale: This study meets EPA approved requirements for assessing acute pesticidal risks to earthworms.

MATERIALS & METHODS: The methods used in conducting the study followed procedures

specified in the Organization for Economic Cooperation and Development (OECD) Guideline 207.

The test material, consisted of purified Cry3Bb1 protein (*E. coli* -produced) in purified water suspension as prepared by the Sponsor. The test substance was described as a pale yellow solid, and identified as Cry3Bb1 11098 (Q349R); Cry3Bb1 Protein Variant; Lot 6554026; Batch: 6705604 with a purity of 75.5% Cry3Bb1 Protein Variant. There was no expiration date and the protein was frozen at -80 °C .

The control substance was a 10 mM solution of Phosphate Buffer, pH 7.0; Lot number 6974939-A, and received frozen from the Sponsor with an expiration date of October 1, 2002.

The reference substance was chloroacetamide, received from Chem Service and described as a crystalline solid, identified as 2-chloroacetamide 0-258; Lot no. 212-63B, with a reported purity of 98%, expiration date: July, 2004. The reference substance was stored under ambient conditions.

The test organisms were adult earthworms with clitellum (*Eisenia fetida*) supplied by Worm Man's Worm Farm, Monroe Township, New Jersey. The worms were placed in a container of artificial "soil" mixture (70% quartz sand, 20% kaolin clay, 10% sphagnum peat carbonate, adjusted to ~ 33% moisture by weight for the acclimation period (24 hrs). The pH of the artificial soil mixture was adjusted to 6.0 with calcium carbonate. On the day of test initiation, worms were rinsed with deionized water, and impartially distributed by pairs into groups of 10 worms. Each group of worms was weighed and placed on the soil mixture in the test chamber. Worms were not fed during the test.

The test chambers consisted of 1-L glass beakers covered with plastic wrap and perforated for air exchange. There were four chambers, with 10 adult worms for each of 7 test groups (a total of 40 worms per treatment).

The test soils. To prepare the test soils, the appropriate test, control or reference substance was mixed with deionized water and then mixed into the dry artificial soil and adjusted to a moisture content of 33 %. To ensure homogeneity, the soil was mixed for 20 min with the appropriate test, reference or control substances. The tests were performed in 2 separate experiments (both of 14-day duration), but utilized worms from the same lot number. The first experiment included 2 levels of the Cry3Bb protein variant (10X and 20X maximum EC), a control substance (phosphate buffer) and an assay control (water only); the second experiment included two levels of chloroacetamide, 10 mg/kg soil, and 20 mg/kg soil, and an assay control. Seven hundred fifty grams of the test soils were added to each test chamber.

The maximum estimated environmental concentration of Cry3Bb1 protein variant in soil was calculated as 8.33 mg per kg dry weight soil, based on the assumption of a maximum plant tissue concentration of 93.0 μg Cry3Bb1/g fresh weight, and a planting of 25,000 maize plants /acre.

Environmental conditions for the test chambers were maintained in an environmental

chamber set to maintain a temperature of 20 ± 2°C. Air temperature was measured at least once daily in the chamber; the soil temperature was measured in one replicate of each treatment and control group on Days 0 and 14 of the test. The photoperiod during the test was 24 hrs of continuous light each day provided by overhead fluorescent bulbs. The intensity was approximately 400 to 800 lux.

Soil samples and analyses were collected for measurements of pH, moisture content and test initiation and termination. Measurements of pH were made with a waterproof hand-held pH meter. At the start and end of the test, soil samples were collected from each test, control and reference substance group and were used to measure the initial and final pH and moisture content of the soils. Moisture content was determined by measuring the initial weight of the soil sample, and weighing the soil sample after drying for 18 hrs at 105°C. Percent moisture was calculated using the formula:

%moisture = [(net wet weight - net dry weight) ÷ net wet weight] x100

Samples of treated soils were also collected to verify test concentrations and to confirm the homogeneity and stability of the test substances in the soil. On Day 0, samples for homogeneity (or verification) were collected from the top, middle and bottom of the mixing vessel. Larvae of the Colorado Potato Beetle were used in "[d]ose confirmation bioassays"(via agar diet overlays) conducted by the Sponsor based on Monsanto Company procedures SOP No.BR-ME-0044-02, entitled "Diet incorporation insect bioassay for the biological activity measurement of *Bacillus thuringiensis* and other insecticidal proteins."

Other observations and statistical analyses were recorded on Days 0, 7 and 14. Worms were placed on the surface of the soil in each test chamber and were observed for burrowing behavior on Days 0 and 7. The number of surviving worms were counted and observed for pathological or behavioral abnormalities on Days 7 and 14 of the test after the contents of each test chamber were emptied onto clean paper. Mortalities, signs of toxicity or abnormal behavior were noted. On Day 14, all worms were euthanized after observations; and all carcasses were disposed of by incineration. Body weights by group for each replicate were determined before placement in the test chambers on Day 0. Surviving worms were removed from each replicate test chamber, rinsed with de-ionized water and weighed as a group at test termination, Day 14. Individual body weights were calculated on the basis of the group average.

Body weights and changes in body weights were compared statistically with the Dunnett's test, after testing the data for homogeneity of variance and normal distribution.

REPORTED RESULTS:

A. Dose Confirmation: The Cry3Bb1 protein variant was found in soil aliquots collected from the top, middle and lower portions of the test chamber, indicating that the test material was distributed throughout the soil profile in the chamber at test initiation. Little bioactivity was observed in the Cry 3Bb1 amended soils (low and high doses) ; the low bioactivity was attributed

to rapid degradation of Cry3Bb1 protein variant and other research (Martin et al. 2000. Aerobic soil degradation of the B.t. protein 11098 as a component of insected protected corn. Monsanto Company MSL-16440). Soils amended with the phosphate buffer controls were not bioactive towards CPB.

B. **Environmental Conditions**: For the 14-day study, air temperatures in the chamber ranged from 21 to 22 °C; and under continuous lighting at an average intensity of 499 ±74.8 lux. Worms in the reference substance test were maintained under continuous lighting at an average intensity of 531 ± 65.6 lux (range of surface of test chambers: 443 to 631 lux).

There were little to no noticeable changes in soil pH, temperature and moisture content of test soils for the protein experiment and the reference substance tests. In the protein experiment, soil pH ranged from 8.1 to 8.3 soil temperature ranged from 21-22 °C, and soil moisture ranged from 32 .4% to 31.0% at test initiation and test termination, respectively.

C. Mortalities and Clinical Observations.

Protein variant toxicity test. There were no earthworm mortalities in the any of the controls or protein variant-treated soils during the 14 day study. Changes in average body weights were not statistically different (p>0.05) among the controls and protein-amended soils. Other than a slight loss in body weight (expected), there were no other remarkable observations. An LC50 could not be statistically defined, therefore the LC50 value is greater than the highest concentration tested (166.6 mg Cry3Bb1 protein variant/kg dry soil).

Reference substance toxicity test. Although not run concurrently with the protein variant toxicity test, results were as expected, and average individual body weights, and changes in body weights from test initiation to test termination were reported consistent with those observed in other studies with chloroacetamide. At the end of the study, mortality in the 10 and 20 mg chloroacetamide/kg soil was 2.5%(1 of 40) and 85% (34 of 40), respectively.

DISCUSSION: The 14-day LC_{50} for earthworms exposed to Cry3Bb1 protein in an artificial soil substrate was determined to be greater than 166.6 mg Cry3Bb1 protein variant/kg dry soil, or greater than 20 times the maximum EEC of the protein. There was no apparent effect of the phosphate buffer on the earthworms. Percent mortality of earthworms in the reference substance (chloroacetamide) groups was consistent with historical results, and further confirmed the adequacy and consistency of the protocol used in the definitive test.

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.

Regulatory Compliance: This study was conducted according to OECD Guideline 207 and is in regulatory compliance with GLP Standards of 40 CFR Parts 160 and 792 (USEPA, 1989); OECD Principles of Good Laboratory Practice, ENV/MC/CHEM (98) 17 (OECD, 1998) and Japan MAFF, Notification No. 3850, Agricultural Production Bureau (1999) except that verification of the protein test concentrations, stability and homogeneity of chloroacetamide in the soil were not determined, and the purity and concentration of the protein test substance were not determined. These exceptions did not affect the overall integrity of the study.

B. **Discussion/Hazard Assessment:** The study was procedurally sound and the data show that no adverse effects to earthworms are expected from exposure to twenty times the maximum environmental concentration of Cry3Bb1 protein variant 11098 (Q349R).

C. **Adequacy of the Study:**

1. Validation Category: Acceptable .
2. Rationale: This study meets EPA Guideline requirements for assessing acute pesticidal risks to earthworms.

Reviewer: Gail Tomimatsu, Ph.D. Date: Jan 14, 2003
Microbial Pesticides Branch
Secondary Reviewer: Zigfridas Vaituzis, Ph.D. Date: 1/14/03
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Phil Hutton, Chief
Biopesticides and Pollution Prevention Division (7511C)

DATA EVALUATION REPORT

STUDY TYPE: Acute Toxicity Study- Earthworm (OECD Guideline 207)

CITATION: Hoxter, K.A., S.J. Palmer, H.O. Kreuger. *Bacillus thuringiensis* Protein 11231: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate. Wildlife International Ltd., 8598 Commerce Drive, Easton, MD 21601. Proj. No.139-443A. Monsanto Study No. WL-99-013, July 30, 1999. MRID 449043-16.

DP BARCODE: D262045 **CASE**:066221
REG./FILE#:000524-LRA **CHEMICAL/BIOL#**: 006482 Cry3Bb (Vector ZMIR14L)

COMPANY/SPONSOR: Monsanto Co., Regulatory Sciences, 700 Chesterfield Pkwy North, St. Louis, MO 63198

TEST MATERIALS: The test substance was Cry3Bb1 protein (also known as *B.t.* protein 11231; or CryIIIB2, Cry3B2 or CryIIIC). The negative control was a buffer salt; the positive control was chloroacetamide.

REVIEW CONCLUSION: The study was procedurally sound and the data show that no adverse effects to earthworms are expected at ten times the maximum environmental concentration of Cry3Bb1 protein.

ADEQUACY OF STUDY:

1. Validation Category: Acceptable .
2. Rationale: This study meets EPA Guideline requirements for assessing acute pesticidal risks to earthworms.

MATERIALS & METHODS: The methods used in conducting the study followed procedures specified in the Organization for Economic Cooperation and Development (OECD) Guideline 207.

The test material, consisted of purified Cry3Bb1 protein in purified water suspension as prepared by the Sponsor. The frozen liquid test substance was received from Monsanto Company and was identified as: Cry3B2.11231; Lot no. 6312812; and reported purity of 96 %. The reported activity was 16.8 mg active protein/mL water (adjusted for purity). The test substance was assigned Wildlife International Ltd. identification number 4799, and frozen at -80° C. Cry3Bb1 is the current accepted designation for the nomenclature of this insecticidal protein (Crickmore, N., et al. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62:807-13.)

100

A buffer salt was used in preparing and isolating the test substance. It was received from Monsanto and assigned Wildlife International Ltd. identification number 4800. The frozen liquid was identified as Sodium Bicarbonate Salt Solution from Lyophilized solution; Lot no. 6436620. The control substance contained 33.6 mg sodium bicarbonate/mL deionized water. The control substance was stored at -80° C.

The reference substance was chloroacetamide, known to be toxic to earthworms and received from Chem Service and assigned Wildlife International Ltd. identification number 4058. It was described as a crystalline solid, identified as 2-chloroacetamide; Lot no. 167-54B, with a reported purity of 98%, expiration date: Feb. 2002. The reference substance was stored under ambient conditions.

The test organisms were earthworms (*Eisenia fetida*) supplied by Willingham Worm Farm, Butler, Georgia. Prior to the acclimation period and test, the worms were placed in culture chambers containing a mixture of moist peat and manure for 19 days. During this time, they were fed saturated alfalfa. About 24 hours before test initiation, 325 adult worms with clitellum were selected and placed in a container of prepared artificial soil (peat, sand and Kaolin based), adjusted to about 33% soil moisture. On the day of test initiation, worms were rinsed with deionized water, carefully blotted dry and impartially distributed by pairs into groups of 10 worms. Each group of worms was weighed and placed on the soil mixture in the test chamber. Worms were not fed during the test.

The test chambers consisted of 1-L glass beakers covered with plastic wrap and perforated for air exchange. There were four chambers, with 10 adult worms for each of 7 test groups (a total of 40 worms per treatment).

The test soils were prepared with a bulk artificial soil, which consisted of 70% sand, 20% Kaolin clay, and 10% sphagnum peat. Prior to hydration, the pH of the artificial soil was adjusted to 5.8 with calcium carbonate and stored in a sealed container under ambient conditions until needed for test soil preparation. To prepare the test soils, the appropriate test, control or reference substance was mixed with deionized water and then mixed into the moistened artificial soil and adjusted to a moisture content of 33 %. To ensure homogeneity, the soil was mixed for 15 min with the appropriate test, reference or control substances.

Concentrations of the test, control and reference substances were calculated based on the dry weight of the soil. The test substance and buffer control substance concentrations were based on the reported concentration of the protein or salt solution. The reference substance concentrations were adjusted for the purity of the reported purity of chloroacetamide. The assay control soil was mixed also for 15 minutes but without the addition of the test or control substances. Seven hundred fifty grams of the test soils were added to each test chamber.

Environmental conditions for the test chambers were maintained in an environmental chamber set to maintain a temperature of 20 ± 2°C. Air temperature was monitored continuously; the soil temperature was measured in each group on Days 0 and 14 of the test. The photoperiod during the test was 24 hrs of continuous light each day provided by overhead fluorescent bulbs. The intensity was approximately 400 to 800 lux.

Soil samples and analyses were collected for measurements of pH, moisture content and verification of test concentrations and homogeneity of test substances in the soil. At the start and end of the test, soil samples were collected from each test, control and reference substance group and

were used to measure the initial and final pH and moisture content of the soils. Moisture content was determined by measuring the initial weight of the soil sample, and weighing the soil sample after drying for 18 hrs at 105°C. Percent moisture was calculated using the formula:

%moisture = [(net wet weight - net dry weight) ÷ net wet weight] x100

Samples of treated soils were also collected to verify test concentrations and to confirm the homogeneity and stability of the test, buffer control or reference substances in the soil. On Day 0, samples for homogeneity (or verification) were collected from the top, middle and bottom of the mixing vessel with the 570 mg/kg test soil; and verification samples also were collected from the assay control, the 57.0 mg/kg test, and the control substance soils at preparation. Upon termination of the study, one sample was collected from the assay, and each of the test and control substance soils for stability analyses. All samples were stored in 4-oz polypropylene bottles and frozen (-80 °C) for a maximum of 5 days before shipment to the Sponsor for analysis. Analyses of the samples were based on Monsanto Company procedures SOP No. BR-ME-0044-02, entitled "Diet incorporation insect bioassay for the biological activity measurement of *Bacillus thuringiensis* and other insecticidal proteins."

Other observations and statistical analyses were recorded on Days 0, 7 and 14. Worms were placed on the surface of the soil in each test chamber and were observed for burrowing behavior on Days 0 and 7. The number of surviving worms were counted and observed for behavioral abnormalities on Days 7 and 14 of the test after the contents of each test chamber were emptied onto clean paper. Mortalities, signs of toxicity or abnormal behavior were noted. On Day 14, all worms were euthanized after observations; and all carcasses and soil substrate were disposed of by incineration. Body weights by group for each replicate were determined before placement in the test chambers on Day 0. Surviving worms were removed from each replicate test chamber, rinsed with de-ionized water and weighed as a group at test termination, Day 14. Individual body weights were calculated on the basis of the group average.

Statistical analyses of the mortality and body weight data for the test substance, control substance and assay control groups were conducted using Fisher's LSD test. Comparisons were made between the test substance and the respective control substance groups; and between the control substance and the assay control groups. The reference substance groups were not included in the statistical analyses of the test and control substances.

REPORTED RESULTS:

A. Dose Confirmation: Bioactivity of Cry3Bb1 protein in the earthworm soil samples was confirmed using a bioassay with the Colorado potato beetle (CPB), a known susceptible insect. At test initiation, the bioactivity reported in the collected samples was about 37% of the expected level of activity, whereas the activity was estimated to be between 77 to 96% of the expected level of activity. The cause of the variation was not reported in the study. The difference between Day 0 and Day 14 samples was not significant based on overlap of the 96% confidence intervals. Overall, the data demonstrated that bioactive Cry3Bb1 protein was detected "[t]hroughout the duration of the earthworm study." *Reviewers' Comment: Bioactive Cry3Bb1 protein data for Days 0 and 14 were the only two sampling times provided in the report.*

B. Environmental Conditions: For the 14-day study, air temperatures in the chamber ranged from 19.5 to 20.5 °C; and under continuous lighting at an average intensity of 600 ± 93 lux (range of surface of test chambers: 459 to 780 lux).

The pH of all soils ranged from 7.8 to 8.5 on Day 0 , and from 7.9 to 8.7 on Day 14. The soil temperature "[d]uring the 14-day test remained within the desired range of 20 ± 2 °C, and the moisture content of the soils remained relatively constant during the test." *Reviewers' Comment: Soil temperatures and moisture data were reported only for two times – Days 0 and 14.*

C. Mortalities and Clinical Observations.

Assay (Negative Control) Group. In the assay control group, there were 7 deaths (of 40; 18%). One worm was reported as remaining on the soils surface almost 2 hrs after Day 7 observations, and one worm was found dead on top of the soil surface on the last day (Day 14). All other surviving worms were normal in appearance and behavior throughout the test.

57.0 mg a.i./kg soil Group. The percent mortality reported was 8% (3 of 40) on Day 14. One worm in the 57.0 mg/kg group inadvertently was injured during the Day 7 observation procedure; and other surviving worms appeared normal. Worms in the 57.0 mg /kg group burrowed beneath the surface within ½ hr on Day 0, and within one hr on Day 7. The study authors attributed such behavior as signs of aversion to the soil. The number of mortalities in this group was less than that in the assay control group, and was not significantly different ($p>0.05$) from the [a]ppropriate buffer salt control group (117.5 mg/kg). Accordingly, the mortalities were considered not to be related to treatment with the test substance.

570 mg a.i./kg soil Group. The percent mortality reported was 38% (15 of 40) at the end of the test. A marked increase in the number of mortalities and in the number of worms exhibiting signs of toxicity were noted in the 570 mg/kg test substance group. Reduced reaction to mechanical stimuli and worms which appeared soft, thin and necrotic were considered signs of toxicity. There was no indication of an aversion to the soil on Day 0. Two worms were noted to remain on the soil surface for about 1 hr on Day 7 in this test group. The increase in mortalities in this group was statistically significant [($p<0.05$)] when compared to the appropriate buffer salt control group (117.5 mg/kg); 38% and 3% mortalities, respectively. The mortalities were likely due to a miscalculation of salt concentrations in the test soil [The actual concentration of sodium bicarbonate salt in the 57.0 and 570 mg Cry3Bb1 protein/kg treatment groups was 70 and 699 mg/kg, respectively.]

Buffer salt control Groups. In both of the buffer salt control groups, mortality on Day 14 was 3% (1 out of 40). The mortality in these groups was not significantly different ($p>0.05$) from the assay control group. Surviving worms appeared normal and exhibited no signs of toxicity or aversion to the soils for the test duration.

Reference substance Groups. At the end of the study, mortality in the 10 and 20 mg chloroacetamide/kg soil was 3%(1 of 40) and 100% (40 of 40), respectively. All surviving worms in the soils treated with the lower concentration of chloroacetamide were normal in appearance and behavior throughout the test; all worms had died by Day 7 in the 20 mg chloroacetamide/kg group.

D. Body Weight and Feed Consumption. Average individual body weights for surviving worms were calculated from the measurements taken on Days 0 and 7. There was no significant

difference ($p>0.05$) in the average individual body weights for the 57.0 mg/kg test substance group ($\bar{x}_{n=40} = 0.30$ g), compared to the appropriate buffer control substance group (117.5 mg salt/kg soil control group; $\bar{x}_{n=40} = 0.31$ g). However, the average individual body weights of worms exposed to 570 mg/kg test substance ($\bar{x}_{n=40} = 0.20$ g) was significantly different [($p<0.05$)] from the appropriate buffer control substance group (117.5 mg buffer salt/kg; $\bar{x}_{n=40} = 0.32$ g). *Reviewers' Comment: Body weight comparisons were statistically significant; probability of this significance is likely p>0.05, and not as reported.* Comparison of the control substance treatment levels with the assay control indicated a relatively slight, yet statistically significant increase in body weight.

DISCUSSION: This study was conducted according to EPA approved guideline procedures for acute toxicity testing of pesticidal substances to earthworms. The 14-day LC_{50} for earthworms exposed to Cry3Bb1 protein in an artificial soil substrate was determined to be greater than 570 mg Cry3Bb1 protein/kg dry soil, or greater than 10 times the maximum EEC of the protein. Based on the effects observed among worms in the 570 mg Cry3b1 protein/kg dry soil treated group, the NOEC was 57.0 mg Cry3Bb1 protein/kg dry soil. There was no apparent effect of the buffer on the earthworms. Percent mortality of earthworms in the reference substance (chloroacetamide) groups was consistent with historical results, and further confirmed the adequacy and consistency of the protocol used in the definitive test.

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. It was noted in the study design that the levels of buffer salt in the control substance treatment groups were higher than the buffer salt levels in the test substance treatment groups, because of a miscalculation. The actual concentration of sodium bicarbonate salt in the 57.0 and 570 mg Cry3Bb1 protein/kg treatment groups was 70 and 699 mg/kg, respectively. The higher concentrations did not appear to have any influence on the overall conclusions of the study.

Regulatory Compliance: The study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. EPA in 40 CFR Parts 160 and 792; OECD Principles of Good Laboratory Practice; and Japan MAFF, except:

*Reference substance soils were not analyzed to verify concentrations, homogeneity, or stability of the reference substance in the carrier. This did not affect the results of this study.

*The test and control substance characterization, derivation of the maximum hazard dose, and dose confirmation portions of the study were conducted by the Sponsor in accordance with the GLPs, except:

–Egg receipt, sterilization and incubation to the first instar larvae stage for the insect bioassay portion of the analytical procedure was not conducted in accordance with the GLPs. There was no impact on the outcome of the bioassay.

–SDS-PAGE analysis was performed to reevaluate the molecular weights of the test substance after the initiation of the study. The resulting values were a more accurate assessment of the molecular weights than reported previously. This did not affect the integrity of the study.

104

B. **Statistical Analysis:** The LC_{50} value could not be subjected to statistical analysis because only one test concentration yielded adverse effects attributable to the test substance. The no-mortality and no-observed-effect concentrations were determined using a visual examination of the mortality and clinical observation data.

C. **Discussion/Hazard Assessment:** The study was procedurally sound and the data show that no adverse effects to earthworms are expected at ten times the maximum environmental concentration of Cry3Bb1 protein.

D. **Adequacy of the Study:**

1. Validation Category: Acceptable .
2. Rationale: This study meets EPA Guideline requirements for assessing acute pesticidal risks to earthworms.

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

JUL 23 2002

OFFICE OF PREVENTION, PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Preliminary risk assessment for soil, soil surface and foliar invertebrates for *Bacillus thuringiensis* Cry3Bb protein, EPA Reg. No. 524-LEI; Barcode No. D262045; Case No. 066221; Submission No. S572997, submitted by Monsanto Co. for corn containing *Bacillus thuringiensis* Cry3Bb protein and the genetic material necessary for its production (vector ZMIR13L) in corn for control of corn root worm (*Diabrotica* spp.)

FROM: Zigfridas Vaituzis, Ph.D., Senior Scientist
Phil Hutton, Chief
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511C

PEER REVIEW: Robyn Rose, Entomologist
Biopesticides and Pollution Prevention Division, 7511C

TO: Michael Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division 7511C

Pesticide Name: Modified *Bacillus thuringiensis* Cry3Bb1 insecticidal protein and genetic material necessary for its expression in corn developed with event MON 863 (vector ZMIR13L).

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

The Monsanto Company has requested a registration for *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material (ZMIR13L) necessary for its production in all corn lines and varieties. The Cry3Bb1 protein is intended to control the corn rootworm (CRW, *Diabrotica* spp.), a coleopteran pest of corn. The CRW is a primary pest of corn in the U.S., it feeds on corn roots as larvae leading to a reduction in the plant's ability to absorb water and nutrients from soil and lodging. In areas where the CRW is a pest (e.g., corn belt), significant financial losses are realized from a decrease in production and chemical insecticide usage. Significant acres of corn are treated annually to control CRW with organophosphate, carbamate and pyrethroid insecticides.

Non-target insect studies are required to register a new pesticide (40 CFR §158.590). Monsanto Co. submitted studies on adult and larval lady beetles (MRID Nos 449043-14, 455382-04, 453613-01, 453613-02), green lacewing larvae (MRID No 449043-12), a parasitic Hymenoptera (MRID No 449043-13), adult and larval honey bees (MRID Nos 449043-11, 449043-10), Collembola (MRID No 449043-17) and earthworm (MRID No 449043-16). Additional studies were submitted including field census evaluations (MRID No 455382-06), a soil degradation study (MRID No 451568-04) and an endangered species risk assessment (MRID No 455770-03). In March 10, 2000, the adult lady beetle study was reviewed. This review concluded that tests demonstrating the effect of adult and larval lady beetles feeding on corn pollen containing Cry3Bb1 should be conducted. These studies were conducted by Monsanto and submitted to the Agency. Although Cry3Bb1 is active against coleopterans, a study was also conducted to alleviate any potential public concerns on potential effects of MON863 expressing pollen to monarch butterflies (MRID.No 455382-05). The Agency has used these data to perform a risk assessment to the invertebrate corn field fauna.

The form of the test substances used in the studies for this assessment are plant material such as leaves, pollen or purified bacterially-produced Cry protein incorporated into the diet. The Scientific Advisory Panel (SAP) has provided guidance to EPA that while actual plant material is the preferred test material, bacterially-derived protein is also a valid test material.

A. Non Target Insect Hazard Assessment

Based on the evaluation of the submitted data no unreasonable adverse effects of Cry3Bb1 protein on the invertebrate fauna of the corn field are expected. Preliminary field data suggest that some subtle population shifts may occur, but these may not be as substantial as those resulting from conventional chemical pesticide use. Specific data are cited for concerns related to Cry protein fate in soils, potential effects on soil biota represented by the earthworm and field data, effects on non-target soil Coleoptera species, foliar insects, and endangered or threatened coleopteran species. The results of these studies are presented here in both tabular and more detailed descriptive format. The complete record of the submitted data can be found in the attached Data Evaluation Reports (DERs).

The FIFRA Scientific Advisory Panel (October, 2000) has recommended that non-target testing be focused on species exposed to the crop being registered. The Agency has determined that the non-target organisms most likely to be exposed to the protein in transgenic corn fields were beneficial insects feeding on corn pollen and nectar, and soil invertebrates, particularly Coleoptera ssp. In lieu of extensive and difficult single species soil coleopteran toxicity testing followed by an extrapolation from the results to a community risk assessment, direct field data on coleopteran insect effects and abundance were requested, received and evaluated. Toxicity tests on representative beneficials from other taxa were also performed. The toxicity of the Cry3Bb1 protein has been evaluated following challenge of several species of invertebrates, including: adult and larval honeybees, a parasitic hymenopteran (*Nasonia*), green lacewings, lady beetles, collembola, monarch butterfly and earthworms. Reproductive and developmental effects

evaluations were based on the collembola and the honey bee and lady beetle larva maturation studies. Based on worst-case soil concentration, soil degradation studies show that Cry Bb1 protein in corn tissue is no longer detectable in agricultural field soil in 22 to 28 days.

1. Summary of Non-Target Organism Toxicity Testing

Generally the adult insect studies were of 30 days duration or until the negative control mortality reached 20%. Larval studies went through pupation and adult emergence.

Guideline No	Study	Results	MRID NO
885.4380	Honey Bee Larva Testing	The NOEC for honey bee larvae maturation to adult bees was determined to be >1,790 ppm Cry3Bb1 protein, (100X the concentration in pollen)	449043-10
885.4380	Honey Bee Adult Testing	An adult honey bee feeding study showed the NOEC of the Cry3Bb1 protein to be >360µg/mL. (20X the concentration found in pollen). Therefore, no risk from the Cry3Bb1 protein to honey bees is expected.	449043-11
885.4340	Parasitic Hymenoptera Larva Testing	The NOEC for parasitic Hymenoptera was determined to be >400 ppm Cry3Bb1 protein. Although 400 ppm Cry3Bb1 protein is only 1X field concentration in plants rather than 10X, parasitic Hymenoptera are not expected to feed directly on corn plant tissue. Therefore, minimal exposure and no hazard to parasitic Hymenoptera from Cry3Bb1 protein is expected.	449043-13
885.4340	A Dietary Toxicity Study with Green Lacewing Larvae	The NOEC for green lacewing larvae was determined to be >8,000 ppm Cry3Bb1 protein (20X field exposure). Based on these results it can be concluded that green lacewing will not be adversely affected when exposed to Cry3Bb1 in the field.	449043-12
885.4340	Effects of Bt Protein 11231 on Lady Beetles	This study showed that the NOEC for Cry3Bb1when fed to adult *H. canvergens* is >8,000 µg Bt protein/mL diet., equivalent to 20X the maximum Bt protein concentration in plant tissue. A follow-up pollen feeding study was requested.	449043-14
885.4340	Lady Beetle Larval Pollen Feeding Study	The NOEC for Cry3Bb1 expressed in pollen is >93 µg/g fresh pollen weight. The larvae were observed through pupation to adult emergence. It can be concluded from this study that *Coleomegilla maculata* lady beetle larvae will not be adversely affected by Cry3Bb1 field corn pollen.	455382-04
885.4340	Ladybird Beetle, *Coleomegilla maculata* Pollen feeding study	No significant adverse effects were noted in a 30day 50% pollen feeding study. Based on these results , no hazard to *Coleomegilla maculata* is expected when feeding on MON 863 corn pollen in the field.	453613-01

108

Guideline No	Study	Results	MRID NO
885.4340	Ladybird Beetle, *Hippodamia convergens* Pollen feeding study	No significant adverse effects were noted in a 15 day 50% pollen in honey water feeding study. Based on these results , no hazard to *Hippodamia convergens* is expected when feeding on MON 863 corn pollen in the field.	453613-02
885.4340	Chronic Collembola Toxicity Study	The NOEC of the Cry3Bb1 protein for Collembola was found to be >872.5µg (50% corn leaf tissue in the diet). No adverse reproductive effects were noted. It can be concluded from this test that Cry3Bb1protein does not pose a hazard to Collembola, a representative soil inhabiting species.	449043-17
885.4340	Monarch Butterfly Larval Pollen Feeding Study	Neonate monarch survival, development, or weight gain were not affected after feeding on milkweed dusted with event MON 863 corn pollen up to 3200 grains/cm^2 for 2, 4 or 10 days of pollen exposure. Pollen densities in the field average 150 grains/cm^2. This study has demonstrated that corn pollen expressing the Cry3Bb1 protein will not result in acute toxic or developmental effects to monarch larvae.	455382-05
154-35	Field evaluation of Cry3Bb1corn exposure on non-target organisms	Preliminary results indicate that there are no valid MON 863 corn related data to show an adverse effect on non-target and beneficial invertebrate abundance in the field. Planting MON 863 corn may result in increased numbers of natural enemies in CRW protected Bt corn.	455382-06 456530-03
850.6200	Earthworm Testing	The 14-day LC_{50} for earthworms exposed to Cry3Bb1 protein in an artificial soil substrate was determined to be greater than 570 mg Cry3Bb1 protein/kg dry soil, or greater than 10 times the maximum EEC of the protein. The NOEC was >57.0 mg Cry3Bb1 protein/kg dry soil. The data show that no adverse effects to earthworms are expected from exposure to Cry3Bb1 protein producing corn plants.	449043-16
885.5200	Aerobic Soil Degradation of the *B.t.* Protein 11098	Finely ground corn leaf tissue in sandy loam field soil degradation data at worts-case field concentrations show that the Cry3Bb1 protein DT_{50} based on insect bioassays and ELISA were 2.37 and 2.76 days respectively. The DT_{90} estimates for the insect bioassays and ELISA were 7.87 and 9.16 days respectively. At ≤28 days the CryBb1 protein was below the detection level These results verify that the Cry3Bb1 protein degrades rapidly and does not accumulate in the soil. Additional testing in different soil types is requested.	451568-04
None	Endangered Species Impact Assessment	Monsanto conducted a hazard assessment, exposure assessment and risk characterization to demonstrate that Cry3Bb1 does not pose a risk to endangered Coleoptera. EPA performed an independent assessment and determined that no adverse affects from Cry3Bb1event MON 863 are expected to endangered/threatened Coleoptera species listed by the USFWS.	455770-03

109

2. Non-Target Arthropod Invertebrate Testing

a) Honey Bee Larvae

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

Testing was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (purity 96%; 1.79 mg active protein/mL water; current nomenclature refers to this protein as Cry3Bb1.) inoculated directly into larval brood cells prior to capping. Within 18 days after treatments were administered, all larvae emerged from capped brood cells. All of the larvae (100%) treated with Cry3Bb1 protein survived to pupation or "capping"; whereas, 97.5% (2.5% mortality) of the honey bee larvae in the control group survived to pupation. There was no statistical difference (p=0.05) in total percent mortality during the larval development or adult emergence stages between treated and control groups. Based on the results presented in this study, it can be concluded that honey bee development and survival are not affected by exposure to the Cry3Bb1 protein. There was 88.8% mortality of larvae treated with the reference substance potassium arsenate which indicated that bees were exposed to the treatments. The NOEC for honey bee larvae was determined to be >1,790 ppm Cry3Bb1 protein.

According to the guidelines, non-target insects should be tested with does of 10-100X the field dosage. This test was conducted at an acceptable level 100X the concentration in pollen or 1,790 ppm Cry3Bb1 protein. Since potential exposure of honey bees to Cry3Bb1 will be from pollen, this test was conducted at an appropriate maximum hazard dose.

It can be concluded from this test that the NOEC of the Cry3Bb1 protein for honey bee larvae is >1,790 ppm. Therefore, no hazard to honey bee larvae and their development is expected from exposure to the Cry3Bb1 protein in corn pollen.

b) Adult Honey Bee Testing

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

The testing consisted of a control group fed 30% sucrose in deionized water, a reference group fed 100µg/mL potassium arsenate, and a test group fed 360 µg/mL of Cry3Bb1 protein and a water only group. The study concluded that 360 µg/mL Cry3Bb1 protein did not affect survival or behavior of adult honey bees. The >360 µg/mL NOEC is 20X the concentration found in

11

pollen. Therefore, no hazard to adult honey bees is expected from exposure to the Cry3Bb1 protein in corn pollen.

c) **Parasitic Hymenoptera Testing**

This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not affect the integrity of the test. This study was conducted based on OPPTS Series 885-4340 Non-target Insect Testing, Tier 1.

A dietary toxicity study with the parasitic Hymenoptera (*Nasonia vitripennis*) was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (purity 96%; 34.5 mg active protein/mL water). Wasps were tested at rates of 400 and 8,000 ppm Cry3Bb1 protein which is approximately equivalent to 1X and 20X the maximum protein concentration in plant tissue. The NOEC for parasitic Hymenoptera was determined to be >400 ppm Cry3Bb1 protein. When an adjustment for mortality in the control group is considered, mortality in the 8,000 ppm treatment group is 45%. Although differences in mortality between the control and treatment groups were not significantly different ($p>0.05$), a treatment effect could not be precluded in this study. At test termination mortality for the 100 ppm potassium arsenate reference group was 33% (24 of 73) and 100% mortality (70 of 70) in the 1,000 ppm reference group.

Based on this test, the NOEC for adult parasitic Hymenoptera exposed to Cry3Bb1 in diet is >400 ppm and the LC_{50} is 8,000 ppm. The risk assessment is based on 400 ppm Cry3Bb1 protein which is 1X rather than 10X the field concentration in plants because parasitic Hymenoptera do not feed directly on corn plant tissues. Therefore, minimal exposure of parasitic Hymenoptera to Cry3Bb1 protein is expected. As a result, no hazard to *Nasonia vitripennis* is expected from exposure to MON 863 Cry3Bb1 corn.

d) **Green Lacewing Larva Testing**

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

Green lacewing larvae were fed the Cry 3Bb1 protein in a moth egg (*Sitotroga* sp.) and water meal diet at rates of 400 and 8,000 ppm which is approximately equivalent to 1X and 20X the maximum protein concentration in plant tissue. There was 20% mortality in the negative control group on Day 10. Compared to the negative control, at day 10, there was no significant increase in green lacewing larval mortality when fed 1X (400 ppm) and 20X (8,000 ppm) the maximum Cry3Bb1 protein concentration found in plant tissue. At test termination mortality for the 1,000 ppm reference group was 43% (13 of 30) and 100% mortality in the 10,000 ppm reference group (potassium arsenate).

The data show that the NOEC for green lacewing larvae exposed to Cry3Bb1 in diet is >8,000 ppm . Based on these results it is not expected that the green lacewing will be adversely effected when exposed to Cry3Bb1 in the field.

e) **Lady Beetle Testing**

EPA routinely requests studies on the effects of Bt proteins on beneficial non-target insects such as lady beetles (Coccinellidae), green lacewings (*Chrysoperla carnea*), parasitic Hymenoptera and honey bees (*Apis mellifera*). Since the Cry3Bb1 protein specifically targets coleopteran (beetle) insects, particular attention is warranted regarding potential effects of MON 863 on lady beetles. In a memorandum from Robyn Rose to Mike Mendelsohn dated March 10, 2000, in addition to a dietary exposure to the purified Cry protein (44903-14), the Agency requested a test demonstrating the effect of lady beetles feeding on corn pollen containing Cry3Bb1. Monsanto conducted three laboratory studies (MRID Nos 453613-01, 453613-02 and 455382-04) on two different lady beetle species (*Coleomegilla maculata* and *Hippodamia convergens*) in response to this request.

Adult protein dietary study
A diet containing purified Cry protein and honey was fed to the adult lady beetle (*H. convergens)* at rates one and 20 times the maximum protein concentration found in corn leaf tissue (MRID NO.:449043-14) . When the negative control group reached 20% mortality (10 days), the results showed no significant differences in mortality the rate between lady beetles fed 400 and 8,000 µg Cry3Bb1/mL of diet. Results from this study showed that the no-observed-effect-concentration NOEC for Cry3Bb1 when incorporated in diet and fed to *H. convergens* is >8,000 µg Bt protein/mL diet. Mortality for the 1,000 and 10,000 µg potassium arsenate/mL diet groups were 55% and 95% respectively at day 10. This demonstrates that toxicity can be measured by mixing a test substance in the lady beetle diet. Lady beetles do not feed on corn plant tissue. They do, however, prey on pest insects that may feed on corn tissue and contain Cry3Bb1 in their gut, thus exposing lady beetles to the Bt protein. There is approximately 390 µg Cry3Bb1/g fresh weight corn tissue. Lady beetle exposure is expected to be significantly lower than this since the corn tissue would be metabolized, eliminated, or otherwise degraded within the prey species. Since the NOEC was found to be 8,000 µg Cry3Bb1/mL diet which is 20 times higher than maximum expected exposure levels, no risk from Cry3Bb1 in corn plants to adult lady beetles is anticipated.

Larval pollen feeding study
Since corn pollen may comprise up to 50% of lady beetle larvae's diet, the effects of corn pollen containing event MON 863 Cry3Bb1 protein on lady beetle larvae (*Coleomegilla maculata*) was evaluated (MRID NO: 455382-04). Pollen was fed to lady beetle larvae in a diet consisting of equal amounts of lyophilized tephritid fruit fly eggs and bee pollen. Diets contained 50% pollen (93 µg/g fresh pollen weight) since this is the potential level of field exposure and an equal amount of the tephritid fruit fly diet. First instar lady beetle larvae were individually placed in test arenas to avoid cannibalism. There was not a statistically significant difference between

developmental time of larvae to pupae and/or adults; nor was there a difference in adult weight survival between larvae fed bee pollen or corn pollen nor was there a difference between larvae fed Bt and non-Bt pollen. There was a significant difference between the reference group (potassium arsenate) and other test groups since no larvae survived in the reference group. The 100% mortality observed in the reference group verified that the lady beetles were ingesting the diet. This test was conducted with pollen levels greater than or equal to levels lady beetle larvae are expected to be exposed to in the field. Therefore, the NOEC for Cry3Bb1 expressed in pollen is >93 µg/g fresh pollen weight. This study demonstrates that lady beetle larvae will not be adversely affected by Cry3Bb1 field corn.

***Coleomegilla maculata* and *Hippodamia convergens* adult pollen feeding studies**
Coleomegilla maculata lady beetle adults were fed diets of transgenic corn pollen mixed with fruit fly eggs to determine potential effects of transgenic pollen to beetles (MRID NO: 453613-01). The corn (MON 863) test pollen contained the Cry3Bb1 protein at a concentration of 37.4 µg/g pollen. After 30 days of diet exposure, 83.3 and 80.0% of adult *C. maculata* survived in the test and control pollen groups, respectively. While these survival rates were significantly less than that in the assay control group (bee pollen which exhibited 100% survival), there were no significant differences between the test and control pollen groups. All adults in the positive control (arsenate treated corn pollen) died in less than 8 days. Results indicated that transgenic Bt corn pollen expressing the variant Cry3Bb1 protein has no negative effects on the survival of *Coleomegilla maculata* adults.

Hippodamia convergens adults were fed diets of transgenic corn pollen in honey to determine potential effects of transgenic pollen to beetles (MRID NO: 453613-02). The corn (MON 863) test pollen contained the Cry3Bb1 protein at a concentration of 37.4 µg/g pollen. After 15 days of diet exposure, 84% and 81% of adult *Hippodamia convergens* survived in the test pollen and control pollen groups, respectively. There were no significant differences in survival among the test pollen, control pollen and the assay control (honey only) treatment groups. Only 5% of beetles exposed to the positive control (arsenate treated corn pollen) survived. Results demonstrate that transgenic Bt corn pollen expressing the variant Cry3Bb1 protein had no negative effects on the survival of *Hippodamia convergens* adults from dietary exposure.

No adverse effects were detected when *Coleomegilla maculata* and *Hippodamia convergens* were fed MON 863 pollen in diet in the laboratory. Pollen levels fed on by the lady beetles in this study exceeded concentrations that are expected to be encountered in the field. Therefore, it can be concluded the MON 863 will not pose a risk to lady beetle adults in the field.

f) Collembola Feeding study

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

Collembola were fed diets consisting of transgenic corn leaf tissue containing Cry3Bb1 protein mixed with dry granulated Brewer's yeast. Diets contained a ratio of 0.50, 5.0 and 50% corn leaf tissue in Brewer's yeast which was equivalent to 8.73, 87.3 and 872.5 µg Cry protein- per gram diet respectively. Corn leaf tissue contained 1,745 µg Cry3Bb1 protein/g dried leaf tissue. These results show a NOEC of Cry3Bb1 protein in diet for Collembola to be > 872.5 µg/g diet. The study demonstrated that diet containing 50% corn leaf tissue expressing the Cry3Bb1 Bt protein did not adversely affect survival or reproduction of Collembola. This test was conducted at concentration levels much greater than Collembola are expected to be exposed to in the field. The primary route Collembola would be exposed to Cry3Bb1 in the field is from decaying root tissue (and possibly from pollen to a much lesser degree). MON 863 is expressed in corn roots in the range of 3-66 µg/g which is significantly lower than the levels used in this test.

This study adequately addresses potential concerns for Cry3Bb1 protein expressed in transgenic corn to Collembola (*Folsomia candida*) a representative of beneficial soil insect species. The results of this study demonstrate that Cry3Bb1 proteins found in transgenic corn pose no hazard to soil inhabiting Collembola species, and by inference to other beneficial non-coleopteran soil insects.

g) Monarch Butterfly Larval Pollen Feeding Study

This study was not required nor requested for Cry3Bb1 because it is a coleopteran active protein that is not expected to affect lepidopterans such as the monarch butterfly. In addition, extensive research conducted on the potential affects of monarch feeding on lepidopteran-active Bt corn pollen has shown a lack of concern for subchronic toxicity. However, due to recent public concern of possible adverse effects of Bt corn on monarchs, Monsanto sponsored this study and submitted it to the Agency for review. This study has demonstrated that corn pollen expressing the Cry3Bb1 protein will not result in subchronic toxic or developmental effects to monarch larvae. Neonate monarch survival was not affected after feeding on milkweed dusted with up to 3200 pollen grains/cm^2. expressing Cry3Bb1 for 2, 4 or 10 days of pollen exposure. Larval development, weight gain and milkweed leaf consumption were also not affected by feeding on Bt pollen 96 hours and 10 days after exposure.

Pollen densities in the field are not expected to be as great as 3200 grains/cm^2. Pollen densities in the field average 150 grains/cm^2. Levels of 400 and 800 pollen grains/cm^2 would probably be rare. Therefore, results of this study indicate that young monarch larvae (at the most sensitive stage) will not be adversely affected by exposure to Bt corn pollen expressing Cry3Bb1 in the field.

Although this study was not conducted in accordance with FIFRA GLP compliance as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160, a scientifically valid study was performed by a university laboratory and is considered acceptable.

h) **Field Evaluation of Cry3Bb1 Corn Exposure on non-target Invertebrates**

These studies (MRID No 455382-06, 456530-03) are being conducted in Kansas, Nebraska, Illinois, Virginia, South Dakota and New York to evaluate the ecological impact of MON 863 Bt corn grown under different insecticide regimes on abundance of non-target organisms relative to non-transgenic corn. Although these studies are not being conducted according to FIFRA GLP, they are being performed according to scientifically valid, peer reviewed protocols that are acceptable. The data suggest that planting event MON 863 CRW protected corn will not have an unreasonable adverse affect non-target and beneficial invertebrate abundance in the field. On the contrary, a reduction in pesticide use that would result from planting MON 863 corn will probably result in increased numbers of natural enemies in CRW protected Bt corn.

Transgenic Bt corn and non-transgenic corn were monitored for two years (2000-2001) for the stability and abundance of non-target organism communities in soil, soil surface and foliage including arthropods, earthworms and soil microbes as well as plant diseases. Additional data on soil fertility, microbial populations and soil Bt protein was collected during these studies but not reported at this time. Cry3Bb1 corn rootworm (*Diabrotica* spp.; CRW) protected corn event MON 863 (RX670 pedigree) was compared to a non-transgenic control hybrid (RX670) with a similar genetic background.

All experimental plots were managed according to typical cultural practices of commercially grown corn in the region and included the application of the herbicides acetochlor and atrazine after planting and before emergence. Bt and control hybrids were planted in a split-plot design with four replications planted 20 ft apart. Rows were planted 30 in apart, seeded at a rate of approximately 1.7 seeds/ft and planted 1.5 - 1.75 inches deep. Plots (240 ft × 60 ft) were divided into 24 row subplots (60 ft × 60 ft) that served as replications receiving one of 4 insecticide regimes. Insecticide treatments of the Bt and non-Bt plots included: 1. No insecticide; 2. Seed treated with Gaucho (imidacloprid) prior to planting; 3. The granular insecticide Force 3G (teflathtin) applied and incorporated in furrows at planting; and 4. A foliar insecticide, Pounce 3.2 EC (permethrin), applied at the V10 and R2 corn growth stages to control 1st and 2nd generation CRW adults. A 4-row buffer of non-transgenic corn was planted around each plot to minimize edge effects from adjacent subplots.

Invertebrates were sampled from the soil, soil surface and foliage. Soil-dwelling invertebrates were collected using a "pan trap" which utilized a modified Burlese extraction method. Surface-dwelling invertebrate were sampled in the field with pitfall traps. Foliage-dwelling invertebrate were monitored by yellow sticky traps (Pherocon AM™) set in the field at canopy level and adjusted as the season progressed. Sampling for lady beetles was also done using a drop cloth technique.

The 7 most abundant species counted and identified in the pan samples were: Araneae (spiders), Carabidae (immature and adult ground beetles), Chilopda (centipedes), Staphylinidae (rove beetles), Japygidae (diplurans) and Oligochaeta (earthworms). Preliminary results show no

statistically significant difference between the Bt and non-Bt hybrids in the number of soil-dwelling organisms collected from pan samples. There was also no significant effect on abundance of organisms from the insecticide treatments and their interaction with hybrids. The number of carabid larvae collected in the pan samples varied among Bt and non-Bt plots treated with different insecticides. For example, the abundance of Aranaea (spiders) were less in the non-Bt plots treated with soil insecticide and the number of Oligochaeta (earthworms) and Japygidae (diplurans) were very variable but with no trends observed. On the other hand, the number of Chilopods (centipedes) and Staphaylinds (rove beetles) were somewhat consistent among insecticide treatments. Since the number of pest species (e.g., scarab beetles, sap beetles, wireworms/click beetles and immature CRW) in the pan samples were low, no comparison between treatments was made.

The most abundant surface-dwelling species collected in pitfall traps were Araneae (spiders), Carabidae (immature and adult ground beetles), Gryllidae (crickets), Cicindellidae (tiger beetles), Chilopoda (centipedes), Diplopoda (millipedes), Formicidae (ants), Staphylinidae (rove beetles) and Silphidae (carrion beetles). Of these organisms, spiders, Carabids (ground beetles), and crickets were the predominant species sampled respectively and were not significantly different between the two hybrids. There was also no interaction effect of insecticide and hybrid found for any of the species evaluated. Insecticide treatments did not significantly affect the number of Carabids or crickets but did affect spider abundance. There were 30% fewer spiders found in soil treated plots and 50% fewer in foliar treated plots than the seed and no insecticide treated plots.

In addition to non-pest species, the abundance of pest insects including Nitidulidae (sap beetles), Scarabaeidae (scarab beetles), Chrysomelidae (corn flea beetles) and a minimal number of Elateridae (click beetles) was determined in the pitfall traps. There were no differences in the number of pest insects between hybrids but there were differences between insecticide treatments. There was a large number of sap beetles collected and abundance was highly variable in all treatments. Scarab beetle abundance was consistent among plots except for foliar treatments which resulted in fewer beetles. Soil and foliar insecticides resulted in fewer corn flea beetles.

The 5 most abundant pest species captured on sticky traps were *Diabrotica barberi* (northern corn rootworm), *Diabrotica virgifera* (western corn rootworm), *Chaetocnema pulicaria* (corn flea beetle), Nitidulidae (sap beetle) and *Rhopalosiphium maidis* (corn leaf aphids). The predominant foliage-dwelling beneficial insects collected on sticky traps were lady beetle species including *Harmonia axyridis* (Asian lady beetle), *Coccinella septempunctata* (7-spotted lady beetle), *Hippodamia convergens* (convergent lady beetle) and *Cycloneda munda* (lady beetle). Other non-target beneficial invertebrate found on sticky traps were spiders, parasitic Hymenoptera, Syrphidae (syrphid flies), *Chrysoperla carnea* (green lacewing), Hemerobiidae (brown lacewings), Carabidae (ground beetles), Fomicidae (ants) and Nabidae (damsel bugs).

The key natural enemies in corn fields *Coleomegilla maculata* (12-spotted lady beetle), *Orius insidiosus* (minute pirate bug) and the parasitic Hymenoptera *Macrocentrus grandii*

(Macrocentrus), were also abundant in the treated and control plots. The number of beneficial insects was not significantly different between the Bt and non-Bt plots nor was the interaction between insecticide treatment and hybrid. Although *C. maculata*, a generalist lady beetle predator abundance was not significantly effected by the chemical insecticide treatments, there were less observed in the foliar treated plots. In general, the number of *C. maculata* increased during the growing season. The number of *M. grandii* were significantly less in plots receiving foliar insecticide treatments. This affect was probably due to the insecticide directly affecting the parasitoid or from reducing ECB populations. There were no differences between treated and control plots in the abundance of *O. insidiosis*, a generalist predator. However, foliar insecticide treatments reduced the number of *O. insidiosis* by 50% and the population never fully recovered. *O. insidiosis* numbers also differed among sample dates; abundance increased from the first to the third date and dropped on the final sample date.

The second year preliminary report shows some possible effects of MON 863 on corn field insects. Preliminary data are being analyzed which suggesting an increase in Coccinellid larvae, an increase in abundance of mites on MON 863 residues, a laboratory study showing significantly reduced root population and egg production of the plant pathogenic nematode *M. incognita*, a reduction in the population of the non-pathogenic *E. elegans* in root extracts (no effects in soil leachate), and no effects were noted on the non-pathogenic nematode *S. carpocapsae*. It was also reported that of 14 species of Collembola, a decrease in one species was noted, although no effect was seen on total Collembola populations. One inconclusive study found a decrease in Carabids, but the data are suspect because of limitations in movement of ground dwelling insects due to plot layout problems. These studies are being repeated in more adequately rearranged and randomized plots.

Conclusions:
According to the pan, pitfall and sticky trap sample methods used in this study, abundance of the predominant species were not significantly different between the Bt treated and non-Bt control plots. The most abundant soil-dwelling organisms found in pan and pitfall traps were spiders and Carabids. The abundance of most important foliage-dwelling natural enemies captured on sticky traps, *C. maculata*, *O. insidiosis* and *M. grandii*, did not differ between treated and control plots. These preliminary results indicate that planting event MON 863 does not negatively impact the abundance of several beneficial non-target invertebrates. Data also indicated that planting event MON 863 results in less impact on non-target invertebrate than conventional pest management practices. Preliminary data from the second year studies noted increases in mite populations and Coccinellid larvae, a possible shift in Collembola diversity but not abundance, an apparently detrimental effect on a plant pathogenic nematode, and a variable effect on two non-pathogenic nematodes. Further analysis of the data already collected, as well as additional results from the ongoing trials are needed before a final assessment can be fully developed. However, the preliminary data do not point to any unreasonable adverse effects on soil invertebrate populations.

3. Nontarget Invertebrate - Earthworm Testing

The study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Parts 160 and 792; Organization for Economic Development (OECD) Principles of Good Laboratory Practice; and Japan Ministries of Agricultural Forestry and Fisheries (MAFF), with certain exceptions that did not affect the integrity of the test. The testing was conducted based on OPPTS Series 850.6200 Earthworm Subchronic Toxicity Test and OECD Guideline 207. This study meets current testing requirements for assessing subchronic risks to earthworms from plant-incorporated protectants derived from *Bacillus thuringiensis*.

The 14-day LC_{50} for earthworms exposed to Cry3Bb1 protein in an artificial soil substrate was determined to be greater than 570 mg Cry3Bb1 protein/kg dry soil, or greater than 10 times the maximum EEC of the protein. Based on the inconclusive effects (due to procedural error) observed among worms in the 570 mg Cry3b1 protein/kg dry soil treated group, the NOEC was determined to be >57.0 mg Cry3Bb1 protein/kg dry soil. Percent mortality of earthworms in the reference substance (chloroacetamide) groups was consistent with historical results, and further confirmed the adequacy and consistency of the protocol used in the definitive test.

The study was procedurally sound and the data show that no adverse effects to earthworms are expected from the growing of Cry3Bb1 protein containing corn plants. Field study data (MRID NO.: 455382-06) confirm this assessment.

4. Soil Degradation Studies

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

An insect bioassay and an ELISA was conducted to measure the level of functional and non-functional Cry3Bb1 protein present in field soil samples. The amount of lyophilized corn tissue added to the field soil in this study was based on the amount of plant tissue that could potentially be incorporated into the top six inches of soil under field conditions. Since field incorporation of plant tissue will not take place until the Fall, this amount of Cry3Bb1 protein represents the worst-case scenario during the growing season. Based on this calculation 0.03 g (rounded up from 0.028) dry weight plant tissue was added to each gram of dry sandy loam field soil (from Fayette County of Lexington, KY.); therefore, 3% of the dry weight of soil was dry weight of lyophilized plant tissue. An additional test was conducted with 10% of the soil containing lyophilized dry weight plant tissue (0.10 g leaf tissue/g soil). Insect bioassays included a mixture of test and control substances with an agar-based insect diet added to wells of bioassay trays. Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) larvae were added one larva/well and each treatment bioassay was replicated twice for a total of 16 CPB/replicate. CPB are more sensitive to the Cry3Bb1 protein then CRW and were, therefore, expected to result in a more

measurable response then CRW, the target species. In addition to an insect bioassay, an ELISA was conducted to measure the level of Cry3Bb1 protein present in samples. However, the ELISA test will only show extractable protein and does not distinguish between functional and non-functional proteins.

Results from this study show the DT_{50} and DT_{90} for Cry3Bb1 protein in sandy loam soil based on ELISA test are 2.76 and 9.16 days. The 21 day ELISA sample was the last to show traces of Cry protein. At 28 days the CryBb1 protein was below the detection level. However, the value of these results need to be considered with regard to biological activity because it is unknown if the extractable protein in the ELISA test was functional or non-functional. The DT_{50} and DT_{90} determined by insect bioassays with CPB were 2.37 and 7.87 days respectively. The no-detection level was in the range of the results obtained by ELISA.

Based on these results Cry3Bb1 protein degrades rapidly and does not accumulate in sandy loam soil. However, corn is not necessarily grown in sandy loam soil in all regions. Corn is grown in other soil types such as clay loam and silt loam soils in various regions of the U.S. Testing clay soils would, therefore, be considered a "worst case scenario." In addition, this test does not account for all plant tissue such as roots, or root exudation of the Cry protein in the field. It is possible that root tissue is degrading slower then leaf tissue in the soil which may result in a longer duration of degradation time of the Cry3Bb1 protein. Therefore, field testing should be continued in a variety of soil types including clay and humic acid soils over a three year period of time to determine the long-term degradation rate and accumulation/persistence of Cry3Bb1 in soil.

B. Endangered Species Considerations

Exposure Assessment for Endangered Coleoptera

Cry3 proteins including Cry3Bb1 are known to be highly specific against coleopteran insects and are not hazardous to vertebrate animals. It has been generally demonstrated that Cry3 proteins do not pose a risk to non-target animals or invertebrates. The Cry3Bb1 protein is specifically toxic to Chrysomelid beetles including corn rootworms (*Diabrotica* spp.) and Colorado potato beetles (*Leptinotarsa decemlineata*). Currently, there are no Chrysomelid species listed on the endangered species list and no other species are known to be sensitive to Cry3Bb1. Therefore, no adverse affects from Cry3Bb1 event MON 863 are expected against endangered species. Nevertheless, all endangered/threatened beetle species found in the counties where corn is grown (including the recently listed Stephan's riffle beetle *Heterelmis stephani*) were checked to determine possible exposure to corn pollen. Their habitat (and breeding grounds) were found not to overlap with corn fields. Therefore no endangered beetles will be exposed to potentially harmful levels of corn tissue or pollen containing Cry3Bb1 protein.

Terrestrial and aquatic exposure were considered in this assessment since non-target coleopterans may be exposed to the Cry3Bb1 protein within corn fields or in surrounding areas from plant

tissue (e.g., pollen) movement offsite. However, the distance pollen moves outside of the corn field should be considered. Pleasants *et al.* showed that <25 grains of pollen per cm^2 are expected 4-5 meters from the corn field edge. A relative comparison of surface ratio of milkweed to other substrates (e.g., other host plants, arthropod prey, animal carrion) can be used as a basis for estimating the amount of pollen that may leave the field. The maximum concentration of Cry3Bb1 protein has been determined to be 93 µg/g fresh weight pollen. Based on this concentration, <0.03 µg Cry3Bb1 protein/g of diet would be expected to be deposited 4-5 meters from the field edge. The potential of aquatic organisms to be exposed to the Cry3Bb1 protein is minimal. Such exposure would occur from runoff of the protein (either free or sequestered in plant debris) into adjacent water bodies or pollen drift. Since movement of Cry3Bb1 in soil into water bodies is expected to be negligible, pollen drift was considered the primary source of potential risk to endangered aquatic Coleoptera. According to estimates based on studies conducted by Pleasants *et al.* (2001), if 100% of the pollen grains leaving the field were deposited in a 1 ha pond with 2 m depth and located ≥1 m from the edge of the corn field, <0.0001 µg Cry3Bb1/mL of water would be expected.. This is a few orders below the toxic level to any insect.

Many of the endangered and threatened beetles occur in cave or aquatic habitats. None of the endangered beetles are expected to occur in or near corn fields. The American burying beetle may occur in old fields or cropland hedge rows. However, based upon the feeding habits of the American burying beetle, it is not expected to occur within corn fields nor will it be exposed to Cry3Bb1 protein. Adult American burying beetles are classified as opportunistic scavengers that feed on anything dead and bury vertebrate carcasses on which their larvae feed. Carrion regurgitated by adults is fed to larvae until they are able to feed directly on a carcass.

In addition, Monsanto conducted a hazard assessment, exposure assessment and risk characterization to demonstrate that Cry3Bb1 does not pose a risk to endangered Coleoptera (MRID NO: 455770-03). This endangered species assessment was based on the Hazard Evaluation Division, Standard Evaluation Procedure, Ecological Risk Assessment (U.S. EPA, 1986). The Agency reviewed this assessment and found it acceptable.

The reviewed non-target data confirm the expectation that CryBb1 corn will have no adverse effect on endangered and/or threatened species listed by the US Fish and Wildlife Service, including mammals, birds, plants, terrestrial and aquatic invertebrate species. Therefore, no consultation with the USFWS is required under the Endangered Species Act.

C. Summary

In evaluating the risks from use of a pesticide, EPA considers the toxicity and the exposure. Where there is no observable adverse effect (especially when the test dose is several times that found in the environment), EPA concludes that there are no unreasonable adverse effects from the use of the pesticide. When a toxic endpoint is found, then the level of field exposure is factored in to arrive at a risk at field exposures. From all of the submitted indicator species test

data on Cry3Bb1 corn, including the preliminary field data, EPA concludes that the levels of Cry3Bb1 protein in corn will not pose unreasonable adverse effects to invertebrate corn field fauna. Available data also indicate that there should be no accumulation of Cry3Bb1 protein in agricultural soil. In addition, no adverse effect on listed endangered/threatened species is expected from the proposed Cry3Bb1 CRW resistant corn registration. However, continued field study data are needed to confirm that there will be no unreasonable adverse environmental effects from long term use of this product. EPA does not believe that this data requirement was reasonably foreseeable, or practically achievable, by the applicant at the time of application.

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

JUL 11 2002

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Review of laboratory pollen feeding studies on two species of lady beetles (*Coleomegilla maculata* and *Hippodamia convergens*) in support of Monsanto's request to register *Bacillus thuringiensis* (Bt) Cry3Bb1 protein (event MON 863) expressed in field corn pollen. EPA Reg.No: 524-LRA, DP Barcode No. 282664, PC Code No 006484, MRID Nos. 453613-01 and 453613-02.

FROM: Robyn I. Rose, Entomologist Robyn Rose 7-11-02
Biopesticides and Pollution Prevention Division (7511C)

TO: Mike Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division (7511C)

CLASSIFICATION: Acceptable

BACKGROUND:
Monsanto Co. has request a registration for *Bacillus thuringiensis* (Bt) Cry3Bb1 protein (event MON 863) expressed in field corn pollen. EPA routinely requests studies on the effects of Bt proteins on beneficial nontarget insects such as lady beetles (Coccinellidae), green lacewings (*Chrysoperla carnea*), parasitic Hymenoptera and honey bees (*Apis mellifera*). Since the Cry3Bb1 protein specifically targets coleopteran (beetle) insects, particular attention is warranted regarding potential effects of MON 863 on lady beetles. In a memorandum from Robyn Rose to Mike Mendelsohn dated March 10, 2000, the Agency requested a test demonstrating the effect of lady beetles feeding on corn pollen containing Cry3Bb1. Monsanto conducted two laboratory studies (MRID Nos 453613-01 and 453613-02) on two different lady beetle species (*Coleomegilla maculata* and *Hippodamia convergens*) in response to this request. In addition, Monsanto conducted a lady beetle larvae pollen feeding study (MRID No 455382-04) that was classified as acceptable by EPA. In this study, lady beetle larvae (*Coleomegilla maculata*) were fed MON 863 corn pollen expressing the Cry3Bb1 protein in the laboratory. According to results of this study, the NOEC for Cry3Bb1 expressed in pollen is ≤93 µg/g fresh pollen weight. It can be concluded from this study that lady beetle larvae will not be adversely affected by Cry3Bb1 field corn.

12

DATA EVALUATION RECORD

Reviewed By: Anthony Q. Armstrong and Eric B. Lewis, Toxicology and Hazard Assessment Group, Life Sciences Division, Oak Ridge National, Laboratory, Oak Ridge, TN 37831

EPA Reviewer: Robyn I. Rose, Biopesticides and Pollution Prevention Division (7511C) RR 7-11-02

STUDY TYPE: NonTarget Insect Testing,Tier I (OPPTS 885.4340); Dietary effects of adult lady beetles feeding on Bt corn pollen.

TEST MATERIAL: *Bacillus thuringiensis* Cry3Bb1 protein (event MON 863) expressed in corn pollen

CITATION:

Authors:	Jian J. Duan, Graham Head, Mike McKee, Thomas E. Nickson
Title:	Dietary Effects of Transgenic *Bacillus thuringiensis* (Bt) Corn Pollen Expressing a Variant of Cry3Bb1 Protein on Adults of Ladybird Beetle, *Coleomegilla maculata*
Study Completion Date:	March 13, 2001
Testing Laboratory:	Monsanto's Biotechnology Regulatory Sciences, 700 Chesterfield Parkway North, St. Louis, MO 63198 and Ecological Technology Center, 800 North Lindbergh, St. Louis, MO 63011
Sponsor:	Monsanto Company, 700 Chesterfield Parkway North, St. Louis, MO 63198
Laboratory Report ID:	MSL-16936
MRID No:	45613-01
EPA Reg.No:	524-LRA

CLASSIFICATION: Acceptable

QUALITY ASSURANCE STATEMENT: Acceptable

GLP COMPLIANCE STATEMENT: Acceptable

STUDY PARAMETERS:

Test Organism:	Lady beetle (*Coleomegilla maculata*)
Age of Test Organisms:	Adult
Definitive Study Duration:	August 26, 2000 - September 30, 2000

12

STUDY SUMMARY:

Objective: To evaluate the potential hazards of *Bacillus thuringiensis* endotoxins Cry3Bb1 to adult lady beetles *(Coleomegilla maculata)*.

Materials & Methods: This study was based upon procedures outlined in Series 885 of US EPA's, Office of Prevention, Pesticides and Toxic Substances Microbial Pesticide Test Guidelines (OPPTS Number 885.4340, February 1996). Treatments included test pollen from Bt corn pollen, control pollen, bee pollen provided by a commercial source and served as an assay control, and potassium arsenate treated pollen which served as a positive control. The test substance was transgenic corn pollen which contained the Cry3Bb1 protein from *Bacillus thuringiensis* produced by corn event MON 863. The Cry3Bb1 protein has insecticidal activity against the corn rootworm (*Diabrotica* spp.). Non-transgenic corn pollen from event MON 846 was used as control pollen. An ELISA was conducted to verify the level of Cry3Bb1 protein in the test and control pollen. The ELISA indicated that there was 37.4 µg Cry3Bb1 protein/g fresh pollen weight and no Cry3Bb1 detected in the control pollen. An additional ELISA was conducted at test termination to evaluate stability of the variant Cry3Bb1 protein. The estimated level of Cry3Bb1 protein verified by ELISA at test termination was 49.3µg/g fresh weight pollen.

The test organisms, lady beetles (*Coleomegilla maculata*), were reared in test chambers maintained at 25-30°C and fed a diet consisting of equal parts of insect diet and bee pollen. An insect diet consisting of equal amounts of dried fruit fly eggs and pollen was used for testing. Diets contained 50% pollen since this is the potential level of field exposure and an equal amount of the tephritid fruit fly diet. The dried fruit fly egg and test diet mixtures were stored in the freezer at -20°C until use. Adults that were less than three days old were randomly placed in test chambers with diet and treatments. The test chambers were polystyrene petri dishes (10 cm in diameter, 1.5 cm depth) with filter paper (8.5 cm diameter) placed on the bottom and covered with a ventillated top. Each test chamber contained one randomly selected beetle. A total of ten adults were tested in each assay and the assay was replicated three times. Water and diet mixtures were presented *ad libitum* to the beetles on glass microscope slides. The test chambers were maintained in an incubator at 27°C with a photoperiod of 14 hours light and 10 hours of darkness.

Daily observations of the adult beetles for mortality and number of eggs laid continued for 30 days after test initiation. However, egg production data was not evaluated because the male:female ratio varied between treatments. Concentration of Cry3Bb1 protein in the test and control substances was verified at test termination by counting pollen grains in sub-samples of the diet mixes. However, this data was rejected because of a missing data entry o n the amount of the test pollen diet weighed for pollen counting. Statistical analysis on the percentage of surviving larvae in the test was conducted using Analysis of variance (ANOVA) and Tukey's studentized range (HSD).

Reported Results: Survival of beetles exposed to transgenic corn pollen, containing the Cry3Bb1 protein, in dietary form was 83.3% after 30 days of exposure and was numerically greater than the 80% survival exhibited by beetles exposed to the control corn pollen diet. There

was no statistical difference between survival of *C. maculata* fed MON 863 pollen and non-transgenic pollen. Beetles exposed to bee pollen had a survival rate of 100% while beetles exposed to arsenate treated corn pollen did not survive longer than 8 days (0% survival verifies lady beetles are ingesting pollen in this study). There were significantly more *C. maculata* surviving on bee pollen the corn pollen (Bt and nonBt) suggesting that a corn pollen diet is probably not as optimal as a bee pollen diet.

Study Authors Conclusions: Results from this study demonstrated that when offered as a dietary component, transgenic Bt corn pollen containing the Cry3Bb1 protein had no effect on survival of adult lady beetles (*Coleomegilla maculata*).

Reviewer's Comments: This study was performed according to established protocols and guidelines making it acceptable for fulfilling FIFRA Guideline 153A-23. Both summarized and raw data were included in the study report (Table 1, pp.19-20). No deviations were noted in the study report or identified in this data evaluation record. Results demonstrate that transgenic Bt corn pollen expressing the variant Cry3Bb1 protein had no negative effects on the survival of *C. maculata* adults. Based on results of this study, no adverse effects are expected when *Coleomegilla maculata* feed on level of MON 863 pollen they will potentially encounter in the field.

Table 1. Number of adult lady beetles surviving after dietary exposures to test corn pollen (Cry3Bb1 protein), control corn pollen, arsenate treated corn pollen and bee pollen.

Treatment Group	*Day*														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Test pollen (MON 863 containing Cry3Bb1)	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30
Control pollen (MON 846)	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30
Bee pollen	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30
Arsenate treated corn pollen	30/30	30/30	30/30	29/30	24/30	17/30	2/30	1/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30

Treatment Group	*Day*												
	15	16	17	18	19	20	21	22	23	24	25	26	27
Test pollen (MON 863 containing Cry3Bb1)	29/30	29/30	29/30	29/30	29/30	29/30	29/30	29/30	29/30	29/30	29/30	28/30	27/30
Control pollen (MON 846)	29/30	28/30	28/30	28/30	27/30	27/30	26/30	25/30	25/30	25/30	25/30	25/30	24/30
Bee pollen	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30	30/30
Arsenate treated corn pollen	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30

Table 1. (Cont). Number of adult lady beetles surviving after dietary exposures to test corn pollen (Cry3Bb1 protein), control corn pollen, arsenate treated corn pollen and bee pollen.

Treatment Group	***Day***			
	28	29	30	Survival at Day 30 (%)
Test pollen (MON 863 containing Cry3Bb1)	27/30	26/30	25/30	83.3[a]
Control pollen (MON 846)	24/30	24/30	24/30	80.0[a]
Bee pollen	30/30	30/30	30/30	100
Arsenate treated corn pollen	0/30	0/30	0/30	0

[a] Means followed by the same letter are not significantly different at 0.05 error level as determined by ANOVA and Tukey's studentized range tests.

12

DATA EVALUATION RECORD

Reviewed By: Anthony Q. Armstrong and Eric B. Lewis, Toxicology and Hazard Assessment Group, Life Sciences Division, Oak Ridge National, Laboratory, Oak Ridge, TN 37831

EPA Reviewer: Robyn I. Rose, Biopesticides and Pollution Prevention Division (7511C) RL 7-11-

STUDY TYPE: NonTarget Insect Testing,Tier I (OPPTS 885.4340); Dietary effects of adult lady beetles feeding on Bt corn pollen.

TEST MATERIAL: *Bacillus thuringiensis* Cry3Bb1 protein (event MON 863) expressed in corn pollen

CITATION:

Authors:	Rebecca L. Bryan, John R. Porch, Henry O. Krueger
Title:	Dietary Effects of Transgenic Bt Corn Pollen Expressing a Variant of Cry3Bb1 Protein on Adults of Ladybird Beetle, *Hippodamia convergens*
Study Completion Date:	March 15, 2001
Testing Laboratory:	Wildlife International, Ltd., 8598 Commerce Dr., Easton, MD 21601
Sponsor:	Monsanto Company, 700 Chesterfield Pkwy, North, Chesterfield, MO 63198
Laboratory Report ID:	139-453
MRID No:	45613-02
EPA Reg.No:	524-LRA

CLASSIFICATION: Acceptable

QUALITY ASSURANCE STATEMENT: Acceptable

GLP COMPLIANCE STATEMENT: Acceptable

STUDY PARAMETERS:

Test Organism:	Lady beetle (*Hippodamia convergens*)
Age of Test Organisms:	Adult
Definitive Study Duration:	September 25, 2000 - November 21, 2000

STUDY SUMMARY:

Objective: To evaluate the potential hazards of *Bacillus thuringiensis* endotoxins Cry3Bb1 to adult lady beetles *(Hippodamia convergens)*.

128

Materials & Methods: This study was based upon procedures outlined in Series 885 of US EPA's, Office of Prevention, Pesticides and Toxic Substances Microbial Pesticide Test Guidelines (OPPTS Number 885.4340, February 1996). The test diets consisted of test pollen, control pollen and a positive control (potassium-arsenate treated pollen) were mixed with honey in a 1:1 ratio. The test substance was transgenic corn pollen which contained the Cry3Bb1 protein from *Bacillus thuringiensis* produced by corn event MON 863. The Cry3Bb1 protein has insecticidal activity against the corn rootworm. Diets contained 50% pollen since this is the potential level of field exposure and an equal amount of honey. The assay control consisted of honey without pollen. The control pollen was a non-genetically modified corn MON 846. The test and control pollen were characterized by molecular analysis and ELISA as documented in Monsanto SOP BR-ME-0059-05. Concentrations of Cry3Bb1 protein determined by Monsanto in the transgenic corn pollen ranged from 55.86 to 72.45 µg/g pollen when supplied to Wildlife International for lady beetle testing. No Cry3Bb1 was detected in the control corn pollen. Stability analysis of CryBb1 indicated the presence of the protein in the corn pollen/honey diet supplied to the lady beetles; however, quantification of CryBb1 in the diet was not provided by Monsanto.

The test organisms, lady beetles (*Hippodamia convergens*), were adults obtained from A- Unique Insect Control (Citrus Heights, CA) and stored in a cold room for three months prior to test initiation. Beetles were placed in an incubator set at 26-28°C and 40% relative humidity and allowed *ad libitum* access to honey/water for four days prior to test initiation. Beetles were randomly selected and placed in test chambers consisting of disposable one-pint rolled paper containers approximately 9 cm in diameter and 9 cm high covered with a 10 cm in diameter petri dish and equipped with a 20-mL glass vial containing deionized water. Twenty-five beetles were placed in each chamber. A cotton swab containing the appropriate diet was inserted through the side of each chamber. Beetles were allowed *ad libitum* access to the diets and water. The chambers were maintained in an incubator where temperature averaged 26.0 ± 0.2°C and relative humidity averaged 75% ± 6% for the test period. The photoperiod during the test was 12 hours of light and was controlled with an automatic timer.

The beetles were observed ½ hour and 1 ½ hours after test initiation and then daily to evaluate signs of clinical toxicity, abnormal behavior and mortality for the 14 day test period. Analysis of Variance (ANOVA) was used to analyze differences between treatments in the mean number of dead beetles.

Reported Results: Survival of beetles exposed to transgenic corn pollen, containing Cry3Bb1 protein, in a honey diet was 84.0% after 15 days of exposure (Table 1). Likewise, survival of the beetles exposed to control corn pollen diet was 81.3% and survival of the beetles exposed to honey was 78.6%. Survival in these three groups were not significantly different indicating no impact of the transgenic corn pollen on mortality of lady beetles. Beetles exposed to arsenate treated corn pollen had a low survival rate of 5% which verifies that the *H. convergens* were ingesting pollen.

Study Authors Conclusions: Results from this study demonstrated that when offered as a dietary component, transgenic Bt corn pollen containing the Cry3Bb1 protein had no effect on survival of adult lady beetles *(Hippodamia convergens)*.

Reviewer's Comments: This study was performed according to established protocols and guidelines making it acceptable for fulfilling FIFRA Guideline 153A-23. Both summarized and raw data were included in the study report (Table 1, pp.14-17). No deviations were noted in the study report or identified in this data evaluation record. Based on results of this study, no adverse effects are expected when *Hippodamia convergens* feed on level of MON 863 pollen they will potentially encounter in the field.

Table 1. Number of adult lady beetles surviving after dietary exposures to test corn pollen (Cry3Bb1 protein), control corn pollen, arsenate treated corn pollen and bee pollen.

Treatment Group	*Day*											
	1	2	3	4	5	6	7	8	9	10	11	12
Test pollen (MON 863 containing Cry3Bb1)	73/75	73/75	70/75	70/75	70/75	68/75	68/75	67/75	67/75	67/75	67/75	67/75
Control pollen (MON 846)	75/75	75/75	74/75	70/75	70/75	70/75	70/75	70/75	69/75	69/75	68/75	66/75
Honey only	74/75	73/75	73/75	72/75	71/75	69/75	69/75	66/75	64/75	64/75	63/75	62/75
Arsenate treated corn pollen	24/75	19/75	15/75	11/75	10/75	9/75	9/75	9/75	6/75	4/75	4/75	4/75

Treatment Group	*Day*		
	13	14	Survival at Day 15 (%)
Test pollen (MON 863 containing Cry3Bb1)	65/75	63/75	84.0[a]
Control pollen (MON 846)	65/75	61/75	81.3[a]
Honey only	61/75	59/75	78.6[a]
Arsenate treated corn pollen	4/75	4/75	5.3

[a] Means followed by the same letter are not significantly different as determined by ANOVA(p=0.8039).

DATA EVALUATION RECORD

Bt Corn Pollen (Cry3Bb1 Protein)
(*Bacillus thuringiensis* Cry3Bb1)

STUDY TYPE: NONTARGET INSECT TESTING, TIER I (885.4340)
MRID 45361301

Prepared for

Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Toxicology and Hazard Assessment Group
Life Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37831
Task Order No. 135

Primary Reviewer:
Anthony Q. Armstrong, M.S.

Signature: Anty Q Armstrong
Date: JUN 27 2002

Secondary Reviewers:
Eric B. Lewis, M.S.

Signature: Eric B. Lewis
Date: JUN 27 2002

Robert H. Ross, M.S., Group Leader

Signature: Robert H. Ross
Date: JUN 27 2002

Quality Assurance:
Susan S. Chang, M.S.

Signature: SS Chang
Date: JUN 27 2002

Disclaimer

This record may have been altered subsequent to the contractor's signatures above.

Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the U.S. Department of Energy under contract number DE-AC05-00OR22725

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

DEC 23 2002

MEMORANDUM

Subject: Review of aerobic soil degradation study submitted by Monsanto Co. in support of registering *Bacillus thuringiensis* Cry3Bb1 protein expressed in field corn. EPA Reg. No. 524-LEI; Barcode No. D285418; Case No. 069017; Submission No. S621576; MRID No 457571-02..

From: Robyn Rose, Entomologist Robyn Rose
Biopesticides and Pollution Prevention Division, 7511C

To: Mike Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division, 7511C

Classification: Supplemental to conducting additional aerobic soil degradation studies in additional soils particularly a clay soil. Additional studies should include parameters outlined by the August 2002 FIFRA SAP and found in the conclusions section below.

Background

An aerobic soil degradation study has been submitted to EPA by Monsanto Company in support of their request to register *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material (ZMIR13L) necessary for its production in corn event MON 863. This study is intended to be supplemental to MRID No. 451568-04 titled *Aerobic Soil Degradation of the B.t. Protein 11098 as a Component of Insect Protection Protected Corn* which was reviewed in a memorandum from Robyn Rose to Mike Mendelsohn dated July 10, 2002. This study was classified as "[s]upplemental to conducting the study in the field with all plant tissue incorporated into the soil in fields that have had MON 863 corn grown for one to three consecutive years. Studies should also be conducted in a variety of soil types particularly soil high in clay and humic acids."

The previously submitted aerobic soil degradation study (MRID No 451568-04) utilized plant tissue from Cry3Bb1 transformation event MON 859 rather than MON 863. The recent submission (MRID No 457571-02) utilized MON 863 corn root and shoot tissue. According to Monsanto, "[t]his supplemental data provides further evidence that the Cry3Bb1 protein produced in corn event MON 863 produces no risk of unreasonable adverse effects for the environment."

Since the submission and review of MRID No. 451568-04, EPA convened a FIFRA Scientific

Internet Address (URL) • http://www.epa.gov
Recycled/Recyclable • Printed with Vegetable Oil Based Inks on Recycled Paper (Minimum 20% Postconsumer)

Advisory Panel (SAP) on August 27, 2002 to address corn rootworm plant incorporated protectant (PIP) non-target issues including the degradation of the Bt protein in soil. This memorandum includes a review of MRID No 457571-02 and addresses comments made by the August SAP.

Conclusions

It is difficult to determine a DT50 or DT90 for Cry3Bb1 expressed in event MON 863 corn based on the aerobic soil degradation study found in MRID No 457571-02. In this study, corn shoot and root tissue were analyzed separately and not all plant material was included. Therefore, methods utilized in this study do not represent actual field conditions. It is unknown whether these laboratory results can be adequately correlated to the field. Field studies should be conducted that include the incorporation of all non-harvested plant tissue in a variety of soil types particularly areas high in clay and humic acids. These studies should be conducted for at least one growing season after harvest and continue until no Cry3Bb1 protein is detected. In addition the persistence of the Cry3Bb1 protein under less than optimum conditions (e.g., high or low temperatures; high or low soil moisture content) should be examined. Additional studies conducted to address the degradation of Cry3Bb1 protein in the soil should include an insect bioassay utilizing a known sensitive species such as the Colorado potato beetle (*Leptinotarsa decemlineata*).

Conclusions of the aerobic soil degradation study previously submitted by Monsanto recommended conducting field tests in a variety of soil types including clay and humic acid soils. This study only utilized a silt loam soil; therefore, these results would be considered supplemental to MRID No 451568-04. Additional field studies in an area with high clay and/or humic acids should be conducted in addition to test conducted under field conditions with silt loam soils.

Reviewer's comments and conclusions to MRID No 57571-02 are based on comments received from the August 27, 2002 FIFRA SAP. The Panel concluded that several different soils should be examined and monitored for a minimum of one growing season after harvest and continued until the Cry3Bb1 protein can no longer be detected. The Panel also recommended that an additional sample or two should be examined to verify that an analytical error was not the cause for the lack of detection. According to the Panel, at least two additional soil types should be evaluated for Cry3Bb1 persistence. Soils that are high in organic matter and clay should be concentrated on since there is the highest potential of persistence in these soil types. However, additional soils should also be considered. In addition, the Panel recommended that the soil degradation studies be conducted under less than optimum conditions such as high or low temperatures or high or low moisture content. Since corn roots grow deep into the soil to areas with reduced microbial activity, degradation rates may be reduced. Therefore, degradation of Cry3Bb1 from deep sites should also be examined.

The Panel also addressed the protein source that is appropriate for the soil degradation studies. Future studies should utilize plant material that is representative of actual field conditions. For

example, whole plant tissue should be incorporated. Plant tissue should not be ground prior to incorporation because it artificially increases the surface area exposed to microorganisms which may lead to an increase in the rate of degradation of the protein.

Since more protein may be present than is detected by an ELISA, an insect bioassay using a sensitive species such as the Colorado potato beetle (*Leptinotarsa decemlineata*) should be conducted. The SAP concluded that "[r]eal life or true persistence is likely to be equal to or less than that measured with ELISA." If an ELISA is conducted, the results should be compared to results from a beetle bioassay.

The use of the term DT50 is not appropriate for the soil degradation tests currently conducted. Theses tests only verify the time to 50% degradation of detectable protein. Another term should be considered. For example, the results of the soil degradation studies may be presented as the time to degradation of detectable protein.

DATA EVALUATION RECORD

Reviewed by: Anthony Q. Armstrong and Eric B. Lewis, Oak Ridge National Laboratory, Oak Ridge, TN 37830
Secondary Reviewer: Robyn I. Rose, Entomologist

STUDY TYPE:	Aerobic Soil Degradation
MRID NO:	457571-02
DP BARCODE:	D285418
CASE NO:	069017
SUBMISSION NO:	S621576
TEST MATERIAL:	*Bacillus thuringiensis* Cry3Bb1 Protein in Corn (*Zea mays*)
PROJECT NO:	01-01-39-01
SPONSOR:	Monsanto Company, Biotechnology Regulatory Sciences, 700 Chesterfield Parkway North, St. Louis, MO 63198
TESTING FACILITY:	Monsanto Company, Environmental Science Technology Center, 800 North Lindbergh Boulevard V2C, St. Louis, MO 63167
TITLE OF REPORT:	Aerobic Soil Degradation of the *Bacillus thuringiensis* Cry3Bb1 Variant Protein Produced by Corn Rootworm Protected Corn Event MON 863
AUTHORS:	Samuel Dubelman, Bonnie Ayden, Michael Mueth, Jo Anne Warren, Changjian Jiang, Jeff Bookout and Yelena A. Dudin
STUDY COMPLETED:	September 6, 2002
GOOD LABORATORY PRACTICE:	GLP Compliant
CLASSIFICATION:	**Supplemental** to conducting additional aerobic soil degradation studies

---

I. TEST MATERIAL:

The test substance consisted of finely ground and lyophilized root and shoot tissue of the corn event MON 863 containing the Cry3Bb1 insecticidal protein. The concentration of Cry3Bb1 protein in the lyophilized corn tissue, determined by ELISA, was 487 µg/g in the root and 468 µg/g in the shoot.

The control substance was finely ground and lyophilized root and shoot tissue of the corn event RX670 which does not contain the Cry3Bb1 insecticidal protein. There was no detection of Cry3Bb1 in RX670 tissues determined by ELISA.

The second control was purified Cry3Bb1 protein obtained from a genetically modified *E. coli* strain containing the identical sequence as the MON 863 corn.

II. METHODS:

Soil Samples and Incubation

MON 863 and RX670 corn shoot and root tissue were collected from field grown plants in Richland, IA. The test matrix consisted of three soils collected from the top six inches, Horizon A, of corn fields in Carlyle, IL, Monmouth, IL and Richland, IA. Soil properties for these three soils (Table 1) and a microbial analysis (Table 2) were characterized by AGVISE Laboratories, Norhtwood, ND. Soil viability was confirmed at test initiation and at approximately four and eight weeks of incubation. Soils were shown to remain active and viable throughout the study (144-228 microbial biomass carbon/50 g soil).

To determine representative amounts of corn tissue and corresponding Cry3Bb1 concentrations expected in field conditions, the highest measured amounts of Cry3Bb1 in previously grown corn plants were used. It was assumed: 1. The corn plant residue would be incorporated into the top six inches of soil; 2. There is 616 m^3 of soil three inches deep in one acre; 3. 40,000 plants/acre; 4. An average corn plant weighs 650 grams; 5. Bulk density of soil is 1.0 g/cm^3; 6. The highest concentration of Cry3Bb1 protein in both shoot and leaf tissue 21-days after planting is 93µg/g; 7. The highest concentration of Cry3Bb1 protein in roots 35-days after planting is 66 µg/g. Based on these assumptions, the maximum field loading of Cry3Bb1 in corn shoots is 3.93 µg/g soil (equivalent to 8 mg shoots/g soil) and for corn root tissue the loading is 2.79 µg/g soil (equivalent to 6 mg of roots/g soil).

TABLE 1. Physicochemical characteristics of soils collected from Horizon A (top 6 inches).

Parameter		*Soil Source*		
		Carlyle, IL	Monmouth, IL	Richland, IA
USDA Textural Class		Silt Loam	Silt Loam	Silt Loam
Particle Size Distribution (%)	Sand Silt Clay	21 58 21	18 56 26	13 62 25
Bulk Density (g/cm^3)		1.00	1.03	1.07
% Organic Matter		2.5	4.6	4.0
CEC (meq/100g)		16.6	23.9	20.7
Field Moisture Capacity	@ 1/3 Bar @ 15 bar	28.2 15.7	30.7 17.9	30.3 17.2

Table copied from page 29 of 80 of MRID No 457571-02

TABLE 2. Microbial Characterization of Soil During the Incubation Period				
Microbial Media		CFU/g		
		Carlyle, IL	Monmouth, IL	Richland, IA
Study Initiation	Aerobic Bacteria	2.61×10^6	4.82×10^6	4.93×10^6
	Actinomycetes	6.14×10^5	1.04×10^6	1.11×10^6
	Fungi	8.01×10^4	1.39×10^4	2.74×10^4
13 Weeks Post Study	Aerobic Bacteria	1.96×10^6	4.54×10^6	2.02×10^6
	Actinomycetes	6.26×10^5	1.20×10^6	1.01×10^6
	Fungi	1.11×10^5	1.29×10^4	2.41×10^4

Results of an ELISA assay demonstrated 468 µg/g Cry3Bb1 in lyophilized corn shoot tissue and 487 µg/g Cry3Bb1 in lyophilized root tissue. Based on these results, soil dosing with corn shoot tissue was determined to be 3.93µg Cry3Bb1/g soil ÷ 468 µg Cry3Bb1/g shoots = 0.0084 g shoots/g soil ≅ 8 mg root/g soil. Soil dosing with corn root tissue was determined to be 2.79 µg Cry3Bb1/g soil ÷ 487 µg Cry3Bb1/g root = 0.0057 g root/g soil ≅ 6 mg root/g soil. For additional conservatism, the maximum values were exaggerated 3X for dosing of soils. Therefore, 24 mg of shoot tissue and 18 mg of root tissue was added to soils. Also, the purified Cry3Bb1 control dosing concentration was exaggerated ~25X to model the unlikely scenario that large amounts of protein would be exuded by roots into the soil throughout the growing season. Approximately 48 µg of purified protein was added to 0.5 g of soil.

Soils from the three test locations were passed through a 2-mm screen and 1 gram or 0.5 gram of each representative soil was added to sterile glass vials. Next, soils were air dried and dosed with the Cry3Bb1 test substance at rates of 24 mg of lyophilized corn shoot tissue or 18 mg lyophilized corn root tissue per gram of soil. Also, purified Cry3Bb1 protein was tested at a concentration of 48 µg of protein per 0.5 gram of soil. An additional set of sample vials served as a control and only contained soil. All vials were mixed thoroughly and soil moisture adjusted with deionized water to obtain soil moisture of 75% field capacity at 0.33 bar. Vented caps were placed on the vials. At time zero, all vials were placed in an incubator and maintained at 25°C for 59 days. Twice weekly vials were removed and weighed to determine volume of water needed to make up for loss of soil moisture. Soil moisture was adjusted to 75% field moisture capacity at 0.33 bar with deionized water. Four vials for each treatment and eight vials of the pure protein control were removed at nine sampling intervals over a two month incubation period. After 59 days, all soil samples were analyzed for Cry3Bb1 degradation.

ELISA
Direct ELISA was performed on incubated soils for analysis of total extractable and immunologically active Cry3Bb1 protein in soil. Soils were extracted and material prepared for a double antibody sandwich ELISA with protein-G purified goat polyclonal anti-Cry3Bb1 antibodies. Biotin goat anti-Cry3Bb1 antibodies were added to complete the sandwich. ELISA was performed by addition of standards, soil samples or quality control solutions in triplicate to 96-well plates. Plates were incubated, washed and developed using specific ELISA procedures developed for Cry3Bb1 (see study report page 21). The concentration of the Cry3Bb1 protein was interpolated by reference to a standard curve developed from each ELISA plate. The ELISA limit of quantitation (LOQ) was 0.313 nanograms of Cry3Bb1 protein per milliliter or 0.16 µg Cry3Bb1 per gram soil (µg/g).

The SAS (version 8.1) computer program was used to calculate degradation times for 50% (DT50) and 90% (DT90) loss of original Cry3Bb1 protein concentration based on the ELISA results (see study report pages 22 and 23 for an explanation of the model).

III. RESULTS:

ELISA
Cry3Bb1 protein levels in soil sample extracts determined by ELISA are reported in Tables 3, 4 and 5 which were reproduced from pages 33, 34 and 35 of the study submission. Data in Tables 3, 4 and 5 show that Cry3Bb1 protein concentrations were near or below the ELISA LOQ (0.16 µg/g) after 2 months of incubation. After two months of soil incubation, Cry3Bb1 concentrations were at least an order of magnitude below the initial concentration of Cry3Bb1 in the dosed samples.

TABLE 3. Concentration of Cry3Bb1 protein in Carlyle, Illinois soils.

Incubation Time (day)	Average ELISA Value for Cry3Bb1 Protein in Soil (µg/g)		
	Purified Protein[a]	Corn Root Tissue[b]	Corn Shoot Tissue[c]
0	35.46	5.06	3.32
2	8.42	1.05	1.88
3	5.91	0.54	0.75
4	3.64	0.70	0.64
5	2.86	0.39	0.39
8	1.51	0.37	0.35
12	0.82	0.32	0.28
21	0.58	0.25	0.24
37	0.16	0.21	0.18
59	<LOQ[d]	0.23	0.16

[a]Soil dosed with a solution of purified Cry3Bb1 protein
[b]Soil dosed with MON 863 corn root tissue
[c]Soil dosed with MON 863 corn shoot tissue
[d]Level of quantitation of Cry3Bb1 by ELISA = 0.16 µg/g

TABLE 4. Concentration of Cry3Bb1 protein in Monmouth, Illinois soils			
Incubation Time (day)	Average ELISA Value for Cry3Bb1 Protein in Soil (µg/g)		
	Purified Protein[a]	Corn Root Tissue[b]	Corn Shoot Tissue[c]
0	53.45	4.20	4.81
1	24.66	2.04	2.16
2	15.85	1.58	1.62
3	10.16	1.22	1.32
5	7.46	0.54	0.62
8	2.90	0.48	0.31
12	2.08	0.35	0.27
21	0.84	0.24	0.22
39	0.39	0.19	<LOQ[d]
58	0.20	<LOQ[d]	<LOQ[d]

[a]Soil dosed with a solution of purified Cry3Bb1 protein
[b]Soil dosed with MON 863 corn root tissue
[c]Soil dosed with MON 863 corn shoot tissue
[d]Level of quantitation of Cry3Bb1 by ELISA = 0.16 µg/g

TABLE 5. Concentration of Cry3Bb1 protein in Richland, Iowa soils.			
Incubation Time (day)	Average ELISA Value for Cry3Bb1 Protein in Soil (µg/g)		
	Purified Protein[a]	Corn Root Tissue[b]	Corn Shoot Tissue[c]
0	65.97	4.75	2.84
1	30.78	2.06	1.51
2	24.34	1.45	0.99
3	17.24	1.45	1.09
5	12.47	0.99	0.78
12	4.16	0.74	0.40

20	2.38	0.56	0.30
30	1.06	0.55	0.20
41	0.61	0.34	0.20
58	0.45	0.35	0.20

[a]Soil dosed with a solution of purified Cry3Bb1 protein
[b]Soil dosed with MON 863 corn root tissue
[c]Soil dosed with MON 863 corn shoot tissue

Calculation of Cry3Bb1 Degradation Rates

The DT50 values for all dosing regimes and soil types ranged from 0.6 days to 2.3 days and the DT90 values ranged from 4.03 days to 50 days (Table 6 reproduced from page 36 of the study submission). Cry3Bb1 purified protein dissipated faster than when corn shoot or root tissue was applied. Visual observation verified that root tissues are slower to degrade in soil than shoot tissue. According to the study author, these results also indicate, as expected, that the longer DT50 and DT90 values for corn tissues are due to the time required for the Cry3Bb1 protein to move from tissue to soil.

TABLE 6. DT50 and DT90 estimates for the dissipation of Cry3Bb1 protein in soils

Protein Source	Soil Source	DT50[a] (days)	DT90[b] (days)
Purified Protein	Carlyle, IL	0.63 (0.49, 0.78)[c]	4.03 (3.67, 4.42)
	Monmouth, IL	0.64 (0.50,0.80)	4.18 (3.77, 4.62)
	Richland, IA	0.73 (0.50, 1.00)	5.23 (4.48, 6.07
MON 863 Corn Root Tissue	Carlyle, IL	1.74 (0.78, 3.20)	27.29 (14.52, 50.58)
	Monmouth, IL	1.19 (0.86, 1.57)	12.48 (9.90,15.67)
	Richland, IA	2.27 (1.68, 2.98)	50.02 (34.57, 72.18)
MON 863 Corn Shoot Tissue	Carlyle, IL	1.45 (0.82, 2.30)	18.68 (12.61, 27.46)
	Monmouth, IL	0.90 (0.61, 1.24)	7.43 (6.01, 9.12)
	Richland, IA	1.77 (1.40, 2.21)	28.59 (23.16, 35.23)

[a]DT50=Time to 50% dissipation of original protein concentration
[b]DT90=Time to 90% dissipation of original protein concentration
[c]Lower and upper 95% confidence interval on the DT value

IV. STUDY AUTHOR'S CONCLUSIONS:

These results indicate a rapid degradation of Cry3Bb1 protein derived from MON 863 tissues. Results demonstrated a DT50 of 0.9 to 2.3 days and a DT90 of 7.4 to 50 days for corn plant tissue. In addition, the rapid degradation (DT90 4.0 to 5.2 days) of the purified Cry3Bb1 protein suggest that any Cry3Bb1 protein reaching the soil by root exudation would be >90% degraded in less

DATA EVALUATION RECORD

Bacillus thuringiensis Cry3Bb1 Protein in Corn (*Zea mays*)

STUDY TYPE: Aerobic Soil Degradation

MRID 45757102

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by
Toxicology and Hazard Assessment Group
Life Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 157

Primary Reviewer:
Anthony Q. Armstrong, M.S.

Signature: Anthony Q. Armstrong
Date: NOV 20 2002

Secondary Reviewers:
Eric B. Lewis, M.S.

Signature: Eric B. Lewis
Date: NOV 20 2002

Robert H. Ross, M.S., Group Leader

Signature: Robert H. Ross
Date: NOV 20 2002

Quality Assurance:
Lee Ann Wilson, M.A.

Signature: L. A. Wilson
Date: NOV 20 2002

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

Oak Ridge National Laboratory managed and operated by UT-Battelle, LLC., for the U.S. Department of Energy under Contract No. DE-AC05-00OR22725.

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

JUL 10 2002

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

Subject: Review of aerobic soil degradation study submitted by Monsanto Co. in support of registering *Bacillus thuringiensis* Cry3Bb1 protein expressed in field corn. EPA Reg. No. 524-LRA; Barcode No. D262045; Case No. 066221; Submission No. S572997. MRID No 451568-04.

From: Robyn Rose, Entomologist Robyn Rose 7-10-02
Biopesticides and Pollution Prevention Division, 7511C

Through: Chris Wozniak, Biologist Chris A. Wozniak 7/10/02
Biopesticides and Pollution Prevention Division, 7511C

To: Mike Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division, 7511C

Classification: Supplemental to conducting the study in the field with all plant tissue incorporated into the soil in fields that have had MON 863 corn grown for one to three consecutive years. Studies should also be conducted in a variety of soil types particularly soil high in clay and humic acids.

Background

Monsanto Company has requested a registration for *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material (ZMIR13L) necessary for its production in all corn lines and varieties. The Cry3Bb1 protein is intended to control corn rootworms (CRW, *Diabrotica* spp.), a coleopteran pest of corn. Studies were submitted to support registration of the transformation event MON 863. CRW, a primary pest of corn in the U.S., feeds on corn roots as larvae leading to a reduction in the plant's ability to absorb water and nutrients from soil and lodging. In areas where CRW is a pest (e.g., Corn Belt), significant financial losses are realized from a decrease in production and chemical insecticide usage. Significant acres of corn are treated annually to control CRW with organophosphate, carbamate and pyrethroid insecticides.

This soil degradation study was submitted as part of the ecological risk assessment in support of registering CRW protected Bt corn event MON 863. However, Cry3Bb1.11231 (event MON 859) rather than Cry3Bb1.11098 (event MON 863) was utilized in this study. According to Monsanto, Cry3Bb1.11231 "demonstrates an eight-fold increase in activity against the SCRW as

Internet Address (URL) • http://www.epa.gov
Recycled/Recyclable • Printed with Vegetable Oil Based Inks on Recycled Paper (Minimum 30% Postconsumer)

a result of four strategically placed amino acid substitutions in the wild type protein sequence." Cry3Bb1.11098 "demonstrates an eight-fold increase in activity against the SCRW as a result of five strategically placed amino acid substitutions in the wild type protein sequence." Cry3Bb1.11098 has the same amino acid sequence as Cry3Bb1.11231 with an additional substitution. It is acceptable that this test was conducted with event MON 859 rather than MON 863 because they produce nearly identical Cry3Bb1 protein variants.

Conclusions

Results from this study show the DT_{50} and DT_{90} for Cry3Bb1 protein in sandy loam soil based on ELISA test are 2.76 and 9.16 days. However, the value of these results are not necessarily correlated with activity in insect guts because it is unknown if the extractable protein in the ELISA test was functional or non-functional. The DT_{50} and DT_{90} determined by insect bioassays with CPB were 2.37 and 7.87 days respectively.

Based on these results it is likely that Cry3Bb1 protein degrades rapidly in sandy loam soil. However, corn is not necessarily grown in sandy loam soil in all regions. Corn is grown in other soil types such as clay loam and silty loam soils in various regions of the U.S. In addition, this test does not account for all plant tissue such as roots. It is possible that root tissue is degrading slower then plant tissue in the soil which may result in a longer duration of degradation time of the Cry3Bb1 protein. Therefore, field tests should be conducted in a variety of soil types including clay and humic acid soils over a three year period of time to determine the long-term degradation rate of Cry3Bb1 in soil. It is important to include tests using clay soils since it has been determined that Bt Cry proteins may readily bind to clay and humic acid soil particles potentially leading to increased accumulation and persistence of the protein (Crecchio and Stotzky 1998, Koskella and Stotzky 1997, Tapp and Stotzky 1995, Tapp and Stotzky 1998) . Testing clay soils would, therefore, be considered a "worst case scenario."

142

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist RR Biopesticides and Pollution Prevention Division
PEER REVIEWER:	Chris Wozniak, Ph.D., Biologist CW Biopesticides and Pollution Prevention Division
STUDY TYPE:	Aerobic soil degradation study
MRID NO.:	451568-04
DP BARCODE NO.:	D262045
STUDY NO.:	XX-99-015
SUBMISSION NO.:	S572997
SPONSOR REPORT NO.:	MSL-16440
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	PTRL East, Inc., 3945 Simpson Lane, Richmond, KY 40475 & Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198.
TEST MATERIAL:	*Bacillus thuringiensis* Cry3Bb1 protein
AUTHORS:	John W. Martin, Michael J. McKee, Samuel Dubelman and Yelena A. Dudin
STUDY DATED:	April 26, 2000
GLP:	Good Laboratory Practice statement signed
CLASSIFICATION:	

Title: "Aerobic Soil Degradation of the *B.t.* Protein 11098 as a Component of Insect Protection Protected Corn."

Objective: This study was conducted to evaluate the rate of Cry3Bb1 degradation in a soil common to corn growing areas.

Study Summary

Materials and Methods:

Materials

Test Substance: Finely ground lyophilized field corn leaf tissue from event MON 859 plants grown in the greenhouse expressing Cry3Bb1 insecticidal protein. Tissue from the youngest whorl leaf of two to four week old corn plants (e-mail communication from Michael McKee to Robyn Rose on 7-10-02) was lyophilized in plastic bags sealed with a rubber stopper until sample weights were invariate. Leaf tissue from individual bags was combined

and mixed before removing 200 g of each corn event/line to be ground in a blender with liquid nitrogen. The lyophilized plant material was stored at -80°C until test initiation.

Enzyme-linked immunosorbent assay (ELISA) was used to determine the Cry3B1 protein concentration as 1745 µg/g dry weight in lyophilized leaf tissue. The amount of lyophilized corn tissue added to the soil in this study was based on the amount of plant tissue that could potentially be incorporated into the top six inches of soil under field conditions. According to Monsanto's submission there is 616 m^3 of soil in one acre six inches deep. The calculation provided by Monsanto to determine the amount of plant tissue incorporated in the soil was:

$$\frac{(650 \text{ g dry wt/plant}) \times (40{,}000 \text{ plants/acre})}{(616 \text{ m}^3\text{/acre}) \times (10^6 \text{ cm}^3\text{/m}^3) \times (1.5 \text{ g soil/cm}^3)} = 0.028 \text{ g dry wt plant tissue/g soil}$$

Based on this calculation 0.03 g (rounded up from 0.028) dry weight plant tissue was added to each gram of dry soil; therefore, 3% of the dry weight of soil was dry weight of lyophilized plant tissue. An additional test was conducted with 10% of the soil containing lyophilized dry weight plant tissue (0.10 g leaf tissue/g soil).

Control Substance: Finely ground lyophilized field corn leaf tissue from event MON 846 that does not express the Cry3Bb1 insecticidal protein. ELISA was used to determine that no detectable levels (limit of detection = 0.10 µg/g dry weight) of Cry3Bb1 protein were present in the leaf tissue.

Based on this calculation 0.03 g (rounded up from 0.028) dry weight plant tissue was added to each gram of dry soil; therefore, 3% of the dry weight of soil was dry weight of lyophilized plant tissue. An additional test was conducted with 10% of the soil containing lyophilized dry weight plant tissue (0.10 g leaf tissue/g soil).

Test System: The top 6 in (horizon A) of sandy loam soil which is commonly found in corn growing areas was collected by PTRL East, Inc. near the testing facility in Fayette County of Lexington, KY. Soil properties were determined by A & L Great Lakes and are listed in Table 1. In addition, a soil microbial analysis was conducted by PTRL East, Inc. (Table 2.).

Soil was prepared for testing by PTRL East, Inc. by passing moist soil through a 2 mm screen and incubating it at 25 ± 1°C. Soil remained moist until testing when it was air-dried. After drying, soil was remoistened to 75% field capacity (0.33 bar) with deionized water.

145

Table 1. Physicochemical Characteristics of Soil

Parameter	Results/Units
PTRL East, Inc. Log. No.	Q-2
USDA Textural Class:	Sandy Loam
Soil Series:	Huntington Series
pH	7.7
Particle Size Distribution:	
Sand	61.6%
Silt	28.0%
Clay	10.4%
Water Holding Capacity (at 0.33 bar)	14.63%
Organic Matter	2.32%
Cation Exchange Capacity (meq/100g)	6.72
Bulk Density (disturbed) gm/cc	1.24

* Table 1 was copied from page 22 of MRID451568-04

Table 2. Microbial Characterization Evaluated by Enumerating the Total Colony Forming Units (CFU) Per Gram of Soil

Microbial Media	CFU/g soil
Pre-Study	
Aerobic Bacteria	1.3 E + 07
Actinomycetes	1.8 E + 07
Fungi	5.0 E + 04
Week 3	
Aerobic Bacteria	9.3 E + 06
Actinomycetes	9.8 E + 06
Fungi	3.1 E + 04
Week 6	
Aerobic Bacteria	8.7 E + 06
Actinomycetes	1.0 E + 07
Fungi	3.4 E + 04

Table 2. Continued

Microbial Media	CFU/g soil
Week 9	
Aerobic Bacteria	6.4 E + 06
Actinomycetes	8.6 E + 06
Fungi	3.2 E + 04
Post-Study	
Aerobic Bacteria	6.5 E + 06
Actinomycetes	8.4 E + 06
Fungi	7.7 E + 03

* Table 2 was copied from page 23 of MRID451568-04

Methods

Two levels of test and control substances including lyophilized transgenic and non-transgenic plant tissue were added to sandy loam soil at the testing facility, PTRL Inc. A high concentration and low concentration of test and control substances were added to 22 mL sterile, glass vials (Table 3). The 10% Cry3Bb1 vials contained 174.5 µg Cry3Bb1 protein/g of soil and the 10% control vials contained 0.30 g control substance/g of soil. The 3% Cry3Bb1 vials contained 0.09 g of the test substance per 2.91 g of dried sandy loam soil for a Cry3Bb1 protein concentration of 52.4 µg/g soil. The 3% concentration control vials contained 0.09 g of the control substance. There were also tests conducted on "control-only", "soil-only" and "microbial characterization" samples. All samples contained enough deionized water to achieve 75% field capacity at 0.33 bar and vials were covered by rubber caps that were vented with three holes to allow air flow. This was achieved by adding 0.33 mL or 0.36 mL deionized water to the 10% (2.7 g soil) or 3% (2.91 g soil) test concentrations.

Table 3. Application of Test and Control Substance to Soil

# of Samples	Soil (g)	Test Substance (g)	Test Substance (g)	Cry3Bb1 (µg/g)	Designation
High Concentration (10%)					
40	2.7	0.3	0	174.5	"test-soil"
40	2.7	0	0.3	0	"control soil"
4	0	0.3	0	175.5	"test only"
4	0	0	0.3	0	"control only"

147

Table 3. Continued

# of Samples	Soil (g)	Test Substance (g)	Test Substance (g)	Cry3Bb1 (µg/g)	Designation
4	2.7	0	0	0	"microbial characterization"
4	2.7	0	0	0	"soil only"
Low Concentration (3%)					
20	2.91	0.09	0	52.4	"test-soil"
20	2.91	0	0.09	0	"control soil"
4	0	0.09	0	52.4	"test only"
4	0	0	0.09	0	"control only"
4	2.91	0	0	0	"microbial characterization"

* Table 3 was copied from page 24 of MRID451568-04

At test initiation, samples of test and control substances were sent to Monsanto to determine if any protein degradation occurred during handling and transport. Soil-only samples were adjusted for FMC twice a week and incubated until microbial activity was evaluated on weeks 3, 6, 9 and at test termination. On days 1, 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, 71, 77, 84, 91, 99, 105, 112 and 119 high dose samples were removed from the incubator and on days 1, 3, 7, 14, 28, 42, 56, 71 and 80 low dose samples were removed from the incubator and shipped to Monsanto on dry ice.

The concentration of active Cry3Bb1 protein in the soil samples was determined by conducting insect bioassays. Insect bioassays included a mixture of test and control substances with an agar-based insect diet added to wells of bioassay trays. Standard diet concentrations were prepared from purified protein. Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) larvae were added to each well (one larva/well) and each treatment bioassay was replicated twice for a total of 16 CPB/replicate. CPB are more sensitive to the Cry3Bb1 protein then CRW and were, therefore, expected to result in a more measurable response then CRW, the target species. According to Monsanto, CPB are also preferred for this test because an adequate insect diet is available, whereas, a dependable commercial diet is not available for CRW. During the test, bioassay trays were kept in an incubator set at approximately 27°C for seven days. Survivorship and weight of surviving CPB were evaluated and recorded.

Of the four insect bioassays conducted, the first two were low dose (3%) with a target concentration of 2.3 µg Cry3Bb1 protein/mL insect diet. This rate was calculated from: (1.745 µg Cry3Bb1 protein/g tissue) × (0.03 g lyophilized corn tissue/mL soil) × (0.5 g soil/10 mL diet) × 0.893 (adjustment for 10.7% soil moisture) = 2.3 µg Cry3Bb1 protein/mL diet. The third

bioassay contained 2.6 µg Cry3Bb1 protein/mL insect diet and was calculated from: (1.745 µg Cry3Bb1 protein/g tissue) × (0.03 g lyophilized corn tissue/g soil) × (0.56 g soil/10 mL diet) × 0.893 (adjustment for 10.7% soil moisture) = 2.6 µg Cry3Bb1 protein/mL diet. The fourth bioassay contained 3.9 µg Cry3Bb1 protein/mL insect diet and was calculated from: (1.745 µg Cry3Bb1 protein/g tissue) × (0.01 g lyophilized corn tissue/g soil) × (0.25 g soil/10 mL diet) × 0.90 (adjustment for 10.7% soil moisture) = 3.9 µg Cry3Bb1 protein/mL diet.

A bioassay was not conducted for "shipping control" samples at the 3% leaf tissue level because a lack of sensitivity resulted in inconclusive results. While attempting to analyze the 3% dosing level, a large portion of the "shipping control" samples were depleted which prevented analysis of the 10% leaf in soil level.

In addition to an insect bioassay, an ELISA was conducted to measure the level of functional and non-functional Cry3Bb1 protein present in samples. However, the ELISA test will only show extractable protein and does not distinguish between functional and non-functional proteins. A soil extraction buffer adopted from Palm *et al.* 1994 was used in the ELISA which was based on a one-step, two-site sandwich. According to Monsanto, the "ELISA consisted of a simultaneous incubation of 50 µL per well of standards, samples or quality control solutions, and 50 µL per well of a 1:5,000 dilution of Protein-A purified rabbit anti-Cry3Bb1 conjugated to horseradish peroxidase (HRP; Sigma Chemical, St. Louis, MO)." A detailed description of the ELISA methodology can be found on page 17 of MRID No. 451568-04.

The DT_{50} (time to 50% degradation of the bioactivity or concentration of the protein) and DT_{90} (time to 90% loss of bioactivity or concentration) degradation rates for the Cry3Bb1 protein were determined from a DISSFIT Excel Computer Program Analysis. A calculation was done for the percent insect mortality from bioassay data and an additional calculation was conducted from the concentration of extractable Cry3Bb1 protein remaining in the soil.

Results:

Data from the 3% lyophilized corn plant tissue were not reported because the samples taken at test initiation in the first two bioassays did not show a response by the CPB. The third bioassay which included a 3% lyophilized plant tissue sampled resulted in high contamination and high mortality so these results were not reported. A Cry3Bb1 dissipation rate for the 10% lyophilized plant tissue samples was calculated based on insect survival. CPB mortality in the control treatments ranged from 3.1% to 12.5% ($\bar{x}$ = 7.3%) during the 42 day testing period (Table 4). The DT_{50} was calculated from data collected on days 0, 1, 3 and 7 (Table 5). On day 7, percent mortality reached and remained at zero. The average background mortality of 7.3% observed in the control group was used for the day 7 time point in the DT_{50} calculation rather than zero. The **DT_{50}** based on linear plots from calculations in Table 4 (see Figure 1 on page 29 of MRID No. 451568-04) was determined to be **2.37 days** and **DT_{90}** was **7.87 days**.

Table 4. Percent Mortality of Colorado Potato Beetle Larvae as a Function of Time after exposure to samples containing Control and Test Substances added to soil at 10% (w:w).

Treatment	Cry3Bb1 starting concentration in diet mg/mL	Soil Incubation (days)	# of Insects at Start	# of Insects Dead	% Mortality
Test Substance (10%)	3.9	0	32	16	50
Test Substance (10%)	3.9	1	32	15	46.8
Test Substance (10%)	3.9	3	32	4	12.5
Test Substance (10%)	3.9	7	32	0	0
Test Substance (10%)	3.9	14	32	0	0
Test Substance (10%)	3.9	21	32	3	9.4
Test Substance (10%)	3.9	28	31	1	3.2
Test Substance (10%)	3.9	42	32	1	3.1
Control Substance (10%)	0	0	32	2	6.3
Control Substance (10%)	0	1	32	1	3.1
Control Substance (10%)	0	3	32	4	12.5
Control Substance (10%)	0	7	32	2	6.3
Control Substance (10%)	0	14	32	3	9.4
Control Substance (10%)	0	21	32	1	3.1
Control Substance (10%)	0	28	32	3	9.4
Control Substance (10%)	0	42	32	3	9.4

* Table 4 was copied from page 25 of MRID451568-04

Table 5. Soil Dissipation Analysis Based on Insect Bioassay Results

		Curve Fitting Parameters				
Days	% Morality	LN % Mortality	Trnsfmd Days	LN Pred Conc.	Pred Conc.	% Error
0	50	3.91202	0	4.03920	56.7807	13.56
1	46.8	3.84588	0.85811	3.52369	33.9093	-27.54
3	12.5	2.52573	2.057882	2.80394	16.5096	32.08
7	7.3	1.98787	3.555999	1.90469	6.7173	-7.98

* Table 5 was copied from page 26 of MRID451568-04

The extractable Cry3Bb1 protein of the 10% plant tissue soil samples were below the limit of

150

quantification (LOQ) of an ELISA test. All values were <2 ng Cry3Bb1/mL which is the lowest detectable protein standard. Days 0, 1 and 3 sample values exceeded the range of the ELISA curve so the test was repeated at a high dilution rate (Table 6). On day 7 samples were analyzed at the same dilution rate to allow for data bridging. Data on day 7 showed values of 2578 and 2304 ng/mL ($\bar{x}$ = 2441 ng/mL) which were considered equivalent and consistent by Monsanto. The final value used for day 7 was 2441 ng/mL and a quality control sample utilizing known concentrations of Cry3Bb1 protein resulted in 59.4 ng/mL and 52.6 ng/mL on the two respective sample dates (8/24/99 & 9/8/99) (Table 6). The extractable protein on days 28 and 42 were <LOQ. The DT_{50} determined by the ELISA was 2.76 days and the DT_{90} was 9.16 days (Table 7).

Table 6. ELISA Results for Concentration of Cry3Bb1 Protein in Soil Samples at Various Incubation Times

Day #	ELISA 8/24 Total µg/mL[1]	ELISA 9/8 Total ng/mL[2]	Results (µg/mL)	Results (µg/g soil)
0	–[3]	22,744 / 23,062	22,903	115[5]
1	–[3]	20,448 / 19,682	20,065	100
3	–[3]	7,226 / 7,469	7,348	36.7
7	2578	2304	2,441	12.2
14	997	N/A	997	4.99
21	87.2	N/A	87.2	0.44
28	<LOQ[4]	N/A	<LOQ[4]	<LOQ[4]
42	<LOQ[4]	N/A	<LOQ[4]	<LOQ[4]

* Table 6 was copied from page 27 of MRID451568-04

(1) 1:16 dilution rate used for all samples

(2) Days 0 and 1 dilution rates were 1:100 and 1:200; Day 3 dilution rates were 1;50 and 1:100; Day 7 dilution rate was 1:16 which was repeated as a bridging control.

(3) Could not be quantified because samples exceeded the highest standard concentration

(4) LOQ = 0.1 µg/g dry weight

(5) Initial protein concentration on day zero was 115 µg/g which represents 66% of 174.5 µg/g (theoretical initial concentration)

Table 7. Soil Dissipation Analysis Based on ELISA Results

		Curve Fitting Parameters				
Days	Conc. (µg/g)	LN Conc.	Trnsfmd Days	LN Pred Conc.	Pred Conc.	% Error
0	115	4.74493	0	4.63445	102.9715	-10.46
1	100	4.60517	1	4.38302	80.0794	-19.92
3	36.7	3.60278	3	3.88015	48.4316	31.97
7	12.2	2.50144	7	2.87442	17.7151	45.21

Table 7. Continued

Days	Conc. (µg/g)	LN Conc.	Trnsfmd Days	LN Pred Conc.	Pred Conc.	% Error
14	4.99	1.60744	14	1.11438	3.0477	-38.92
21	0.44	-0.82098	21	-0.64565	0.5243	19.16

* Table 7 was copied from page 28 of MRID451568-04

Monsanto's Conclusions:

According to the data and results presented by Monsanto the DT_{50} based on insect bioassays and ELISA were 2.37 and 2.76 days respectively. The DT_{90} estimates for the insect bioassays and ELISA were 7.87 and 9.16 days respectively. These results verify that the Cry3Bb1 protein degrades rapidly in the soil.

Reviewer's Comments:

This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with two exceptions that did not affect the integrity of the test. According to the signed GLP statement, "1. Equipment SOPs were not available for the incubators used for the insect bioassay. However, appropriate maintenance and calibration was conducted and documented in the study data or facility records. There was no impact on the results of the bioassay. 2. Due to a machine malfunction at PTRL East, Inc., temperature data are not available for a portion of the incubation period. This had no impact on the study."

Results from this study show the DT_{50} and DT_{90} for Cry3Bb1 protein in sandy loam soil based on ELISA test are 2.76 and 9.16 days. However, the value of these results need to be considered with regard to biological activity because it is unknown if the extractable protein in the ELISA test was functional or non-functional. The DT_{50} and DT_{90} determined by insect bioassays with CPB were 2.37 and 7.87 days respectively.

Based on these results it is likely that Cry3Bb1 protein degrades rapidly in sandy loam soil. However, corn is not necessarily grown in sandy loam soil in all regions. Corn is grown in other soil types such as clay loam and silt loam soils in various regions of the U.S. In addition, this test does not account for all plant tissue such as roots. It is possible that root tissue is degrading slower then leaf tissue in the soil which may result in a longer duration of degradation time of the Cry3Bb1 protein. Therefore, field tests should be conducted in a variety of soil types including clay and humic acid soils over a three year period of time to determine the long-term degradation rate of Cry3Bb1 in soil. It is important to include tests using clay soils since it has been determined that Bt Cry proteins may readily bind to clay and humic acid soil particles potentially leading to increased accumulation and persistence of the protein (Crecchio and Stotzky 1998, Koskella and Stotzky 1997, Tapp and Stotzky 1995, Tapp and Stotzky 1998) . Testing clay soils

would, therefore, be considered a "worst case scenario."

References Cited:

Crecchio C. and G. Stotzky (1998) Insecticidal activity and biodegradation of the toxin from *Bacillus thuringiensis* subspecies *kurstaki* bound to humic acids in soil. Soil Biol. Biochem. 30(4), 463-470.

Koskella J. and G. Stotzky (1997) Microbial utilization of free and clay-bound insecticidal toxins from *Bacillus thuringiensis* and their retention of insecticidal activity after incubation with microbes. Appl. Environ. Microbiol. 63, 3561-3568.

Palm, C. J., K. Donegan, D. Harris, and R. J. Siedler. 1994. Quantification in soil of *Bacillus thuringiensis* var. *krustaki* d-endotoxin from transgenic plants. Mol. Ecol. 3: 145-151.

Tapp H. and G. Stotzky (1995) Dot blot enzyme-linked immunoabsorbent assay for monitoring the fate of insecticidal toxins from *Bacillus thuringiensis* in soil. Appl. Environ. Microbiol. 61(2), 602-609

Tapp H. and G. Stotzky (1998) Persistence of the insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* in soil. Soil Biol. Biochem. 30(4), 471-476.

15

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

MAY 20 2002

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Review of ecological non-target insect studies for *Bacillus thuringiensis* Cry3Bb1 protein. EPA Reg. No. 524-LRA; Barcode No. D262045; Case No. 066221; Submission No. S572997. MRID Nos 449043-10, 449043-11, 449043-12, 449043-13, 449043-17, 455382-04, 455382-05, 455382-06, 455770-03.

TO: Mike Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division, 7511C

FROM: Robyn Rose, Entomologist Robyn Rose
Biopesticides and Pollution Prevention Division, 7511C

PEER REVIEW: Zigfridas Vaituzis, Ph.D., Microbiologist Zigfridas Vaituzis 5/16/02
Biopesticides and Pollution Prevention Division, 7511C

CLASSIFICATION: **Supplemental** to submitting the final field survey study. All other studies are **Acceptable**.

BACKGROUND:
Monsanto Company has requested a registration for *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material (ZMIR13L) necessary for its production in all corn lines and varieties. The Cry3Bb1 protein is intended to control corn rootworms (CRW, *Diabrotica* spp.), a coleopteran pest of corn. Studies were submitted to support registration of the transformation event MON 863. CRW, a primary pest of corn in the U.S., feeds on corn roots as larvae leading to a reduction in the plant's ability to absorb water and nutrients from soil and lodging. In areas where CRW is a pest (e.g., Corn Belt), significant financial losses are realized from a decrease in production and chemical insecticide usage. Significant acres of corn are treated annually to control CRW with organophosphate, carbamate and pyrethroid insecticides.

Nontarget insect studies are required to register a new pesticide (40 CFR §158.~~590~~ 740 RR). Monsanto Company submitted studies on nontarget adult lady beetles (MRID No 449043-14), green lacewing larvae (MRID No 449043-12), a parasitic Hymenoptera (MRID No 449043-13), adult and larval honey bees (MRID Nos 449043-11, 449043-10) and Collembola (MRID No 449043-17). Additional studies were submitted including field census evaluations (MRID No 455382-

15

06) and an endangered species risk assessment (MRID No 455770-03). In a March 10, 2000 memorandum from Robyn Rose to Mike Mendelsohn, the adult lady beetle study was reviewed. This review concluded that a test demonstrating the effect of lady beetles feeding on corn pollen containing Cry3Bb1 should be conducted. This study was conducted by Monsanto and submitted to the Agency (MRID No 455382-04). Although Cry3Bb1 is active against coleopterans, a study was also conducted to alleviate any potential public concerns on potential effects of MON863 expressing pollen to monarch butterflies (MRID No 455382-05).

CONCLUSIONS:

Green Lacewing Larvae (MRID No 449043-12)
An acceptable study was conducted according to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I. The NOEC for green lacewing larvae was determined to be >8,000 ppm Cry3Bb1 protein.

Parasitic Hymenoptera (449043-13)
An acceptable test was conducted according to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I. The NOEC for parasitic Hymenoptera was determined to be 400 ppm Cry3Bb1 protein. Although 400 ppm Cry3Bb1 protein is only 1X field concentrations in plant rather than a 10-fold safety factor, parasitic Hymenoptera are not expected to feed directly on corn plant tissue. Therefore, minimal exposure of parasitic Hymenoptera to Cry3Bb1 protein is expected.

Honey Bee Larvae (MRID No 449043-10)
An acceptable test was conducted according to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I and OPPTS 885.4380 Honey Bee Testing, Tier I. The NOEC for honey bee larvae was determined to be 1,790 ppm Cry3Bb1 protein.

Honey Bee Adults (MRID No 449043-11)
An acceptable test was conducted according to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I and OPPTS 885.4380 Honey Bee Testing, Tier I. It can be concluded from this test that the NOEC of the Cry3Bb1 protein for adult honey bees is 360μg/mL.

Collembola (MRID No 449043-17)
An acceptable test was conducted. It can be concluded from this test that the NOEC of the Cry3Bb1 protein for Collembola is ≤872.5μg.

Lady Beetle Larval Pollen Feeding Study (MRID No 455382-04)
the NOEC for Cry3Bb1 expressed in pollen is ≤93 μg/g fresh pollen weight. It can be concluded from this study that lady beetle larvae will not be adversely affected by Cry3Bb1 field corn.

15

Monarch Butterfly Larval Pollen Feeding Study (MRID No 455382-05)
This study was not required nor requested for Cry3Bb1 because it is a coleopteran active protein that is not expected to affect lepidopterans such as the monarch butterfly. In addition, extensive research conducted on the potential affects of monarch feeding on Bt corn pollen has shown a lack of concern of acute toxicity. However, due to recent public concern of possible adverse effects of Bt corn on monarchs, Monsanto sponsored this study and submitted it to the Agency for review. This study has demonstrated that corn pollen expressing the Cry3Bb1 protein will not result in acute toxic or developmental effects to monarch larvae.

Field Evaluation of Ecological Impacts (MRID No 455382-06)
Although this study was not conducted according to FIFRA GLP compliance, this study was conducted according to a scientifically valid, peer reviewed protocol that is acceptable. Results reported in this submission suggest that planting event MON 863 CRW protected corn will not adversely affect non-target beneficial invertebrate abundance in the field. On the contrary, a reduction in pesticide use that may result from planting MON 863 corn will probably result in increased numbers of natural enemies in CRW protected Bt corn.

This submission only reported results from the 2000 growing season. A final report will be submitted to the Agency after the 2001 data is collected and analyzed. The final report should include the location of the 2000 and 2001 test sites as well as the additional data on soil fertility, microbial populations and soil Bt protein collected during this study but not reported at this time. This study should also be conducted at additional test sites in different geographic locations to account for regional differences in insect populations.

Endangered Species Impact Assessment (MRID No. 455770-03)
Based on a 10-fold safety factor for terrestrial species and a 20-fold safety factor for aquatic species and a lack of exposure, no unreasonable adverse effects of MON 863 to endangered Coleoptera are expected. According to Monsanto's submission, the LC_{50} for the CPB, the most sensitive species to Cry3Bb1 protein, is 2.4 µg/mL diet. A maximum exposure of 0.23 µg/g (10-fold safety margin) is expected 1 m from the field edge, 0.09 µg/g (28-fold safety margin) is expected 2 m from the field edge and 0.03 µg/g (82-fold safety margin) is expected 4-5 m from the field edge. A 1-ha pond with a 2 m depth located 1 m from the edge of the corn field that has 500 grains/cm^2 would have a maximum exposure of 5.81250×10^{-5} which represents a 41290 safety margin. These data indicate that pollen drift from MON 863 corn fields will not pose a terrestrial or aquatic exposure risk.

Many of the endangered and threatened beetles occur in cave or aquatic habitats. None of the endangered beetles are expected to occur in or near corn fields. The American burying beetle may occur in old fields or cropland hedge rows. However, based upon the feeding habits of the American burying beetle, it is not expected to occur within corn fields nor will it be exposed to Cry3Bb1 protein. Adult American burying beetles are classified as opportunistic scavengers that feed on anything dead and bury vertebrate carcasses which larvae feed on. Carrion regurgitated by adults is fed to larvae until they are able to feed directly on a carcass.

151

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target effects of Bt Protein 11231 on green lacewing larvae
MRID NO.:	449043-12
STUDY NO.:	WL-98-298
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	Wildlife International Ltd., 8598 Commerce Dr., Easton, MD 21601
TEST MATERIAL:	*Bacillus thuringiensis* Protein 11231
AUTHOR:	Susan J. Palmer and Henry O. Krueger, Ph.D
STUDY COMPLETED:	July 28, 1999
CLASSIFICATION:	**Acceptable**

Study Summary

Title: "*Bacillus thuringiensis* Protein 11231: A Dietary Toxicity Study with Green Lacewing Larvae (*Chrysoperla carnea*)"

Authors: Susan J. Palmer and Henry O. Krueger, Ph.D (Wildlife International Ltd.).

Objective: This study was conducted to evaluate the toxicity of *Bacillus thuringiensis* Cry3Bb1 (analogous to Protein 11231) in diet to green lacewing larvae (*Chrysoperla carnea*).

Methods:

This test was based on protocols outlined in EPA's Series 154A *Pesticide Assessment Guidelines, FIFRA Subdivision M, Microbial Pest Control Agents* that are superceded by OPPTS 885.4340 Nontarget insect testing, Tier I and conducted according to a protocol titled "Cry3Bb2 Protein: A Dietary Toxicity Study with Green Lacewing larvae (*Chrysoperla carnea*)." Testing was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (Lot no. 6312812; purity 96%; 34.5 mg active protein/mL water) held at -80°C prior to test initiation. Current nomenclature refers to this protein as Cry3Bb1. During the test, green lacewing larvae were fed the Cry 3Bb1 protein in a moth egg (*Sitotroga* sp.) and water meal diet at rates of 400 and 8,000 ppm which is approximately equivalent to 1X and 20X the maximum protein concentration in plant tissue. Test concentrations were confirmed by a bioassay using Colorado potato beetles, s known sensitive species to the Cry3Bb1 protein.

This study included 30 test chambers (1 oz semitransparent plastic cups with lids) containing 1 newly emerged lacewing larvae that were allowed *ad libitum* access to Cry3Bb1 in diet, 1 control

15

group given water prepared by reverse osmosis and 2 reference groups given 1,000 and 10,000 ppm potassium arsenate (41.7% arsenic). Larvae were transferred to new chambers with fresh diet weekly and kept in incubators set at an average of 20.6°C, 74% ±9% relative humidity and a photoperiod of 12 hours of light. Rates of mortality, pupation and other clinical signs of abnormal behavior or toxicity were recorded within 2 to 4 hours after test initiation and continued daily until there was 20% mortality in the control group on Day 10. Significant differences of mortality at test termination between treated and control groups were determined using Fisher's exact test.

Results:
No pupation occurred in any of the treated, control or reference groups at test termination. There was 27% mortality (8 of 30) in the control group and no signs of clinical abnormalities at test termination. There was 27% mortality (8 of 30) in the 400 ppm Cry3Bb1 treatment group and 23% mortality (7 of 30) in the 8,000 ppm Cry3Bb1 treatment group. Differences in mortality between the control and treatment groups were not significantly different ($p>0.05$). The 1,000 and 10,000 ppm potassium arsenate groups resulted in 10% (3 of 30) and 53% mortality (16 of 30) respectively on Day 1 of testing. At test termination mortality for the 1,000 ppm group was 43% (13 of 30) and 100% mortality in the 10,000 ppm reference group.

Study Conclusions:
There was no significant increase in green lacewing larval mortality when fed 1X (400 ppm) and 20X (8,000 ppm) the maximum Cry3Bb1 protein concentration found in plant tissue. Based on this test, the NOEC for green lacewing larvae exposed to Cry3Bb1 in diet is >8,000 ppm .

Reviewer's Comments:
This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not effect the integrity of the test. According to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I, nontarget insects should be tested with does of 10-100X the field dosage. This test was conducted at an acceptable level 20X field concentrations in plant tissue or >8,000 ppm Cry3Bb1 protein. These guidelines also recommend testing bacteria against nontarget insects for 21 to 30 days. If it is not possible to continue the test for 21 to 30 days, then testing should cease when control mortality reaches 20%. Although this test could have continued for a longer duration, terminating at 20% mortality in the control group is acceptable.

This study was submitted in support of registering CRW protected Bt corn event MON 863. However, green lacewing were fed Cry3Bb1.11231 (event MON 859) rather than Cry3Bb1.11098 (event MON 863) in this study. According to Monsanto, Cry3Bb1.11231 "demonstrates an eight-fold increase in activity against the SCRW as a result of four strategically placed amino acid substitutions in the wild type protein sequence." Cry3Bb1.11098 "demonstrates an eight-fold increase in activity against the SCRW as a result of five strategically placed amino acid substitutions in the wild type protein sequence." It is acceptable that this test

was conducted with event MON 859 rather than MON 863 because they produce nearly identical Cry3Bb1 protein variants.

It can be concluded from this test that the NOEC for green lacewing larvae fed Cry3Bb1 protein is >8,000 ppm.

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Robyn Rose Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Zigfridas Vaituzis 5/16/2 Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target effects of Bt Protein 11231 on parasitic Hymenoptera
MRID NO.:	449043-13
STUDY NO.:	WL-98-300
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	Wildlife International Ltd., 8598 Commerce Dr., Easton, MD 21601
TEST MATERIAL:	*Bacillus thuringiensis* Protein 11231
AUTHOR:	Susan J. Palmer and Henry O. Krueger, Ph.D
STUDY COMPLETED:	July 28, 1999
CLASSIFICATION:	**Acceptable**

Study Summary

Title: "*Bacillus thuringiensis* Protein 11231: A Dietary Toxicity Study with the Parasitic Hymenoptera (*Nasonia vitripennis*)"

Authors: Susan J. Palmer and Henry O. Krueger, Ph.D (Wildlife International Ltd.).

Objective: This study was conducted to evaluate the toxicity of *Bacillus thuringiensis* Cry3Bb1 (analogous to Protein 11231) in diet to a representative parasitic wasp (*Nasonia vitripennis*).

Methods:

This test was based on protocols outlined in EPA's Series 154A *Pesticide Assessment Guidelines, FIFRA Subdivision M, Microbial Pest Control Agents* that are superceded by OPPTS 885.4340 Nontarget insect testing, Tier I and conducted according to a protocol titled "Cry3Bb2 Protein: A Dietary Toxicity Study with the Parasitic Hymenoptera (*Nasonia vitripennis*)." Testing was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (Lot no. 6312812; purity 96%; 34.5 mg active protein/mL water) held at -80°C prior to test initiation. Current nomenclature refers to this protein as Cry3Bb1.

Twenty five adult wasps were kept in ½ pt rolled paper containers (9 cm in diameter and 5 cm high) covered with a plastic petri dish and a 20 mL glass vial with deionized water inserted through it during testing. Diet, including honey combined with Cry3Bb1 protein, was administered to wasps on a cotton swab inserted through the side of test chambers. Test diets were prepared at test initiation and weekly thereafter. Wasps were tested at rates of 400 and 8,000 ppm Cry3Bb1 protein which is approximately equivalent to 1X and 20X the maximum

160

protein concentration in plant tissue. Test concentrations were confirmed by a bioassay using Colorado potato beetles, a known sensitive species to the Cry3Bb1 protein. In addition to Cry3Bb1 treated groups, one control group given water prepared by reverse osmosis and two reference groups given 100 and 1,000 ppm potassium arsenate (41.7% arsenic). Wasps were kept in incubators set at an average of 26.7±0.7°C, 54% ±4% relative humidity and a photoperiod of 12 hours of light and allowed *ad libitum* access to diets. Rates of mortality, pupation and other clinical signs of abnormal behavior or toxicity were recorded within four hours of test initiation and continued daily until there was 20% mortality in the control group on Day 16. Significant differences of mortality at test termination between treated and control groups were determined using Dunnett's test.

Results:
Some wasps were not included in the data because of mortality in the control, reference and 8,000 ppm treatment group that was due to wasps being trapped under the container lids. There was 23% mortality (17 of 73) in the control group at test termination and one immobile wasp recorded on Day 15. There was 24% mortality(18 of 75) in the 400 ppm Cry3Bb1 treatment group and 58% mortality (42 of 73) in the 8,000 ppm Cry3Bb1 treatment group. Additional wasps were observed to be immobile during the test and one wasp was "near death" in each treatment group at test termination. If the wasps "near death" are included in the mortality group, then mortality rates are increased to 25% and 59% for the 400 and 8,000 ppm treatments respectively. If an adjustment for mortality in the control group is considered, mortality in the 8,000 ppm treatment group is 45%. Although differences in mortality between the control and treatment groups were not significantly different ($p>0.05$), a treatment effect could not be precluded in this study. At test termination mortality for the 100 ppm potassium arsenate reference group was 33% (24 of 73) and 100% mortality (70 of 70) in the 1,000 ppm reference group.

Conclusions:
Based on this test, the authors concluded that the NOEC for adult parasitic Hymenoptera exposed to Cry3Bb1 in diet is 400 ppm and the LC_{50} is 8,000 ppm.

Reviewer's Comments:
This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not effect the integrity of the test. According to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I, nontarget insects should be tested with doses of 10-100X the field dosage. This test was conducted at an acceptable level 20X field concentrations in plant tissue or 8,000 ppm Cry3Bb1 protein. These guidelines also suggest testing bacteria against nontarget insects for 21 to 30 days. If it is not possible to continue the test for 21 to 30 days, then testing should cease when control mortality reaches 20%. Although this test could have continued for a longer duration, terminating at 20% mortality in the control group is acceptable.

It can be concluded from this test that the NOEC for the Cry3Bb1 protein is 400 ppm. Although these results are based on 400 ppm Cry3Bb1 protein which is only 1X field concentrations in plant rather than a 10-fold safety factor, parasitic Hymenoptera are not expected to feed directly on corn plant tissue. Therefore, minimal exposure of parasitic Hymenoptera to Cry3Bb1 protein is expected.

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Robyn Rose Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Zigfridas Vaituzis 5/16/02 Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target effects of Bt Protein 11231 on honey bee larvae
MRID NO.:	449043-10
STUDY NO.:	CA-98-169
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	California Agricultural Research, Inc., 4141 N. Vineland, Kerman, CA 93630
TEST MATERIAL:	*Bacillus thuringiensis* Protein 11231
AUTHOR:	Victor L. Maggi, M.S.
STUDY COMPLETED:	August 2, 1999
CLASSIFICATION:	**Acceptable**

Study Summary

Title: "Evaluation of the Dietary Effects of Purified *Bacillus thuringiensis* Protein 11231 on Honey Bee Larvae"

Author: Victor L. Maggi (California Agricultural Research, Inc.)

Objective: This study was conducted to evaluate the toxicity of *Bacillus thuringiensis* Cry3Bb1 (analogous to Protein 11231) in diet to larval honey bees (*Apis mellifera*).

Methods:
This test was based on protocols outlined in EPA's OPPTS 885.4380, Honey Bee Testing, Tier I and conducted according to a protocol titled *Evaluation of the Dietary Effects(s) of Purified Bacillus thuringiensis Cry3Bb2 Protein in Honey Bee Larvae.* Testing was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (Lot no. 6312812; purity 96%; 1.79 mg active protein/mL water) held at -80°C prior to test initiation. Current nomenclature refers to this protein as Cry3Bb1.

Four honey bee hives with at least 2 frames with large, uniformly-sized larval brood populations were treated with Terabee Mix 2X Terramycin (50 g; 5.5% oxytetracycline) and Apistan strips (10.0% Fluvalinate) to control foulbrood diseases and varroa mites respectively and acclimated 30 days prior to testing. Honey bees (*Apis mellifera lingustica* Spin.) were fed a 30% sucrose solution prior to testing.

Each hive represented a replicate containing each treatment group. Twenty honey bee larvae (2-

3 days old) per replicate (80 bees/treatment) were treated with a reference substance (0.5 mg/mL (500 ppm) potassium arsenate + 3µL deionized water), a control substance (3µL deionized water) and a test substance (1.79 mg/mL (1,790 ppm) Cry3Bb1 protein + 3µL deionized water). The reference substance was administered to separate frames from treatments and controls to avoid cross-contamination. The treatment concentration, 1.79 mg/mL Cry3Bb1 protein, was based on a 100-fold safety factor over the maximum concentration of Cry3Bb1 protein expressed in pollen.

Larval brood cells intended to receive treatments were mapped on an acetate sheet laid over the frame. In the laboratory, treatments were administered to brood cells using a micropipette and frames were left undisturbed for 30 minutes to allow larvae to feed prior to returning frames to their original hive.

Capping and the presence of larvae were evaluated on Days 8 and 12 after treatment. On Day 12 treated frames were removed from hives and placed in emergence cages consisting of 3.2 cm wire mesh cage (3 × 6 cm) fit around each group of treated brood cells. Cages were kept in chambers at 28 to 32°C and 25 to 85% relative humidity. Observations of adult emergence occurred twice a day until all bees emerged. When the test was terminated two samples of the test substance were removed and bioassayed with the Colorado potato beetle, a known sensitive species to Cry3Bb1 protein, to verify bioactivity of the protein. A *t*-Test was conducted using Pesticide Research Manager (PRM) software, version 5.

Results:
Within 18 days after treatments were administered, all larvae emerged from capped brood cells. On Day 18, larvae were 20 to 21 days old. All of the larvae (100%) treated with Cry3Bb1 protein survived to pupation or "capping"; whereas, 97.5% (2.5% mortality) of the honey bee larvae in the control group survived to pupation. There was no statistical difference (p=0.05) in total percent mortality during the larval development or adult emergence stages between treated and control groups.

Although 3 bees among 2 replicates of the test substance were not accounted for, no capped cells remained. This suggests that these bees emerged but were somehow missed during counting. An additional error occurred in the 4th replicate of the reference treatment where an additional bee emerged (#21).

Conclusions:
Based on the results presented in this study, it can be concluded that honey bee development and survival are not affected by exposure to the Cry3Bb1 protein. There was 88.8% mortality of larvae treated with the reference substance which indicated that bees were exposed to the treatments.

Reviewer Comments:
This study was conducted in accordance with Good Laboratory Practice Standards as published

by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not effect the integrity of the test. This test was conducted according to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I and OPPTS 885.4380 Honey Bee Testing, Tier I. According to the guidelines, nontarget insects should be tested with does of 10-100X the field dosage. This test was conducted at an acceptable level 100X the concentration in pollen or 1,790 ppm Cry3Bb1 protein. Since potential exposure of honey bees to Cry3Bb1 will be from pollen, this test was conducted at an appropriate maximum hazard dose.

An unclear result was reported on page 15 in the Results and Discussion section of this report. A 21st emerged bee was noted from the 4th replicate of the reference group; however, Table 1 on page 17 does not report this finding. On the contrary, ≤3 bees emerged from each of the 4 reference group replicates resulting in 88.8% overall mortality. The 21st emerged bee is probably from the treated group (replicate 1 or 2) or the control group (replicate 2 or 3) and was likely counted due to error.

It can be concluded from this test that the NOEC of the Cry3Bb1 protein for honey bee larvae is >1,790 ppm.

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Robyn Rose Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Zigfridas Vaituzis 5/16/02 Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target effects of Bt Protein 11231 on honey bee adults
MRID NO.:	449043-11
STUDY NO.:	CA-98-171
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	California Agricultural Research, Inc., 4141 N. Vineland, Kerman, CA 93630
TEST MATERIAL:	*Bacillus thuringiensis* Protein 11231
AUTHOR:	Victor L. Maggi, M.S.
STUDY COMPLETED:	August 2, 1999
CLASSIFICATION:	**Acceptable**

Study Summary

Title: "Evaluation of the Dietary Effect(s) of Purified *Bacillus thuringiensis* Protein 11231 on Adult Honey Bees (*Apis mellifera* L.)"

Author: Victor L. Maggi (California Agricultural Research, Inc.)

Objective: This study was conducted to evaluate the toxicity of *Bacillus thuringiensis* Cry3Bb1 (analogous to Protein 11231) administered in diet to adult honey bees (*Apis mellifera*).

Methods:

This test was based on protocols outlined in EPA's OPPTS 885.4380, Honey Bee Testing, Tier I and conducted according to a protocol titled *Evaluation of the Dietary Effects(s) of Purified Bacillus thuringiensis Cry3Bb2 Protein on Adult Honey Bees2.* Testing was conducted with *Bacillus thuringiensis* Cry 3B2.11231 protein (Lot no. 6312812; purity 96%; 0.36 mg active protein/mL water). Current nomenclature refers to this protein as Cry3Bb1.

Treatment groups consisted of a control group fed 30% sucrose in deionized water, a reference group fed 100µg/mL potassium arsenate, and a test group fed 360 µg/mL of Cry3Bb1 protein and a water only group. The 360 µg/mL Cry3Bb1 protein used in the treatment group was gotten from mixing 100 mL volume of 0.72 mg/mL purified protein with an equal volume of 60% sucrose:40% deionized water solution. The potassium arsenate and 360 µg/mL Cry3Bb1 protein were fed to bees in a 30% sucrose:70% deionized water solution. Stability and dose of the Cry3Bb1 protein was confirmed from samples collected before test initiation, twice during the study, and after completion via bioassay of Colorado potato beetles, a known sensitive species.

In March and October of each year, honey bee (*Apis mellifera*) hives received ~50 g of 5.5% oxyteteracycline hydrochloride to prevent and control foul brood diseases and were treated with an acaricide containing 10.0% Fluvalinate in November to control Varroa mites. Three hives were allowed to acclimate 30 days prior to treatments. The 9 frames used in this study were chosen from hives that were evaluated 2 months prior to testing for the presence of 2 or more frames with large populations of capped brood about to emerge. Two frames from one hive were treated on August 7, 1998 and the other two hives (2 and 3 frames) were treated on August 13. Since the test began late in the season, hives were fed a 30% sucrose solution to ensure bees would accept treated diets that were fed to hives three times a week during the study. Diet was administered to hives in a 12 mL glass vial.

Frames (12.7 cm cube-shaped 3.2 mesh/cm hardware cloth cage with a 10 × 10 cm cardboard door) with capped brood and no adults were put in the cages that were kept in chambers set at 24 hours dark, 26 to 40°C and 22 to 84% relative humidity prior to administering treatments. Forty bees that appeared normal and were ≤6 days old were taken from these frames and placed in hive boxes to be treated. There were 4 replicates of the 3 treated groups (160 bees each) and one water only group for a total of 13 cages and 520 bees used in this study. Treated cages were kept in a chamber set at 24 hours dark, 16 to 18°C and 41 to 49% relative humidity.

Observations of mortality and agitation of the feeding vials were made 5 hours after test initiation and daily thereafter. Test substance solutions (< 8 days old) were replenished every 48 hours. However, since the reference and water only groups resulted in high mortality rates within 48 hours of test initiation and 100% mortality by Day 4, they did not receive new diet. The study was terminated on Day 11 when there was 40% mortality in the control group.

The cumulative percent morality for each replicate was determined by calculating # of dead bees/# bees at study initiation × 100. Each test groups cumulative mean mortality was determined by calculating the cumulative # of dead bees/# bees at study initiation × 100. The Pesticide Research Manager Software , Version 5 was used con conduct a *t*-Test.

Results:

In the arsenic treated reference group, there was 60% mortality on Day 1, 90% mortality on Day 2, and 100% mortality on Day 3 indicating that bees were consuming the diet. There was 100% mortality of the bees in the water only group suggested bees require a dietary component to survive. In the control group, there was 20% mortality on Day 4 and 40% mortality on Day 11 when the test was terminated. The test was continued beyond 20% mortality in the control for a more comprehensive comparison between treated and control groups.

The dose of the Cry3Bb1 protein was confirmed to be bioactive toward susceptible insects. There was no statistically significant difference (p=0.05) between mortality observed in the treated and control groups on a daily basis. There was also no abnormal behavior of bees observed in the treated and control groups.

167

Table 1. Cumulative mortality from Day 0 (test initiation) to Day 11 (test termination) for the treated and control groups

Treatment	Day											
	0	1	2	3	4	5	6	7	8	9	10	11
Control	0	3.3	6.5	8.3	9.3	9.8	11.0	11.8	14.3	16.3	19.5	19.5
Cry 3Bb1	0.3	3.5	6.0	8.8	10.8	11.5	12.5	14.0	14.5	15.3	16.0	17.3

Table2. Cumulative percent mortality from Day 1 to Day 11 (test termination) for the treated and control groups

Treatment	Day										
	1	2	3	4	5	6	7	8	9	10	11
Control	6.92	14.07	18.08	20.26	21.38	24.24	25.9	31.67	36.37	43.8	43.8
Cry 3Bb1	8.22	14.08	20.50	25.21	26.95	29.29	32.79	33.96	35.7	37.46	40.37

Conclusions:
The study authors concluded that 360 µg/mL Cry3Bb1 protein did not affect survival or behavior of adult honey bees. The 360 µg/mL test concentration is 20X the concentration found in pollen. Therefore, there will be minimal risk from the Cry3Bb1 protein to honey bees.

Reviewer's Comments:
This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not effect the integrity of the test. This test was conducted according to the Microbial Pesticide Test Guidelines OPPTS 885.4340 Nontarget Insect Testing, Tier I and OPPTS 885.4380 Honey Bee Testing, Tier I. According to the guidelines, nontarget insects should be tested with does of 10-100X the field dosage. This test was conducted at an acceptable level 20X the concentration in pollen or 360µg/mL Cry3Bb1 protein. Since potential exposure of honey bees to Cry3Bb1 will be from pollen, this test was conducted at an appropriate maximum hazard dose.

It can be concluded from this test that the NOEC of the Cry3Bb1 protein for adult honey bees is >360µg/mL.

168

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Robyn Rose Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Zigfridas Vaituzis 5/16/ Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target effects of Bt Protein 11098 on Collembola
MRID NO.:	449043-17
STUDY NO.:	SB-98-296
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	Springborn Laboratories, Inc., 790 Main Street, Wareham, MA 02571-1075.
TEST MATERIAL:	*Bacillus thuringiensis* Protein 11098
AUTHOR:	Debra Teixeira
STUDY COMPLETED:	August 13, 1999
CLASSIFICATION:	**Acceptable**

Study Summary

Title: "Assessment of Chronic Toxicity of Corn Tissue Containing the *Bacillus thuringiensis* Protein 11098 to Collembola (*Folsomia candida*)"

Author: Debra Teixeira

Objective: This study was conducted to evaluate the toxicity of *Bacillus thuringiensis* Cry3Bb1 (analogous to Protein 11098) administered in diet to Collembola (*Folsomia candida*).

Methods:

Protocols described in Springborn Protocol # 110398/OECD/123/Monsanto were followed for this study. Treatments consisted of 0.50, 5.0, and 50% Bt corn leaf tissue or non-transgenic corn tissue combined with yeast. Lyophilized leaf tissue was used rather than purified protein because initial results indicated that the buffer salt was toxic to Collembola. The Lyophilized corn tissue expressed the Cry3Bb1 protein from the MON 859 line. The concentration of Cry3Bb1 protein in leaf tissue was determined by ELISA to be 1,745 µg Cry3Bb1 protein/g leaf dry weight. The control groups (non-transgenic lyophilized corn tissue) was from the MON 846 corn line which has a similar genetic background to the MON 859 line. MON 859 and MON 846 corn tissue were kept in a freezer at -80°C prior to testing.

Prior to testing, Collembola (*Folsomia candida*) were placed in 4 oz glass jars with approximately 1 cm of a 8:1 plaster:coal breeding substrate and kept in an incubator set at 20 to 22°C and 70 to 100% relative humidity. Collembola were 10 days old at test initiation. There were 10 Collembola added to each test jar for a total of 40 Collembola per treatment. These

organisms were allowed *ad libitum* access to dry granulated yeast once or twice weekly. Temperature and humidity were similar during testing plus the photoperiod was set at 16 hours of light 8 hours of dark.

Treated Collembola were fed approximately 2 mg of diet every other day which allowed for an excess of food. Diets consisted of transgenic corn leaf tissue containing Cry3Bb1 protein mixed with dry granulated Brewer's yeast. Diets contained a ratio of 0.50, 5.0 and 50% corn leaf tissue in Brewer's yeast which was equivalent to 8.73, 87.3 and 872.5 µg corn leaf tissue per gram diet respectively (Table 1). Corn leaf tissue dilutions were 1,745 µg Cry3Bb1 protein/g dried leaf tissue. Bioactivity of the Cry3Bb1 protein was determined at test initiation by conducting a 28-day bioassay with the Colorado potato beetle (*Leptinotarsa decemlineata*), a sensitive species, at test concentrations.

Table 1. Concentration of corn leaf tissue in diet

Bt or Non-Bt Corn Leaf Tissue	Brewer's Yeast (g)	% Concentration of Leaf Tissue
0.50 g	0.50	50
0.10 g of 50%	0.90	5.0
0.010 g of 50%	.99	0.5

A control consisting of Brewer's yeast and non-transgenic corn leaf tissue at ratios of 0.50, 5.0 and 50% was conducted as well as a control using only Brewer's yeast. An additional reference test was conducted with thiodicarb (1.0, 10, and 100 mg a.i./kg) to demonstrate that the study design is able to detect toxic effects.

From test initiation to test termination on Day 28, observations of mortality and sublethal effects such as lethargic behavior were recorded. Adult and young Collembola (e.g., offspring) were removed and counted at test termination. Collembolans reproductive performance was compared between treated and control groups by conducting a Students t-Test. There was not statistical analysis performed on mortality rates since there was ≤5% mortality of Collembola during the study.

Results:

Table 2. Bioactivity of the test substance was verified by the Colorado potato beetle (CPB) bioassay. Results of the (CPB) bioassay

Test Substance	Week	Day	Concentration (ppm)	Initial/Survivors	Weight (mg)
Control	1	0	0	16/16	131.9
50% TS (872.5 ppm)	1	0	1	16/7	22.2
50% TS (872.5 ppm)	1	0	5	16/1	0.7
Control	4	1	0	16/15	110.4

171

50% TS (872.5 ppm)	4	1	1	16/12	44.9
50% TS (872.5 ppm)	4	1	5	16/1	0.7

TS= test substance

It was verified that Collembola were ingesting the corn leaf tissue in diet on Day 6 when organisms fed 50% control test substance levels were observed to have green digestive tracts. In the reference test, there was 5%, 17%, and 57% mortality of Collembola in the 1, 10 and 100 mg respectively demonstrating that Collembola would be killed if ingesting a toxic substance (e.g., thiodicarb) in diet. There was no significant difference in the mortality rate in the treated and control groups. There was also no significant difference in the cumulative number of offspring produced in the treated and control groups

Table 3. Average % survival and average cumulative number of offspring produced.

Nominal Diet Concentration (% as corn leaf tissue)	Percent Survival	Cumulative # of Offspring Produced
Assay Control	98	191
0.5% Control	98	207
5.0% Control	95	237
50% Control	95	226
0.5% Cry3Bb1	100	20
5.0% Cry3Bb1	98	185
50% Cry3Bb1	100	228

These results show a NOEC of Cry3Bb1 protein in diet for Collembola to be > 872.5 µg/g diet.

Conclusions:
Results of this study demonstrated that diet containing 50% corn leaf tissue expressing the Cry3Bb1 Bt protein or 872.5µg did not adversely affect survival or reproduction of Collembola. Therefore, it can be concluded that the NOEC of Cry3Bb1 protein in diet for Collembola is >872.5µg/g diet.

Reviewer's Comments:
This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not effect the integrity of the test. There are no EPA guidelines developed for Collembola testing. This test was conducted at concentration levels much greater than Collembola are expected to be exposed to in the field. MON 859 is expressed in corn leaves in the range of 323-451 µg/g as compared to MON 863 which expresses 30-93 µg/g. The primary route Collembola would be exposed to Cry3Bb1 in the field is from decaying root tissue

(and possibly from pollen to a much lesser degree). MON 863 is expressed in corn roots in the range of 3-66 µg/g which is significantly lower than the levels used in this test.

It can be concluded from this test that the NOEC of the Cry3Bb1 protein for Collembola is >872.5µg/g diet.

172

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Robyn Rose Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Zigfridas Vaituzis Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target effects of Cry3Bb1 Bt pollen on lady beetle larvae
MRID NO.:	455382-04
STUDY NO.:	00-01-39-26
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	Ecological Technology Center, 800 North Lindbergh, St. Louis, MO 63141
TEST MATERIAL:	Pollen expressing *Bacillus thuringiensis* Cry3Bb1 Protein
AUTHOR:	Jian J. Duan
STUDY COMPLETED:	March 1, 2001
CLASSIFICATION:	Acceptable

Study Summary

Title: "Dietary Effects of Transgenic *Bacillus thuringiensis* (Bt) Corn Pollen Expressing a Variant of Cry3Bb1 Protein on Larvae of the Ladybird Beetle, *Coleomegilla maculata*"

Author: Jian J. Duan

Objective: This study was conducted to evaluate the affect of corn pollen expressing Cry3Bb1 protein on survival and development of lady beetle larvae (*Coleomegilla maculata*).

Methods:

Since corn pollen may comprise up to 50% of lady beetle larvae's diet, the effects of corn pollen containing event MON 863 which encodes a variant of the Cry3Bb1 protein on lady beetle larvae (*Coleomegilla maculata*) was evaluated. ELISA has shown there is 93µg/g fresh weight MON 863 in corn pollen. The corn pollen used in this study was stored in the freezer at -80°C for nine months and then an ELISA was conducted that showed approximately 101 µg MON 863/g fresh weight pollen. The transgenic corn pollen was compared to non-transgenic pollen from the corn line MON 864 and a reference substance containing potassium arsenate (positive control). Pollen was fed to lady beetle larvae in a diet consisting of equal amounts of lyophilized tephritid fruit fly eggs and bee pollen. Diets contained 50% pollen since this is the potential level of field exposure and an equal amount of the tephritid fruit fly diet. Diets were thoroughly mixed in a centrifuge tube by rolling contents until they appeared uniform. Between uses diets were stored at -20°C and stability of the Cry3Bb1 protein was determined at test termination.

Three ~6 mg subsamples of diets were taken and the number of pollen grains were counted at the

end of the experiment to verify test and control concentrations. Pollen grains were counted from a 5 μL subsample of 1 mg of diet samples suspended in 0.5 mL of 0.2% agar solution. The total number of pollen grains was estimated from 15 subsamples of the 5 μL diet-agar suspension. An additional evaluation of the number of pollen grains in 1 mg diet/1 mL agar was conducted to determine if the total number of pollen grains in the test and control substances were at expected levels. An ELISA was also conducted to verify the presence of Cry3Bb1 protein in the diet mixes.

First instar lady beetle larvae were individually placed in test arenas to avoid cannibalism. Beetle larvae were given diet mix containing either test pollen, control pollen, reference pollen (potassium arsenate), or bee pollen. Larvae were fed daily and a water moistened sponge was presented as needed on a microscope slide. Diet and water were never depleted during the test. Arenas consisted of 10 cm in diameter and 1.5 cm deep polystyrene petri dishes with a ventilated lid and filter paper on the bottom. Each assay consisted of 10 larvae per treatment replicated 3 times for a total of 30 larvae per treatment. Test arenas were kept in incubators during the test set at ~27°C and a photoperiod of 14 hours of light and 10 hours of dark. Larvae were kept in the arenas until they died or pupated.

Observations were made daily for developmental stage or mortality and newly emerged adults were weighed. An LSD (least significant difference) was determined from a PROC ANOVA conducted by SAS to compare survival rates among the different treatments. Since all pupae developed adults, the survival rate for pupae and adults were identical. An LSD was determined from PROC MIXED conducted by SAS to compare developmental time and weight among the different treatments. There was 100% morality in the reference group so this group was not included in the developmental time or adult weight analysis.

Results:

ELISA of the Bt corn pollen determined that the level of variant Cry3Bb1 protein was approximately 135 μg/g fresh weight pollen in the test pollen stored at -20°C for 35 days. This verified that there was no reduction in the level of variant Cry3Bb1 protein in test pollen which was previously estimated at 93-101μg/g fresh weight pollen. There was no Bt detected in the control pollen. The number of pollen grains in treated and control groups were similar. Pollen grain counts resulted in 2093-2987 grains/mg diet mix for Bt pollen and 2773-2920 grains/mg diet mix for non-transgenic pollen. The number of pollen grains counted in the pure test and control samples were 3907 and 3387 grains/mg test and control pollen samples respectively. Pollen grain levels in test and control diet mixes were within 54-76% and 82-86% of the expected ranges respectively.

There was not a statistically significant difference between survival of lady beetle larvae fed bee pollen or corn pollen nor was there a difference in survival between larvae fed Bt and non-Bt pollen. There was a significant difference between the reference group (potassium arsenate) and other test groups since no larvae survived in the reference group. The 100% mortality observed in the reference group verified that the lady beetles were ingesting the diet (Table 1).

Table 1. Lady beetle larval survival to pupation and/or adult emergence

Treatment	% Survival
test pollen (MON 863)	96.7
control pollen (MON 864)	90
bee pollen (assay control)	93.3
reference pollen (potassium arsenate)	0

There was also no significant difference between the bee pollen and non-Bt and Bt pollen in developmental time of larvae to pupae and/or adults; nor was there a difference in adult weight (Table 2).

Table 2. Survival and development of lady beetle larvae to pupae and adults.

Treatment	# Larvae Surviving to Pupae &/or Adults	Avg Days to Pupation ± SE	Avg Days to Adult ± SE	Adult Weight ± SE (mg)
test pollen (MON 863)	29	12.7 ± 0.54	16.8 ± 0.55	10.9 ± 0.39
control pollen (MON 864)	27	12.9 ± 0.56	16.9 ± 0.57	10.7 ± 0.40
bee pollen (assay control)	28	13.8 ± 0.55	17.8 ± 0.56	10.4 ± 0.39

Conclusions:
Development and survival of *C. maculata* were not affected by feeding on pollen containing the variant Cry3Bb1 protein at the level of ~93 µg/g fresh pollen weight.

Reviewer's Comments:
This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160 with certain exceptions that did not effect the integrity of the test. This study was conducted at EPA's request. This test was conducted with pollen levels greater than or equal to levels lady beetle larvae are expected to be exposed to in the field. Therefore, the NOEC for Cry3Bb1 expressed in pollen is >93 µg/g fresh pollen weight. It can be concluded from this study that lady beetle larvae will not be adversely affected by Cry3Bb1 field corn.

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Robyn Rose Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Zigfridas Vaituzis 5/14/0 Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target effects of Cry3Bb1 on monarch butterfly larvae
MRID NO.:	455382-05
STUDY NO.:	01-01-39-26
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	Department of Environmental Biology, University of Guelph, Guelph, Ontario, NIG 2W1, Canada
TEST MATERIAL:	Pollen expressing *Bacillus thuringiensis* Cry3Bb1 Protein
AUTHOR:	Mark Sears, PhD and Heather Mattila
STUDY COMPLETED:	July 27, 2001
CLASSIFICATION:	**Acceptable**

Study Summary

Title: "Determination of the Toxicity of Corn Pollen Expressing a Cry3Bb1 Variant Protein to First Instar Monarch Butterfly Larvae (*Danaus plexippus*) via Laboratory Bioassay"

Authors: Mark Sears and Heather Matilla

Objective: This study was conducted to evaluate the affect of corn pollen expressing a Cry3Bb1 variant protein on survival and development of the monarch butterfly.

Methods:

This study was conducted to evaluate the lethal and sublethal effects of Bt corn pollen event MON 863 expressing Cry3Bb1.11098 (referred to as Cry3Bb1) protein on young monarch butterfly larvae (*Danaus plexippus*). Effects of corn pollen expressing the *cry3Bb1* gene was compared to non-Bt corn pollen from the MON 846 corn line. MON 863 and MON 846 identities were confirmed at the molecular level by Monsanto. An ELISA showed that there was 89.2 µg Bt/g pollen for MON 863 and no detectable Bt (<0.55 µg/g pollen) in the MON 846 corn pollen. Pollen was shipped from Monsanto to the University of Guelph and stored at -80°C until it was needed for the study.

Prior to testing, pollen was throughly sifted through a 150 mesh (90 microns) screen and subsequently a 250 mesh (63 microns) screen to remove debris, particularly anthers which may have a higher toxin concentration than pollen. After sieving, Pollen samples were re-analyzed by Monsanto using ELISA which showed a level of Cry3Bb1 protein of 63.1 µg/g fresh weight and no detectable level of Bt in the MON 846 corn.

Common milkweed (*Asclepias syriaca*) plants were grown in the greenhouse. A breeding colony was started from adults purchased from Greathouse Butterfly Farm, Inc. (Earleton, FL) that were confirmed to be free of the parasite *Ophryocystis elektroscirrha*. Rearing included 60 adult monarchs kept in 1×1×1 m screened cages with milkweed plants changed daily for monarchs to oviposit on. Rearing cages were kept in chambers set at 25°C, 75% relative humidity and a photoperiod of 16 hours light and 8 hours of dark.

There were 4 replicates for each bioassay and 18 bioassay arenas per replicate that included a milkweed leaf and 10 neonate monarchs. Replicates were blocked by initiating each bioassay on consecutive days. The 18 bioassays included 7 with Bt pollen, 7 with non-Bt pollen and 4 with no pollen. Pollen was evenly spread on milkweeds by an air pressurized (20 psi) Venturi-shaped tube. Even distribution of pollen was visually verified under a dissecting microscope and the number of grains in a 1 cm^2 area on each side of the upper leaf surface were counted. Leaves with pollen densities 10% above or below the desired testing level were discarded. Doses of the 4 replicated were determined to be 55.7±2.1, 107.9±3.6, 199.1±5.5, 423.4±11.3, 847.4±21.2, 1652.3±48.1 and 3279.0±60.5 grains/cm^2.

Neonate larvae (1st instars <24 hrs old) were used in this bioassay. Bt and non-Bt pollen was applied to milkweed leaves in the laboratory at rates of 50, 100, 200, 400, 800, 1600 and 3200 grains/cm^2. A maximum of 3200 pollen grains/cm^2 was chosen because previous studies conducted by this laboratory demonstrated that high levels of pollen deterred monarch feeding on milkweed leaves leading to artificially inflated mortality. Larvae were weighed prior to placing them on milkweed leaves kept in ventilated plastic arenas placed in a growth room set at 20°C, 50% relative humidity, and 16 hours of light and 8 hours of dark. Larvae were allowed to feed on pollen dusted milkweed leaves for 4 days and then moved to clean leaves for an additional 6 days. Observations of larval survival and development were evaluated 48 hours, 96 hours and 10 days after test initiation. Larval weights were determined on Days 0, 4 and 10 to determine weight gain in mg/larva by calculating [($weight_{timeX}$/larva) - ($weight_{time0}$/larva)]. Leaf consumption was also evaluated 48 and 96 hours after test initiation and reported in cm^2/larva.

Percent survival, development, weight gain and leaf consumption were statistically analyzed (SAS) as a three-factor design (pollen type, dose and time) with a repeated measure on the last factor. A two-way ANOVA (pollen type and dose) was conducted from daily sampling data when the repeated measure model could not be fitted due to significant time interactions. The survival data was log transformed to equalize variance and significant differences (p=.05) were determined with Tukey's test.

Results:

Overall larval survival decreased over time; however, there was no statistical difference in survival of larvae feeding on the Bt or non-Bt pollen at the different doses. This implies that the reduction in larval survival was not due to feeding on Bt. On average, there was 95 to 100% larval survival after 48 and 96 hours of feeding on the milkweed leaves and 87.5 to 100% survival after 10 days (Table 1). Ten days after testing initiation, 71% of the arenas containing

10 larvae had 100% survival.

Table 1. Mean percent survival of larval cohorts ± S.E. after 48 hours, 96 hours and 10 days of exposure to Bt and non-Bt pollen

Pollen Type	Pollen Density (grains/cm²)	48 hour Post-Treatment	96 hour Post-Treatment	10 Days Post-Treatment
Control	0	99.4 ± 0.6 %	98.8 ± 0.9 %	96.3 ± 1.8 %
Bt	50	100 ± 0 %	100 ± 0 %	100 ± 0 %
Non-Bt	50	100 ± 0 %	100 ± 0 %	100 ± 0 %
Bt	100	95 ± 2.9 %	95.5 ± 2.9	95 ± 2.9 %
Non-Bt	100	97.5 ± 2.5 %	95.5 ± 2.9	95 ± 2.9 %
Bt	200	100 ± 0 %	100 ± 0 %	97.5 ± 2.5 %
Non-Bt	200	100 ± 0 %	97.5 ± 2.5 %	92.5 ± 2.5 %
Bt	400	100 ± 0 %	100 ± 0 %	100 ± 0 %
Non-Bt	400	97.5 ± 2.5%	100 ± 0 %	100 ± 0 %
Bt	800	97.5 ± 2.5 %	97.5 ± 2.5 %	95 ± 5 %
Non-Bt	800	97.5 ± 2.5 %	97.5 ± 2.5 %	95 ± 2.9 %
Bt	1600	100 ± 0 %	100 ± 0 %	100 ± 0 %
Non-Bt	1600	100 ± 0 %	100 ± 0 %	92.5 ± 4.8 %
Bt	3200	97.5 ± 2.5 %	95 ± 2.8 %	92.5 ± 4.8 %
Non-Bt	3200	100 ± 0 %	95 ± 5 %	87.5 ± 7.5 %

Mean development of larvae were effected by time; however there was no statistical difference between larvae feeding on Bt and non-Bt pollen. After ten days, average larval instar stage ranged from 1.28 to 1.55, 1.98 to 2.26 and 4.31 to 4.68 at 48 hours, 96 hours and 10 days respectively after test initiation (Table 2).

Table 2. Mean larval development of <24 hour old 1st instar larvae ± S.E. after 48 hours, 96 hours and 10 days of exposure to Bt and non-Bt pollen

Pollen Type	Pollen Density (grains/cm²)	Mean Larval Instar Stage 48 hour Post-Treatment	Mean Larval Instar Stage 96 hour Post-Treatment	Mean Larval Instar Stage 10 days Post-Treatment
Control	0	1.34 ± 0.6	2.12 ± .04	4.53 ± .07
Bt	50	1.55 ± .09	2.00 ± .04	4.53 ± .21
Non-Bt	50	1.28 ± .06	2.10 ± .07	4.40 ± .12

178

Table 2. Continued

Pollen Type	Pollen Density (grains/cm²)	Mean Larval Instar Stage 48 hour Post-Treatment	Mean Larval Instar Stage 96 hour Post-Treatment	Mean Larval Instar Stage 10 days Post-Treatment
Bt	100	1.39 ± .11	2.05 ± .05	4.31± .18
Non-Bt	100	1.31 ± .10	2.05 ± .05	4.50 ± .16
Bt	200	1.45 ± .06	2.08 ± .05	4.60 ± .24
Non-Bt	200	1.30 ± .06	2.05 ± .05	4.38 ± .13
Bt	400	1.43 ± .01	2.18 ± .09	4.68 ± .20
Non-Bt	400	1.40 ± 0.17	2.23 ± .19	4.50 ± .22
Bt	800	1.44 ± .06	2.05 ± .05	4.49 ± .17
Non-Bt	800	1.37 ± 0.13	2.26 ± 0.12	4.66 ± .14
Bt	1600	1.33 ± .06	2.13 ± .10	4.35 ± .13
Non-Bt	1600	1.35 ± 0.19	2.03 ± .10	4.44 ± .23
Bt	3200	1.41 ± 0.11	1.98 ± .05	4.44 ± .16
Non-Bt	3200	1.35 ± 0.17	2.03 ± .03	4.40 ± .23

Although larval weight gain significantly changed over time, there was no statistical difference in larval weight gain or pollen consumption between Bt and non-Bt pollen 96 hours and 10 days after test initiation (Table 3). Additional analysis showed no statistical difference in the pollen and dose interaction.

Table 3. Mean larval weight gain ± S.E. (mg/larva) of <24 hour old 1st instar larvae after 96 hours and 10 days of exposure to Bt and non-Bt pollen

Pollen Type	Pollen Density (grains/cm²)	Mean Larval Instar Stage 96 hour Post-Treatment	Mean Larval Instar Stage 10 Days Post-Treatment
Control	0	9.15 ± 0.62	321.07 ± 13.08
Bt	50	9.40 ± 1.07	341.16 ± 26.76
Non-Bt	50	7.94 ± 1.26	306.67 ± 23.80
Bt	100	7.51 ± 0.34	333.88 ± 36.21
Non-Bt	100	7.88 ± 0.90	294.12 ± 25.00
Bt	200	8.58 ± 1.56	351.12 ± 53.52
Non-Bt	200	6.92 ± 1.24	295.47 ± 42.16
Bt	400	9.65 ± 1.15	307.23 ± 30.04
Non-Bt	400	8.37 ± 1.20	349.21 ± 69.60

Table 3. Continued

Pollen Type	Pollen Density (grains/cm^2)	Mean Larval Instar Stage 96 hour Post-Treatment	Mean Larval Instar Stage 10 Days Post-Treatment
Bt	800	9.28 ± 0.64	330.97 ± 18.98
Non-Bt	800	8.17 ± 1.66	410.94 ± 69.89
Bt	1600	8.56 ± 1.17	319.40 ± 29.21
Non-Bt	1600	7.64 ± 1.68	274.78 ± 32.73
Bt	3200	7.44 ± 0.64	289.35 ± 31.74
Non-Bt	3200	7.03 ± 1.88	305.12 ± 31.66

There was not a statistically significant difference in larval milkweed leaf consumption between Bt and non-Bt treatments. However, there was a downward trend in consumption of both Bt and non-Bt pollen as the dose increased (Table 4). This trend was not statistically significant. In general, results of this study showed that monarch larvae consumed more leaf tissue dusted with Bt pollen than non-Bt pollen.

Table 4. Mean leaf consumption ± S.E. (cm^2/larva) after 96 hours and 10 days of exposure to Bt and non-Bt pollen

Pollen Type	Pollen Density (grains/cm^2)	96 hour Post-Treatment	10 Days Post-Treatment
Control	0	0.37 ± 0.02	1.26 ± 0.07
Bt	50	0.40 ± 0.04	1.37 ± 0.21
Non-Bt	50	0.40 ± 0.04	1.24 ± 0.12
Bt	100	0.37 ± 0.02	1.40 ± 0.13
Non-Bt	100	0.41 ± 0.06	1.11 ± 0.12
Bt	200	0.35 ± 0.03	1.29 ± 0.13
Non-Bt	200	0.31 ± 0.03	1.17 ± 0.20
Bt	400	0.34 ± 0.03	1.27 ± 0.05
Non-Bt	400	0.37 ± 0.02	1.28 ± 0.11
Bt	800	0.35 ± 0.03	1.37 ± 0.13
Non-Bt	800	0.29 ± 0.04	1.13 ± 0.23
Bt	1600	0.32 ± 0.02	1.31 ± 0.15
Non-Bt	1600	0.29 ± 0.04	1.04 ± 0.22
Bt	3200	0.32 ± 0.02	1.11 ± 0.08
Non-Bt	3200	0.30 ± 0.02	0.84 ± 0.07

180

Conclusions:
Neonate monarch survival was not affected after feeding on milkweed dusted with corn pollen expressing Cry3Bb1 event MON 863 for 2, 4 or 10 days of pollen exposure. Larval weight gain and milkweed leaf consumption were also not affected by feeding on Bt pollen 96 hours and 10 days after exposure. In fact, there was an increase in leaf consumption 96 hours after test initiation in the Bt group. In addition, increasing doses of corn pollen (Bt or non-Bt) did not effect larval survival, development, or weight gain during the 10-day bioassay. The only effect seen was an increase in leaf consumption 48 hours after test initiation in both the Bt and non-Bt groups.

Pollen densities in the field are not expected to be as great as 1600 or 3200 grains/cm^2. Levels of 400 and 800 pollen grains/cm^2 would probably be rare. Therefore, results of this study indicate that young monarch larva (the most sensitive stage) will not be adversely affected by exposure to Bt corn pollen expressing Cry3Bb1 in the field.

Reviewer's Comments:
Although this study was not conducted in accordance with FIFRA GLP compliance as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160, a scientifically valid study was performed by a university laboratory and is considered acceptable. This study was not required nor requested to register Cry3Bb1 because it is a coleopteran active protein that is not expected to affect lepidopterans such as the monarch butterfly. In addition, extensive research conducted on the potential affects of monarch larvae feeding on Bt corn pollen has shown a lack of concern of acute toxicity. However, due to recent public concern of possible adverse effects of Bt corn on monarchs, Monsanto sponsored this study and submitted it to the Agency for review. This study has demonstrated that corn pollen expressing the Cry3Bb1 protein will not result in acute toxic or developmental effects to monarch larvae.

181

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Robyn Rose Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Zigfridas Vaituzis 5/16 Biopesticides and Pollution Prevention Division
STUDY TYPE:	Field evaluation of exposure to Cry3Bb1 protein effects on non-target organisms
MRID NO.:	455382-06
STUDY NO.:	01-01-39-16
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	Monsanto Agronomy Center, Monmouth, IL 61462, Monsanto Company, 800 N Lindbergh Blvd., St. Louis, MO 63141
TEST MATERIAL:	*Bacillus thuringiensis* Event MON 863 Cry3Bb1 Protein
AUTHORS:	Muhammad A. Bhatti, Carol L. Pilcher, Michael J. McKee, Thomas E. Nickson, Graham P. Head and Clinton D. Pilcher
STUDY COMPLETED:	July 19, 2001
CLASSIFICATION:	**Supplemental to submitting the full report after completion of this study.**

Study Summary

Title: "Field Evaluation of the Ecological Impact of Corn Rootworm Insect-Protected Corn on Non-Target Organisms"

Authors: Muhammad A. Bhatti, Carol L. Pilcher, Michael J. McKee, Thomas E. Nickson, Graham P. Head and Clinton D. Pilcher

Objective: This study was conducted to evaluate the ecological impact of MON 863 Bt corn grown under different insecticide regimes on abundance of non-target organisms relative to non-transgenic corn.

Methods:

Transgenic Bt corn and non-transgenic corn were monitored for two years (2000-2001) for the stability and abundance of non-target organism communities in soil, soil surface and foliage including arthropods, earthworms and soil microbes as well as plant diseases. Additional data on soil fertility, microbial populations and soil Bt protein was collected during this study but not reported at this time. Cry3Bb1 corn rootworm (*Diabrotica* spp.; CRW) protected corn event MON 863 (RX670 pedigree) was compared to a non-transgenic control hybrid (RX670) with a similar genetic background.

All experimental plots were managed according to typical cultural practices of commercially

grown corn in the region and included the application of the herbicides acetochlor and atrazine after planting and before emergence. Bt and control hybrids were planted in a split-plot design with four replications planted 20 ft apart. Rows were planted 30 in apart, seeded at a rate of approximately 1.7 seeds/ft and planted 1.5 - 1.75 inches deep. Plots (240 ft × 60 ft) were divided into 24 row subplots (60 ft × 60 ft) that served as replications receiving one of 4 insecticide regimes. Insecticide treatments of the Bt and non-Bt plots included: 1. No insecticide; 2. Seed treated with Gaucho prior to planting; 3. The granular insecticide Force 3G applied and incorporated in furrows at planting; and 4. A foliar insecticide, Pounce 3.2 EC, applied at the V10 and R2 corn growth stages to control 1st and 2nd generation CRW adults. A 4-row buffer of non-transgenic corn was planted around each plot to minimize edge effects from adjacent subplots.

Invertebrates were sampled from the soil, soil surface and foliage. Soil-dwelling invertebrates were collected using a "pan trap" which utilized a modified Burlese extraction method. At the V-6, V-10 and R-1 corn growth stages, 3 root balls including an 8 in diameter of root mass and soil were randomly selected from rows 8, 12 and 16 of each subplot. Root balls were transported to the greenhouse in an onion sack where they were suspended 2 in above a 10 in pan containing 500 mL of a 50:50 alcohol: water solution and a small amount of antifreeze to delay alcohol evaporation. Root balls were kept in the greenhouse with the cooling system off to increase invertebrate extraction for 7 days. After samples were sifted through an 80-mesh screen, they were placed in the refrigerator for later counting identification to the family level.

Surface-dwelling invertebrate were sampled in the field with pitfall traps. Four plastic cups (474 mL, 110 mm rim diameter) were placed rows 9 and 16 of each subplot to trap invertebrates. Cups were set inside larger plastic cups (947 mL, 110 mm rim diameter) that stayed in the field and were protected from rain by a 20 cm × 20 cm plastic plate. Invertebrates were trapped and preserved in 100 mL ethylene glycol for 3 day periods during the V-6 to R-4 growth stages. Samples were stored in ethylene glycol in the refrigerator until they were counted and identified to the family level.

Foliage-dwelling invertebrate were monitored by yellow sticky traps (Pherocon AM™) set in the field at canopy level and adjusted as the season progressed. Three traps were placed 15 ft apart in rows 6, 12 and 18 of each subplot from the V-6 to R-4 corn growth stages and switched at approximately 7 day intervals. Sticky traps were stored in the refrigerator until organisms were counted and identified to family and the genus and species level when possible.

Amendments to this study written during the 2000 season included sampling for lady beetles using a drop cloth technique. Three deviations were also noted in the methods of this study. 1) There were deviations in sampling time during the season due to physical constraints. 2) Two soil core samples were collected rather than 4. 3) Insect thresholds were not determined nor was equipment calibrated within 24 hours of insecticide applications.

Data was analyzed by SAS using a mixed linear model (PROC MIXED) to compare transgenic

and non-transgenic hybrids among the 4 insecticide treatments, their interactions and effect of sampling time. Since the number of insects captured depended on the area of the trap, square root transformations to adjust for normality were done prior to data analysis. Hybrid effects were determined from main plot residual variation, insecticide treatment effects and their interaction with hybrids was determined from the subplot residual variation and effects of sample date were evaluated from the residual variation.

Results:
Interim results are reported at this time and a full report will be submitted to the Agency for review when all data is collected and analyzed. Replications of each treatment summed across sampling dates are reported as well as the mean and standard error of the most abundant species collected.

The 7 most abundant species counted and identified in the pan samples were: Araneae (spiders), Carabidae (immature and adult ground beetles), Chilopda (centipedes), Staphylinidae (rove beetles), Japygidae (diplurans) and Oligochaeta (earthworms). Preliminary results show no statistically significant difference between the Bt and non-Bt hybrids in the number of soil-dwelling organisms collected from pan samples. There was also no significant effect on abundance of organisms from the insecticide treatments and their interaction with hybrids. The number of carabid larvae collected in the pan samples varied among Bt and non-Bt plots treated with different insecticides. For example, the abundance of Aranaea were less in the non-Bt plots treated with soil insecticide and the number of Oligochaeta and Japygidae were very variable but with no trends observed. On the other hand, the number Chilopods and Staphaylinds were somewhat consistent among insecticide treatments. Since the number of pest species (e.g., scarab beetles, sap beetles, wireworms/click beetles and immature CRW) in the pan samples were low, no comparison between treatments was made.

The most abundant surface-dwelling species collected in pitfall traps were Araneae (spiders), Carabidae (immature and adult ground beetles), Gryllidae (crickets), Cicindellidae (tiger beetles), Chilopoda (centipedes), Diplopoda (millipedes), Formicidae (ants), Staphylinidae (rove beetles) and Silphidae (carrion beetles). Of these organisms, spiders, Carabids, and crickets were the predominant species sampled respectively and were not significantly different between the two hybrids. There was also no interaction effect of insecticide and hybrid found for any of the species evaluated. Insecticide treatments did not significantly effect the number of Carabids or crickets but did effect spider abundance. There were 30% fewer spiders found in soil treated plots and 50% fewer in foliar treated plots then the seed and no insecticide treated plots. Effects of individual insecticides will be evaluated and presented in the final report.

In addition to non-pest species, the abundance of pest insects including Nititulidae (sap beetles), Scarabaeidae (scarab beetles), Chrysomelidae (corn flea beetles) and a minimal number of Elateridae (click beetles) was determined in the pitfall traps. There was no differences in the number of pest insects between hybrids but there were differences between insecticide treatments. There was a large number of sap beetles collected and abundance was highly

584

variable in all treatments. Scarab beetle abundance was consistent among plots except for foliar treatments which resulted in fewer beetles. Soil and foliar insecticides resulted in fewer corn flea beetles.

The 5 most abundant pest species captured on sticky traps were *Diabrotica barberi* (northern corn rootworm), *Diabrotica virgifera* (western corn rootworm), *Chaetocnema pulicaria* (corn flea beetle), Nititulidae (sap beetle) and *Rhopalosiphium maidis* (corn leaf aphids). The predominant foliage-dwelling beneficial insects collected on sticky traps were lady beetle species including *Harmonia axyridis* (Asian lady beetle), *Coccinella septempunctata* (7-spotted lady beetle), *Hippodamia convergens* (convergent lady beetle) and *Cycloneda munda* (lady beetle). Other non-target beneficial invertebrate found on sticky traps were spiders, parasitic Hymenoptera, Syrphidae (syrphid flies), *Chrysoperla carnea* (green lacewing), Hemerobiidae (brown lacewings), Carabidae (ground beetles), Fomicidae (ants) and Nabidae (damsel bugs).

The key natural enemies in corn fields *Coleomegilla maculata* (12-spotted lady beetle), *Orius insidiosus* (minute pirate bug) and the parasitic Hymenoptera *Macrocentrus grandii* (Macrocentrus), were also abundant in the treated and control plots. The number of beneficial insects was not significantly different between the Bt and non-Bt plots nor was the interaction between insecticide treatment and hybrid. Although *C. maculata*, a generalist lady beetle predator that feeds on ECB eggs and larvae, abundance was not significantly effected by the insecticide treatments, there were less observed in the foliar treated plots. In general, the number of *C. maculata* increased during the growing season. The number of *M. grandii* were significantly less in plots receiving foliar insecticide treatments. This affect was probably due to the insecticide directly affecting the parasitoid or from reducing ECB populations. There were no differences between treated and control plots in the abundance of *O. insidiosis*, a generalist predator that feeds on ECB eggs and larvae. However, foliar insecticide treatments reduced the number of *O. insidiosis* by 50% and the population never fully recovered. *O. insidiosis* numbers also differed among sample dates; abundance increased from the first to the third date and dropped on the final sample date.

Conclusions:

These results are preliminary and this study will be continued for a second year. According to the pan, pitfall and sticky trap sample methods used in this study, abundance of the predominant species were not significantly different between the Bt treated and non-Bt control plots. The most abundant soil-dwelling organisms found in pan and pitfall traps were spiders and Carabids. The abundance of most important foliage-dwelling natural enemies captured on sticky traps, *C. maculata*, *O. insidiosis* and *M. grandii*, did not differ between treated and control plots. These preliminary results indicate that planting event MON 863 does not negatively impact the abundance of several beneficial non-target invertebrate. Data also indicated that planting event MON 863 results in less impact on non-target invertebrate than conventional pest management practices.

Reviewer's Comments:
Although this study was not conducted according to FIFRA GLP compliance as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160, this study was conducted according to a scientifically valid, peer reviewed protocol that is acceptable. Results reported in this submission suggest that planting event MON 863 CRW protected corn will not adversely affect non-target beneficial invertebrate abundance in the field. On the contrary, a reduction in pesticide use that may result from planting MON 863 corn will probably result in increased numbers of non-target invertebrate in CRW protected Bt corn.

This submission only reported results from the 2000 growing season. A final report will be submitted to the Agency after the 2001 data is collected and analyzed. The final report should include the location of the 2000 and 2001 test sites as well as the additional data on soil fertility, microbial populations and soil Bt protein collected during this study but not reported at this time. This study should also be conducted at additional test sites in different geographic locations to account for regional differences in insect populations.

186

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Robyn Rose Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Zigfridas Vaituzis 5/14 Biopesticides and Pollution Prevention Division
STUDY TYPE:	Endangered Species Impact Assessment
MRID NO.:	455770-03
REPORT NO.:	MSL-17614
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TESTING LAB:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TEST MATERIAL:	*Bacillus thuringiensis* Event MON 863 Cry3Bb1 Protein
AUTHORS:	Jian J. Duan, Michael J. McKee, Graham Head, Christopher R. Brown
STUDY COMPLETED:	January 4, 2002
CLASSIFICATION:	**Acceptable**

Study Summary

Title: "Endangered Species Impact Assessment for Cry3Bb1 Protein in Transgenic Corn Event MON 863"

Authors: Jian J. Duan, Michael J. McKee, Graham Head, Christopher R. Brown

Objective: This report evaluated the potential risk Cry3Bb1 corn rootworm protected corn poses to endangered species.

Endangered Species Assessment

Cry3 proteins including Cry3Bb1 are known to be highly specific against coleopteran insects and are not hazardous to vertebrate animals. It has been generally demonstrated that Cry3 proteins do not pose a risk to non-target animals or invertebrates. The Cry3Bb1 protein is specifically toxic to Chrysomelid beetles including corn rootworms (*Diabrotica* spp.) and Colorado potato beetles (*Leptinotarsa decemlineata*). Currently, there are no Chrysomelid species listed on the endangered species list and no other species are expected to be sensitive to Cry3Bb1. Therefore, no adverse affects from Cry3Bb1 event MON 863 are expected against endangered species.

Monsanto conducted a hazard assessment, exposure assessment and risk characterization to demonstrate that Cry3Bb1 does not pose a risk to endangered Coleoptera. This endangered species assessment was based on the Hazard Evaluation Division, Standard Evaluation Procedure, Ecological Risk Assessment (U.S. EPA, 1986).

Hazard Assessment: Most Susceptible Coleoptera

According to research that has been conducted with Cry3Bb1 thus far, Chrysomellids are the most sensitive species. Of species in the Chrysomellid family, the Colorado potato beetle (*Leptinotarsa decemlineata*; CPB) has been identified as the most sensitive species to the Cry3Bb1 protein. Astwood *et al.* (2001) determined the Cry3Bb1 LC_{50} for CPB to be 2.4 µg/mL of diet. Since CPB is the most sensitive species identified to the Cry3Bb1 protein, 2.4 µg/mL of diet will be used for this risk assessment (Table 1).

Table 1. Sensitivity of tested species to the Cry3Bb1 protein.

Order/Family	Genus/Species	Stage Tested	LC_{50} (µg/mL diet)	NOEC* (µg/mL diet)
Coleoptera/Chrysomelidae	*Leptinotarsa decemlineata*	larvae	2.4	-
Coleoptera/Chrysomelidae	*Diabrotica virgifera*	larvae	75	-
Coleoptera/Bruchidae	*Callosobruchus maculatus*	larvae	>200	200
Coleoptera/Coccinellidae	*Hippodamia convergens*	adults	>8000	8000
Coleoptera/Tenebrionidae	*Tribalium castaneum*	larvae	>200	200
Coleoptera/Curculionidae	*Anthonomus grandis*	larvae	>50	50
Coleoptera/Curculionidae	*Anthonomus eugenii*	larvae	>200	200
Coleoptera/Curculionidae	*Sitophilus oryzae*	larvae	>200	200
Lepidoptera/Noctuidae	*Helicoverpa zea*	larvae	>200	200
Lepidoptera/Crambidae	*Ostrinia nubilalis*	larvae	>200	200
Hymenoptera/Brachonidae	*Nasonia vitripennis*	adults	>200	400
Hymenoptera/Aphidae	*Apis mellifera*	adults	>360	360
Hymenoptera/Aphidae	*Apis mellifera*	larvae	not reported	1790
Neuroptera/Chrysopidae	*Chrysoperla carnea*	larvae	>8000	8000
Collembola	*Folsomia candida*	nymphs/adults	>870	870

* NOEC= maximum dose tested

Exposure Assessment for Endangered Coleoptera

Terrestrial and aquatic exposure were considered in this assessment since non-target coleopterans may be exposed to the Cry3Bb1 protein within corn fields or in surrounding areas from plant tissue (e.g., pollen) movement offsite. However, the distance pollen moves outside of the corn field should be considered. Pleasants *et al.* showed that <25 grains of pollen per cm^2 are expected 4-5 meters from the corn field edge. A relative comparison of surface ratio of milkweed to other substrates (e.g., other host plants, arthropod prey, animal carrion) can be used as a basis for estimating the amount of pollen that may leave the field. The maximum concentration of Cry3Bb1 protein in corn tissue has been determined to be 93 µg/g fresh weight pollen or other tissue. Based on this concentration, <0.03 µg Cry3Bb1 protein/g of diet would be

expected to be deposited 4-5 meters from the field edge.

The potential of aquatic organisms to be exposed to the Cry3Bb1 protein is minimal. Such exposure would occur from runoff of the protein (either free or sequestered in plant debris) into adjacent water bodies or pollen drift. Since movement of Cry3Bb1 in soil into water bodies is probably negligible, pollen drift was considered the primary source of potential risk to endangered aquatic Coleoptera. According to Monsanto's estimation based on studies conducted by Pleasants *et al.* (2001), if 100% of the pollen grains leaving the field were deposited in a 1 ha pond with 2 m depth and located ≥1 m from the edge of the corn field, <0.0001 µg Cry3Bb1/mL of water would be expected (Table 2).

Table 2. Pollen grain density

Distance from field edge	Pollen grains/cm^2 on surface H_2O	Total pollen grains in 1 ha surface H_2O	Cry3Bb1 protein (µg/1-ha pond)	Max. Cry3Bb1 protein in 1-ha, 2 m deep pond (µ/mL)
1	500	5.000×10^{10}	1.162500×10^{6}	5.81250×10^{-5}
2	187.5	1.875×10^{10}	4.359375×10^{5}	2.17969×10^{-5}
4-5	62.5	6.250×10^{9}	1.453125×10^{5}	7.26563×10^{-6}

Risk Characterization for Endangered Species

A 10-fold safety factor for terrestrial species and a 20-fold safety factor for aquatic species based on the LC_{50} is recommended by EPA for endangered species (U.S. EPA 1986 Hazard Evaluation Division, Standard Evaluation Procedure, Ecological Risk Assessment). Based on the 2.4 µg/mL diet LC_{50} for the CPB, the most sensitive species to Cry3Bb1 protein, a maximum exposure of 0.23 µg/g (10-fold safety margin) is expected 1 m from the field edge, 0.09 µg/g (28-fold safety margin) is expected 2 m from the field edge and 0.03 µg/g (82-fold safety margin) is expected 4-5 m from the field edge. A 1-ha pond with a 2 m depth located 1 m from the edge of the corn field that has 500 grains/cm^2 would have a maximum exposure of 5.81250×10^{-5} which represents a 41290 safety margin. These data indicate that pollen drift from MON 863 corn fields will not pose a terrestrial or aquatic exposure risk.

Conclusions

According to Monsanto's risk analysis, event MON 863 field corn expressing the Cry3Bb1 protein will not pose a risk to aquatic or terrestrial endangered species. Endangered coleopterans, the most sensitive species to Cry3Bb1 are not expected to occur in or near corn fields. If an endangered coleopteran were to occur in a corn field, exposure and consumption would be minimal because of their habits and feeding biology. Plant feeding Coleoptera from the Cerambycidae and Scarabaeidea would not occur because their host plants are not present in corn fields. The endangered Cerambycid feeds on the elderberry tree (*Sambucus* sp.) which grows in riparian forests along rivers and streams. The endangered Scarab's host plant occurs in sandy habitats where corn is not likely to be grown. Other terrestrial beetles would be minimally exposed to pollen from food items such as prey and carrion.

189

Reviewer's Comments

Based on a 10-fold safety factor for terrestrial species and a 20-fold safety factor for aquatic species and a lack of exposure, no unreasonable adverse effects of MON 863 to endangered Coleoptera are expected. According to Monsanto's submission, the LC_{50} for the CPB, the most sensitive species to Cry3Bb1 protein, is 2.4 µg/mL diet. A maximum exposure of 0.23 µg/g (10-fold safety margin) is expected 1 m from the field edge, 0.09 µg/g (28-fold safety margin) is expected 2 m from the field edge and 0.03 µg/g (82-fold safety margin) is expected 4-5 m from the field edge. A 1-ha pond with a 2 m depth located 1 m from the edge of the corn field that has 500 grains/cm^2 would have a maximum exposure of 5.81250×10^{-5} which represents a 41290 safety margin. These data indicate that pollen drift from MON 863 corn fields will not pose a terrestrial or aquatic exposure risk.

Many of the endangered and threatened beetles occur in cave or aquatic habitats. None of the endangered beetles are expected to occur in or near corn fields (Table 3). The American burying beetle may occur in old fields or cropland hedge rows. However, based upon the feeding habits of the American burying beetle, it is not expected to occur within corn fields nor will it be exposed to Cry3Bb1 protein. Adult American burying beetles are classified as opportunistic scavengers that feed on anything dead and bury vertebrate carcasses which larvae feed on. Carrion regurgitated by adults is fed to larvae until they are able to feed directly on a carcass.

Table 3. Endangered Beetles

Common Name	Scientific Name	Family	Status	States	Host Plant/Habitat
American burying beetle	*Nicrophorus americanus*	Silphidae	E	AK, MA, MI, NE, OH, OK, RI, SD	Cropland/hedgerow; forest (conifer, hardwood,); grassland/herbaceous, old field, shrubland/chaparral; eggs laid in soil adjacent to buried carcass; adults live above ground
Coffin cave mold beetle	*Batrisodes texanus*	Pselaphidae	E	TX	Occurs in very small isolated caves within the Edwards Limestone Formation. Has only been found under rocks ligbtly buried in silt in total darkness
Comal Springs dryopid	*Stygoparnus comalensis*	Dryopidae	E	TX	Occurs in subterranean waters (several outlets of Comal Springs which forms the headwaters of the Comal River).

191

Table 3. Continued

Common Name	Scientific Name	Family	Status	States	Host Plant/Habitat
Comal Springs riffle	*Heterelmis comalensis*	Elmidae	E	TX	Aquatic; Inhabits the gravel substrates & shallow riffles in spring runs
delta green ground beetle	*Elaphrus viridis*	Carabidae	T	CA	Predators preying on softbodied arthropods particularly Collembola. Typically occurs along the margins of vernal pools within 1.5 m of the water but has been found 100 m away. Prefers sandy mud substrate that slopes into the water & low-growing vegetation that provides 25-100% cover.
Hungerford's crawling water beetle	*Brychius hungerfordi*	Halipilidae	E	MI, Canada	Prefers a warm shallow, gravel bottom outflow stream ~1-2 miles below the lake. Found with ~2 ft deep plant roots.
Kretschmarr Cave mold beetle	*Texamaurops reddelli*	Pselaphidae	E	TX	Localized & distinct cave fauna occurring in very small isolated caves within the Edwards Limestone Formation. Found under rocks buried in silt in total darkness.
Mount Hermon June beetle	*Polyphylla barbata*	Scarabaeidae	E	CA	Adults don't feed & larvae feed & occur on roots underground. Occurs in sparsely vegetated, sand parkland & other sandy areas within chaparral & ponderosa pine stands.
Tooth Cave ground beetle	*Rhadine persephone*	Carabidae	E	TX	A subterranean obligate feeding on crickets. Occurs in deep uncompacted silt in small isolated karst caves within the Edwards Limestone Formation.

19

Table 3. Continued

Common Name	Scientific Name	Family	Status	States	Host Plant/Habitat
valley elderberry longhorn beetle	*Desmocerus californicus dimorphus*	Cesambycidae	T	CA	Associated with elderberry trees (*Sambucus* spp.) in California's Central Valley during its entire life cycle
unnamed ground beetle	*Rhadine exilis*	Carabidae	E	TX	Subterranean obligate. A troglobitic ground beetle only found in caves in San Antonio area, Bexar County, TX feeding on cave cricket eggs & nymphs.
unnamed ground beetle	*Rhadine infernalis*	Carabidae	E	TX	Subterranean obligate. A troglobitic ground beetle only found in caves in San Antonio area, Bexar County, TX feeding on cave cricket eggs & nymphs.
Helotes mold beetle	*Batrisodes venyivi*	Pselaphidae	E	TX	Subterranean obligate. Found in caves in San Antonio area, Bexar County, TX
northeastern beach tiger beetle	*Cicindela dorsalis dorsalis*	Cincindelidae	T	CT, MA, MD, NJ, RI, VA	Adults and larvae occur in wide, undisturbed, dynamic, fine sand beaches on the east coast.
Ohlone tiger beetle	*Cincindela ohlone*	Cincindelidae	E	CA	Occurs in CA native grasslands including oatgrass & purple needlegrass where the substrate is poorly-drained clay or sandy clay soil over bedrock of Santa Cruz Mudstone.
Puritan tiger beetle	*Cincindela puritana*	Cincindelidae	E	CT, MA, MD, NH, VT	Occurs near the CT River or Chesapeake Bay. Adults and larvae found on the upper portions of sandy beaches near either fresh or salt water. Larvae found in assoc. with cliffs.

19

Information for Table 3 taken from
http://ecos.fws.gov/webpage/webpage_vip_listed.html?module=undefined&code=I&listings=0#I
http://shanana.berkeley.edu/essig/endins/desmocer.htm
http://www.utexas.edu/depts/tnhc/.www/biospeleology/photos.htm
http://www.dec.state.ny.us/website/dfwmr/wildlife/endspec/nbtbfs.html

References Cited

Astwood, J.D, R.E. Hileman, M.J. Mckee, T.J. Rydel, J.W. Seale and L. English. 2001. Safety assessment of Cry3Bb1 variants in corn rootworm protected corn. (MSL-17225).

Pleasants, J.M., R.L. Hellmich, G. Dively, M.K. Sears, D.E. Stanley-Horne, H.R. Matilla, J.E. Foster, T.L. Clark, and G.D. Jones. 2001. Corn pollen depositionon milkweeds in and near corn fields. Proc Natl Acad Sci USA; 10.1073/pnas.211287498.

19

JUL 09 2002

Reviewed By: Eric B. Lewis and Anthony Q. Armstrong, Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the U.S. Department of Energy under contract number DE-AC05-00OR22725

EPA Reviewer: Robyn Rose, Biopesticides and Pollution Prevention Division (7511C) Robyn R 7-9-0

DATA EVALUATION REPORT

STUDY TYPE: Nontarget Insect Testing Tier I, OPPTS 885.4340

TEST MATERIAL: *Bacillus thuringiensis* Cry3Bb Protein and the Genetic Material Necessary for its Production (Plasmid Insert PHI 8999) in Corn

CITATION:

Author:	G. Head
Title:	Research on the Effects of Corn Rootworm Protected Transgenic Corn Events on Nontarget Organisms: Preliminary Results
Study Completion Date:	March 29, 2002 (Report date - studies not yet completed)
Laboratory:	Various
Sponsor:	Monsanto Co., St. Louis, MO
Laboratory Report ID:	00-CR-032E-7
EPA Reg.No:	Not provided
DP Barcode:	D282664
MRID No:	456530-03

CLASSIFICATION:
Supplemental to completing field studies and submitting final reports to the Agency for review.

ACTIVE INGREDIENT:
Bacillus thuringiensis Cry3Bb1 event MON 863

QUALITY ASSURANCE STATEMENT: No Quality Assurance Statement was included. This MRID is a compilation of progress reports from several different investigators.

GLP COMPLIANCE STATEMENT: A signed GLP compliance statement was submitted that stated this research is not GLP compliant. This MRID is a compilation of progress reports from several different investigators.

REVIEW CONCLUSIONS: Based on preliminary data, MON 863 corn may have the potential to impact some nontarget organisms.

SUBMISSION PURPOSE: To provide progress reports for studies to determine the effects of a transgenic corn rootworm control product (event MON 863) on nontarget organisms. MRID 45653003 contains progress reports for eight ongoing trials being conducted in Kansas, Nebraska, Illinois, Virginia, and South Dakota. A trial is also being conducted in New York, but no progress report was included.

MATERIALS, METHODS & RESULTS: All the trials compared the effects of transgenic corn MON 863 with those of a non-transgenic corn, with or without insecticide. In five of the field trials the corn was planted and monitored at regular intervals to determine the abundance of nontarget insects. Aboveground insect populations were monitored visually and/or by the use of sticky traps and pitfall traps; belowground populations were monitored using Tullgren-type extraction of soil cores. A field trial on plant residue decomposition involved burying bags of corn litter and recovering the bags at regular intervals to determine decomposition rates and litter arthropod populations. One laboratory study fed various combinations of MON 863 or non-transgenic pollen to Coccinellid larvae and measured development and mortality. A second laboratory study infested corn seedlings with plant-pathogenic nematodes and monitored the nematode population for 10 weeks. Details of the individual trials are given below, and a summary is provided in Table 1.

Trial 1, Al-Deeb and Wilde: This study evaluated the effect of MON 863 on non-target arthropods. Field trials were conducted during 2001 in Manhattan (one site) and Scandia (two sites), KS, using MON 863 and appropriate isolines, with or without insecticide. Treatments were conducted in small plots (20 rows x 50 ft or 4 rows x 30 ft). Arthropod abundance was determined using visual counts, pitfall traps, and Tullgren funnels. Visual inspections of 20 randomly-selected plants/treatment were conducted weekly for 3-4 weeks during the silking period, and the number of arthropods was recorded.

Results: Only the visual inspection data have been analyzed. Generally, no significant differences among treatments were found for the abundance (number/plant) of the most common predators, including *Orius insidiosus* (Hemiptera:Anthocoridae) adults/nymphs, *Hippodamia convergens* (Coleoptera:Coccinellidae) adults, *Coleomegilla maculata* (Coleoptera:Coccinellidae) adults, *Scymnus* spp. (Coleoptera:Coccinellidae) adults, or *Hippodamia/Coleomegilla* larvae. The study authors note that these results are consistent with those found in 2000. A table of the 2001 data for each of the three sites was provided in the progress report.

Trial 2, Clark *et al.*: The study was conducted in Nebraska in 2001 to determine the impact of MON 863 on non-target insects, specifically Collembola and Carabids. Treatments included MON 863 and appropriate isolines, with or without insecticide. Plots were 60 x 60 ft. The insects were collected using pitfall traps, and visual observations were made on 10 randomly-selected plants. The timing and duration of sampling were not given in the report.

Results: No significant differences were reported for the number of Carabids or collembolans in the MON 863 and isoline plots. No genus or species identification was provided for the collected insects, and no data were provided in the progress report.

Trial 3, Bitzer and Rice: Studies were conducted during 2000 in Monmouth, IL (one site) and Hampton, NE (three sites) to determine the effect of MON 863 on Collembola (Springtails). Treatments included MON 863 and appropriate isolines, with or without insecticide. Plot size was not provided. In Illinois, soil core samples were taken every two weeks from the end of June until early October. Pitfall traps were installed in June and sampled from mid-July until early October. In Nebraska, pitfall traps were installed (no dates were provided).

Results: MON 863 did not significantly affect the total number of subsurface Collembola found in the Illinois soil core samples, based on analysis of 28 species for which there were at least 20 individuals present. The species were not identified in the report, and the data table referred to in the text was not included. Insecticide use did increase the total number of subsurface Collembola. The Illinois pitfall samples showed that MON 863 did not significantly affect the total number of soil surface Collembola. Insecticide significantly increased the number of surface collembolans, as well as the abundance of several other medium-sized surface insect species. The study authors speculated that the increased numbers were due to the soil insecticide reducing the abundance of a primarily surface-active predator, possibly a Carabid beetle. In the Nebraska trial, 36 surface-active Collembola species (not identified in the report) were captured, and the 14 most common species have been examined for treatment effects. Neither the transgenic corn nor the insecticide significantly affected the total number or species abundance of collembolans, with the possible exception of one Hypogastrid. In that instance, the overall treatment differences were not significant, but the contrast between MON 863 and the isoline suggested that the Hypogastrid was significantly more abundant in the isoline plots (the data table cited in the text was not present in the report). Species richness estimates for the Illinois surface samples were determined using the EstimateS 5.0 program, which examines the cumulative incidence and abundance of species as the number of samples is increased. Results indicate that MON 863 did not reduce species diversity, but insecticide did. Several figures, but no tables, of the data were included in the report.

Trial 4, Zaborski: This study was conducted during 2000 in Champaign, IL to determine the effect of MON 863 on the invertebrate community structure in soil and plant residues. Treatments included MON 863 and appropriate isolines, with or without insecticide. Plot size was 20 x 125 ft. Prior to the 2000 harvest, senesced corn leaves and stalks from each corn line were collected, dried, and reduced to 5-cm pieces. Sub-samples (~3 g) were placed in fiberglass mesh (1 mm) bags and 14 bags of each line were buried 5-10 cm in a non-insecticide, non-transgenic corn plot in February 2001. Two bags from each line were unearthed in April, June, July, August, September, and October, 2001. A modified high gradient extractor was used to extract the microarthropods, and the remaining contents of the bags were oven-dried, ground, and ashed. Soil samples to a depth of 15 cm were also collected from the field, oven-dried, and ashed. A similar experiment using wheat straw was also conducted. Additionally, earthworm populations were sampled by removing one meter of corn plants from the center row at two locations in each plot, placing a 0.5 m^2 metal quadrat over the row, and irrigating the quadrat with two 10 L aliquots of 0.25% formalin solution at 10-minute intervals. Earthworms emerging during the 10 minutes following each irrigation were collected.

Results: Over 26,000 microarthropods representing 62 morphotaxa, including Collembola, mites, and eleven other arthropod taxa have been identified in 87 retrieved litter bags (the taxa were not identified in the report). Only a portion of the data has been partially analyzed, and the only results in the report are for mites. Fungus-feeding Prostigmatid mites initially colonized the material, but populations declined as predatory Mesostigmatid mites increased and fungus-feeding Orabatid mites began to colonize the residues. Late in decomposition, the number of Mesostigmatid declined, while the Orabatid mites persisted, and Astigmatid mites increased (specific data were not provided in the report). The preliminary data suggest that the mite community structure may be slightly different on MON 863 residues than on conventional corn residues, as six morphospecies had an increased abundance (+60 to +450%) on the MON 863

residues. Litter bags from MON 863 contained two and one-half times the number of mites found in litter bags from the non-transgenic isoline. No data were presented for the wheat straw or earthworm studies.

Trial 5, Lundgren and Wiedenmann: In a lab study, *Coleomegilla maculata* larvae were fed either aphids, pollen mixtures containing 0, 25, 50, 75, or 100% MON 863 pollen, or no diet at all. The duration of each instar and the pupal weight were then monitored. Other groups of *C. maculata* larvae were fed one of the pollen mixtures or an artificial diet (not specified), and the duration of larval and pupal stages, pupal weight, adult walking speed, adult flip time, adult survival, and adult fecundity were compared. Similar experiments using Carabids are also being conducted.

Results: There were no significant differences in any of the parameters for *C. maculata* larvae among the pollen-fed treatments. All the pollen-fed treatments had faster larval development, increased survival, and higher pupal weight than the aphid or artificial diet treatments. No data were provided in the progress report. Comparable experiments with Carabids have not been completed.

Trial 6, Lewis et al.: In a non-insect lab study included in MRID 45653003, three-week-old MON 863 and control corn seedlings in pots were infested with 5000, 10,000 or 15,000 *Meloidogyne incognita* (a plant-pathogenic nematode) eggs per plant. Two weeks after infestation, 6 plants per treatment were removed and their roots were stained with fuchsin and pressed between glass plates. *M. incognita* juveniles present were then counted with a dissecting microscope. Five weeks after infestation, 12 plants per treatment were removed and nematode eggs were extracted from the roots using sodium hypochlorite and counted under a dissecting microscope. Ten weeks after infestation, 12 plants per treatment were removed and the nematode egg extraction and counting were again performed. In a separate soil leachate experiment, MON 863 and controls were grown in pots for 4 weeks. The seedlings were then removed and the soil was mixed 1:1 (w/w) with water, shaken for two minutes in plastic bottles, passed through a 125 μ sieve, and centrifuged for 10 minutes at 2000 rpm. The supernatants were transferred to Petri dishes and approximately 100 laboratory-reared *Caenorhabditis elegans* (a bacteriovorous nematode) of mixed life stages or *Steinernema carpocapsae* (an entomopathogenic nematode) juveniles were added to each dish. After 24 hours at ~25°C, the number of live and dead nematodes was determined. In a separate root extract experiment, MON 863 or control roots were finely chopped, blended at high speed for 5 minutes, and passed through 125 μ and 25 μ sieves. The filtered extract was then used to assess the mortality of *C. elegans* and *S. carpocapsae* using the same method as that used for the soil leachates.

Results: Populations of *M. incognita* juveniles were significantly lower in MON 863 roots than in conventional corn roots at all three inoculation rates. The number of eggs was significantly lower in MON 863 roots after both 5 and 10 weeks (significance was specified in the text, but not shown in the accompanying figure). Twenty-four hour survival of *C. elegans* was 10% in MON 863 extracts, compared to 57% in control extracts, a significant reduction. Soil leachates had no effect on survival, indicating that MON 863 corn might be unlikely to affect nematodes not associated with the corn plant itself. Twenty-four hour survival of *S. carpocapsae* was not affected by root extracts from either MON 863 or control seedlings. The study authors speculated

197

that since the *S. carpocapsae* used in the test were nonfeeding stages, the Bt protein from the root extracts may have to be ingested to be lethal.

Trial 7, Fuller and Mc Manus: The study was conducted in Brookings, SD during 2001. Four plots (1.6-ha) of MON 863 and 4 plots of a traditional hybrid were planted with and without soil insecticides and monitored for Coccinellids by whole-plant counts and sticky traps (16/plot). The whole-plant counts were conducted weekly on 100 randomly-chosen plants and included eggs, larvae, pupa, and adults. Below-ground samples were collected using Tullgren-type extraction of soil cores or hand sorting of soil samples. No additional information was provided in the report.

Results: Coccinellid egg density for the overall growing season was not significantly affected by any of the treatments, although a two-fold increase in egg number was seen in the MON 863 fields during the second week of sampling. The study authors speculated that the increase may have been due to the transgenic plants being more attractive at that time to ovipositing females, although no difference was apparent to the sampling crew. The number of Coccinellid larvae was significantly increased (approximately 2-fold) on the MON 863 plots. However, the increased larval numbers did not result in a greater number of pupae or adults. A separate analysis of *Coleomegilla fuscilabrus*, the most abundant adult Coccinellid in each of the fields, found no significant differences among the treatments.

Trial 8, Ellsbury and French: The study was conducted in Brookings, SD during 2001. Treatments included MON 863 and appropriate isolines, with or without insecticide. Carabid density was measured by placing a single pitfall trap inside each of eight corn earworm emergence enclosures per 4-acre plot. Propylene glycol was used as the collection fluid/preservative in the traps. Weekly counts of emerging adult Carabids were taken, and corn rootworm beetle numbers were counted at the end of the season. The density of Carabids was measured separately using 8 pitfall traps placed between corn rows in June and checked weekly through the harvest.

Results: The cumulative mean number of Carabids was lowest for the MON 863 corn, but no statistical analysis of that data was discussed in the progress report. Analysis of the weekly collection data showed that the number of Carabids was significantly lower in the MON 863 plots compared to controls, but not compared to the insecticide-treated plots. However, the study authors state that the results cannot be regarded as definitive due to missing values resulting from animal damage and other unknown factors. Additionally, there was apparently a density gradient across the plots, and a large wetland between the MON 863 and the other treatments which may have limited the movement of ground-dwelling insects and biased the results. The next set of tests is to include a rearrangement of plots to ensure complete randomization.

REVIEWER'S COMMENTS: All these studies are ongoing, and in most cases only very preliminary results have been included in MRID 45653003. Lack of data, especially tables, in the progress reports makes any assessment of most of the studies cursory. Results from several studies include only the number of insects determined, without identifying the genus/species, which would be meaningful. Five of the eight trials found a significant effect due to MON 863. Bitzer and Rice suggest that MON 863 resulted in a decreased population of one of 14 species of Collembola compared to insecticide treated plots, and preliminary results from Zaborski suggest that the mite community structure may be slightly altered on MON 863 plant residues. A third

trial, Ellsbury and French, found a decrease in the number of Carabids in MON 863 plots, but the data are suspect due to plot layout problems. Fuller and McMamis reported that the number of Coccinellid larvae, but not pupae or adults, was increased on MON 863 plots, and Lewis et al. found that MON 863 plants had lower numbers of *M. incognita* eggs and juveniles. While the progress reports do not include enough data to allow any definitive conclusions, the early information appears to indicate that MON 863 corn may affect some nontarget organisms. Further analysis of the data already collected, as well as additional results from the ongoing trials, are needed before an assessment of the effects of transgenic corn on nontarget organisms can be fully developed.

Table 1. Summary of trials to evaluate the effect of MON 863 on nontarget organisms

Investigators (Start date)	Location (Study date) Plot size	Treatment	Type of monitoring	Investigator's conclusion	Comments
Al-Deeb and Wilde (2000)	Manhattan and Scandia, KS (2001) Small plots - 20 rows x 50 ft or 4 rows x 30 ft	MON 863 and isolines with or without insecticide	Weekly visual inspection of leaf, silk, and tassel of 20 randomly selected plants during silking	Generally no significant differences in *Orius* nymphs and adults, *Hippodamia*, *Coleomegilla*, *Scymus* adults, or ladybeetle larvae.	Pitfall and Tullgren funnel data not yet analyzed.
Clark et al. (2000)	Nebraska (2001) 60 x 60 ft	MON 863 and isolines with or without insecticide	Visual inspection of 10 randomly selected plants; pitfall traps	No significant difference in carabid or Collembola numbers.	Other arthropods collected, data not analyzed
Bitzer and Rice (2000)	Illinois, Nebraska (2000) not given	MON 863 and isolines, with or without insecticide	Pitfall traps, Tullgren funnels	No apparent effect of MON 863 on Collembola or species diversity. Soil insecticide increased abundance of some species and reduced species diversity.	Soil insecticide apparently increased abundance of some species due to effect on predatory arthropods.
Zaborski (2000)	Illinois (2001) 20 x 125 ft	MON 863 and isolines, with or without insecticide	Mite populations on leaves and stalks from each line buried in litter bags; mite populations in soil; earthworms	Mite community structure slightly different in MON 863 litter	A study comparing wheat straw with corn litter results and a study on earthworm populations are ongoing.
Lundgren and Wiedenmann (2000)	Lab study (2001)	Pollen (0-100% transgenic), aphids, artificial diet, or no diet fed to *Coleomegilla maculata* larvae	Instar duration and pupal weight; duration of larval and pupal stages; walking speed; flip time; survival; and adult fecundity	MON 863 pollen did not affect the measured parameters.	Feeding studies with Carabidae are underway.

Table 1. Summary of trials to evaluate the effect of MON 863 on nontarget organisms					
Lewis et al. (2001)	Lab study	MON 863 and isoline seedlings infested with plant pathogenic nematode (*M. incognita*) eggs and monitored for 10 weeks; non-plant pathogenic nematodes (*E. elegans*, S. *carpocapsae*) in soil exposed to MON 863 or isoline root extracts or soil leachates	*M. incognita*: root penetration and egg production; *E. elegans*, S. *carpocapsae*: mortality	*M. incognita*: root population and egg production significantly reduced on MON 863 seedings. *E. elegans*: numbers significantly lower in MON 863 root extracts, no difference in soil leachate. S. *carpocapsae*: No difference in root extract or soil leachate.	S. *carpocapsae* used were non-feeding juveniles.
Fuller and McManus (2001)	South Dakota (2001) 1.6 ha	MON 863 and isolines with and without soil insecticides	Whole-plant counts of coccinellid eggs to adults on 100 random plants/week; sticky traps	No significant differences in egg, pupal, or adult density; 2-fold increase in larval density	
Ellsbury and French (2001)	South Dakota (2001) 407 x 407 ft	MON 863 and isoline with and without soil insecticide	Pitfall traps	Significant differences in number of carabids, likely due to plot layout rather than treatment	
Losey (2001)	New York				Preliminary report not available

DATA EVALUATION RECORD

BACILLIS THURINGIENSIS CRY3BB PROTEIN AND THE GENETIC MATERIAL NECESSARY FOR ITS PRODUCTION (VECTOR ZMIR 13L) IN CORN

STUDY TYPE: Nontarget Insect Testing Tier I, OPPTS 885.4340
MRID 45653003

Prepared for

Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Toxicology and Hazard Assessment Group
Life Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Work Assignment No. 128

Primary Reviewer: Eric B. Lewis, M.S.	Signature: Eric B. Lewis Date: JUN 2 1 2002
Secondary Reviewers: Anthony Q. Armstrong, M.S.	Signature: Anthony Q. Armstrong Date: JUN 2 1 2002
Robert H. Ross, M.S., Group Leader	Signature: Robert H. Ross Date: JUN 2 1 2002
Quality Assurance: Lee Ann Wilson, M.A.	Signature: L. A. Wilson Date: JUN 2 1 2002

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the U.S. Department of Energy under contract number DE-AC05-00OR22725

202

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

MEMORANDUM

MAR 10 2000

OFFICE OF PREVENTION, PESTICIDES AND TOXIC SUBSTANCES

TO: Mike Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division, 7511C

FROM: Robyn Rose, Entomologist Robyn Rose 3/10/00
Biopesticides and Pollution Prevention Division, 7511C

PEER REVIEW: Zigfridas Vaituzis, Ph. D., Microbiologist Zigfridas Vaituzis 3/10/
Phil Hutton, Chief
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511C

SUBJECT: Review of ecological non-target lady beetle effects for Monsanto's EUP request to allow testing and further development of their *Bacillus thuringiensis* Cry3Bb1 Field Corn (EPA Reg. No. 524-LRA; Barcode No. D262045; Case No. 066221; Submission No. S572997).

CLASSIFICATION: Acceptable for Experimental Use Permit;
Supplemental for a Section 3 commercial registration.

BPPD CONCLUSIONS:

This study is adequate to address potential concerns for Cry3Bb1 protein expressed in corn to beetle species for the proposed limited duration and acreage EUP. It demonstrates that lady beetles feeding on amounts of Cry3Bb1 protein greater than they are expected to encounter in prey will not be adversely affected. However, lady beetles also routinely feed on corn pollen. Direct exposure to Cry3Bb1 is therefore possible if it is expressed in the pollen. In order to obtain a full commercial registration, Monsanto should determine the effects on lady beetles fed corn pollen containing Cry3Bb1. Field scouting to determine coleopteran and general insect species abundance should also be conducted.

BACKGROUND:

According to 40 CFR §158.590, effects on nontarget insect predators and parasites should be evaluated when registering a new pesticide. The OPPTS 885.4340 guideline titled "Nontarget insect testing" recommends performing tests on three species of insects including parasitic dipterans, predaceous hemipterans, predaceous coleopterans, predaceous mites, predaceous neuropterans, and parasitic hymenopterans. BPPD routinely requests studies on the effects of Bt proteins on lady beetles (coleopteran), green lacewings (neuropteran), and a parasitic Hymenoptera. As part of this requirement, Monsanto sponsored a test on the effects of the Cry3Bb Bt protein against *Hippodamia convergens*, a representative lady beetle.

203

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Biopesticides and Pollution Prevention Division
SECONDARY REVIEWER:	Zigfridas Vaituzis, Ph.D., Microbiologist Biopesticides and Pollution Prevention Division
STUDY TYPE:	Non-target effects of Bt Protein 11231 on lady beetles
MRID NO.:	44903-14
STUDY NO.:	WL-98-299
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
PERFORMING LAB:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198 and Wildlife International Ltd., 8598 Commerce Dr., Easton, MD 21601
TEST MATERIAL:	*Bacillus thuringiensis* Protein 11231
AUTHOR:	Susan J. Palmer and Henry O. Krueger, Ph.D
STUDIES DATED:	August 19, 1999

Study Summary

Title: *Bacillus thuringiensis* Protein 11231: A Dietary Toxicity Study With the Ladybird Beetle (*Hippodamia convergens*)

Authors: Susan J. Palmer and Henry O. Krueger, Ph.D (Wildlife International Ltd.).

Objective: To evaluate the toxicity of *Bacillus thuringiensis* Cry3Bb1 (analogous to Protein 11231) in diet to lady beetles (*Hippodamia convergens*)

Methods: Diet containing *Bacillus thuringiensis* (Bt) and honey was fed to *H. convergens* at rates one and 20 times the maximum protein concentration found in corn leaf tissue. Diets contained 400 and 8,000 μg Cry3Bb1/mL of diet as well as a control group containing a honey/water mixture and two substance reference groups containing 1,000 or 10,000 μg potassium arsenate/mL diet. Four hundred and 8,000 μg Cry3Bb1/mL of diet are approximately equivalent to 1X and 20X the maximum Bt protein concentration in plant tissue. Mortality and other signs of toxicity were recorded twice within four hours after test initiation and daily thereafter until 20% mortality was reached in the control group. Results from groups fed diet containing Cry3Bb1 were compared to control groups.

Results: Results from this study showed that the no-observed-effect-concentration NOEC for Cry3Bb1 when incorporated in diet and fed to *H. convergens* is 8,000 μg Bt protein/mL diet. The study was terminated ten days after initiation because mortality in the control group reached 24%. The was 33% *H. convergens* mortality when fed 400 μg Cry3Bb1/mL diet and 35% *H. convergens* mortality when fed 8,000 μg Cry3Bb1/mL diet. These results demonstrate no significant differences in mortality rate between lady beetles fed 400 and 8,000 μg Cry3Bb1/mL

of diet. At the 400 μg Cry3Bb1/mL diet dose, there was a small number of beetles that became immobile but this was determined not to be related to the treatment. Mortality for the 1,000 and 10,000 μg potassium arsenate/mL diet groups were 55% and 95% respectively at day 10. This demonstrates that toxicity can be measured by mixing a test substance with diet.

Study Author's Conclusions: Lady beetles do not feed on corn plant tissue. They do, however, prey on pest insects that may feed on corn tissue and contain Cry3Bb1 in their gut, thus exposing lady beetles to the Bt protein. According to Monsanto, there is approximately 390 μg Cry3Bb1/g fresh weight corn tissue. Lady beetle exposure is expected to be significantly lower than this since the corn tissue would be metabolized, eliminated, or otherwise degraded within the prey species. Since the NOEC was found to be 8,000 μg Cry3Bb1/mL diet which is 20 times higher than maximum expected exposure levels, risk of Cry3Bb1 to lady beetles is expected to be minimal.

Discussion/Risk Assessment: The study is scientifically sound and no statistically significant treatment mortality was observed between the dosed and control replicates. The NOEC was found to be 8,000 μg Cry3Bb1/mL diet, indicates that Cry3Bb1 protein is practically nontoxic to *Hippodamia convergens* as an indicator species for predatory beetles. The test insects were exposed to a dose of active ingredient higher than the amount that is expected to be ingested by the beetles when feeding on intoxicated aphids or corn pollen under field conditions. As a result, no detectable reduction in the abundance of beetles is expected from the proposed uses of the Cry3Bb1 producing corn. This study adequately address potential concerns for Cry3Bb1 protein expressed in corn to beetle species for the limited duration and acreage of the proposed EUP. However, for a Section 3 registration the results obtained from pure Cry3Bb1 protein feeding should be confirmed by a Cry3Bb1 containing pollen feeding study and by field scouting for soil and phylloplane insect abundance during the duration of the EUP.

Adequacy of the Study:

1. Validation Category: Acceptable for EUP

2. Rationale: This study meets EPA Guideline requirements. However, additional data are required for a Section 3 registration.

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

JUN 25 2002

MEMORANDUM

SUBJECT: Review of efficacy and insecticidal activity data for Event MON 863: Corn Rootworm Protected Corn (Vector ZMIR13L); Permit No. 524-LEI; Chemical No 006484; DP Barcode No D275903, D280086; Case No 065182; MRID # 453613-03, 455382-07, 455382-08.

TO: Mike Mendelsohn, Regulatory Action Leader
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511C

FROM: Alan Reynolds, Entomologist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511C

PEER REVIEW: Sharlene Matten, Ph.D., Biologist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511C
6/25/02

Action Requested:

BPPD has been asked to review efficacy and insecticidal activity data submitted by Monsanto Co. in support of a registration application for MON 863 Corn Rootworm Protected field corn (EPA Reg. No. 534-LEI). Event MON 863 (vector ZMIR13L) contains the Cry3Bb *Bt* gene that produces an insecticidal crystal protein and a *nptII* marker gene. MON 863 is targeted against corn rootworm beetles (*Diabrotica* spp.), a major pest of corn in the U.S. The submitted data consists of three studies: 1) Insecticidal Spectrum of Activity for Cry3Bb Protein *in vitro* (MRID # 455382-07); 2) Comparing the Efficacy of MON 853 and MON 863 to Three Corn Rootworm Species, Northern Corn Rootworm (*Diabrotica barberi*), Southern Corn Rootworm (*D. undecimpunctata howardi*), and Western Corn Rootworm (*D. virgifera virgifera*) (MRID # 455382-08); 3) Efficacy of MON 863 Against Corn Rootworm and Comparison to Insecticide Treatments - Results of Year 2000 Field Trials (MRID # 453613-03). It should be noted that this review will addresses only these three studies and that other benefits and non-target data will be considered separately.

BPPD Conclusions:

1) Insecticidial spectrum studies showed that, of the Coleopteran, Lepidopteran, Hymenopteran, Neuropteran, and Collembola species tested, only beetles from the family Chrysomelidae

Internet Address (URL) • http://www.epa.gov
Recycled/Recyclable • Printed with Vegetable Oil Based Inks on Recycled Paper (Minimum 25% Postconsumer)

(Colorado potato beetle and western corn rootworm) were shown to be susceptible to the Cry3Bb protein. Additional species of corn rootworm, including southern, northern, and Mexican corn rootworm, were not tested. It is noted, however, that a complete review of Cry3Bb effects to non-target, beneficial, and endangered insect species has been conducted separately (see R. Rose memo to M. Mendelsohn, 5/20/02).

2) Comparative efficacy studies showed that both MON 863 and MON 853 (a non-commercialized hybrid expressing Cry3Bb) experienced significantly less root damage from southern, northern, and western corn rootworm than a non-transformed control hybrid. MON 863 also had significantly less root damage from western and northern rootworm than MON 853 (there was no significant difference in southern rootworm damage). In all treatments, damage from southern corn rootworm exceed that from northern and western rootworms.

3) In comparative efficacy studies, MON 863 prevented root damage from rootworm feeding as well or better than commonly used rootworm soil insecticides including Force 3G (tefluthrin), Counter CR (terbufos), and Lorsban 15G (chlorpyrifos). Root damage ratings for MON 863 were typically between 1.2 and 2.0, a high level of control relative to untreated control hybrids. It should be noted that efficacy data such as these (and other studies) will also be reviewed and considered as part of a full benefits assessment for MON 863.

Insecticidal Spectrum of Activity for Cry3Bb Protein in vitro (MRID # 455382-07)

Monsanto has conducted diet bioassays to determine susceptibility to Cry3Bb for a number of insect species. The insects tested included representative species from Coleoptera (families Chrysomelidae, Bruchidae, Coccinellidae, Tenebrionidae, and Curculionidae), Lepidoptera (Pyralidae and Noctuidae), Hymenoptera (Apidae and Pteromalidae), Neuroptera (Chrysopidae), and Collembola (Isotomidae).

The assays were performed with purified Cry3Bb1 (variant 11231) protein obtained from recombinant strains of *Bacillus thuringiensis*. The protein differs from "wild type" Cry3Bb protein by four amino acids. It is unclear from the study report whether the tested Cry3Bb variant is the same as the Cry3Bb protein transformed into the MON 863 corn hybrid. All of the diet bioassays were conducted at Monsanto's laboratories or at contract laboratories.

Diet bioassays for Coleoptera (Colorado potato beetle, western corn rootworm, cotton boll weevil) and Lepidoptera (European corn borer, corn earworm) were performed using serial dilutions of 6-8 concentrations (typically 1 to 200 pprn) in accordance with a Monsanto SOP. Assays for Coleoptera (ladybird beetle), Hymenoptera (honey bee, parasitoid wasp), Neuroptera (green lacewing), and Collembola were preformed by a contractor (GLP) using a single concentration, based on the maximum anticipated exposure in the environment. For honey bee larvae, the dose was delivered via deionized water injected into hive cells and for adult bees the dose was dissolved into a sugar feeding solution. For the parasitoid wasp and ladybird beetle, the Cry3Bb dose was incorporated into a honey mixture for adult feeding. Green lacewings were

tested using a mixture of the Cry3Bb dose and moth eggs fed to larvae. Collembola were tested using lyophilized Cry3Bb leaf tissue mixed with yeast. Testing of other Coleoptera (cowpea weevil, red flour beetle, rice weevil, and pepper weevil) was done by a contractor (non-GLP) using single, maximum concentration doses of Cry3Bb. Where significant toxicity was observed, an LC_{50} was calculated using a nonlinear regression model.

The results from the susceptibility tests are contained in the following table:

Order/Family	Genus/Species	LC_{50} (μg/mL diet)	Max. Conc. (μg/mL)
Coleoptera/Chrysomelidae	*Leptinotarsa decemlineata* Colorado Potato Beetle	2.7	-
Coleoptera/Chrysomelidae	*Diabrotica virgifera* Western Corn Rootworm	75	-
Coleoptera/Bruchidae	*Callosobruchus maculatus* Cowpea Weevil	>200	200
Coleoptera/Coccinellidae	*Hippodamia convergens* Ladybird Beetle	>8000	8000
Coleoptera/Tenebrionidae	*Tribolium castaneum* Red Flour Beetle	>200	200
Coleoptera/Curculionidae	*Anthonomus grandis* Cotton Boll Weevil	>50	50
Coleoptera/Curculionidae	*Anthonomus eugenii* Pepper Weevil	>200	200
Coleoptera/Curculionidae	*Sitophilus aryzae* Rice Weevil	>200	200
Lepidoptera/Noctuidae	*Helicoverpa zea* Corn Earworm	>200	200
Lepidoptera/Crambidae	*Ostrinia nubilalis* European Corn Borer	>200	200
Hymenoptera/Pteromalidae	*Nasonia vitripennis*	>200	400
Hymenoptera/Aphidae	*Apis mellifera* Honey Bee	>360	360
Neuroptera/Chrysopidae	*Chrysoperla carnea* Green Lacewing	>8000	8000
Collembola/Isotomidae	*Folsomia candida*	>870	870

The results from the susceptibility assays show that toxicity was only observed within the Chrysomelid family of beetles (Colorado potato beetle and western corn rootworm). Additional species of corn rootworm, including southern, northern, and Mexican corn rootworm, were not tested (MON 863 is claimed to be effective in controlling corn rootworm (*Diabrotica* spp.) larvae). Other assayed insects, including species from Lepidoptera, Neuroptera, Collembola,

208

Hymenoptera, and the additional families of Coleoptera did not exhibit any susceptibility to Cry3Bb at the tested doses.

While the test included several beneficial species (ladybird beetle, green lacewing, honey bee, and *Nasonia vitripennis* parasitic wasp), these data do not represent a complete assessment of effects to beneficial insects (non-target effects, including beneficial species, have been reviewed separately -- see R. Rose memo to M. Mendelsohn, 5/20/02).

It is also noted that there are a number of endangered beetle species that are of possible concern with the widespread planting of MON 863 corn. These beetle include the American burying beetle (family: Silphidae), delta green ground beetle (Carabidae), and valley elderberry longhorn beetle (Cerambycidae) (previously approved EUPs for MON 863 have been restricted to prevent exposure to these beetles). The submitted data did not include any assays from other species in the Silphidae, Carabidae, and Cerambycidae families. However, it is noted that a separate assessment of MON 863 corn effects on endangered species has been completed (see R. Rose memo to M. Mendelsohn, 5/20/02).

Comparing the Efficacy of MON 853 and MON 863 to Three Corn Rootworm Species, Northern Corn Rootworm (*Diabrotica barberi*), Southern Corn Rootworm (*D. undecimpunctata howardi*), and Western Corn Rootworm (*D. virgifera virgifera*) (MRID # 455382-08)

In this experiment, Monsanto compared the relative efficacy of two transformed corn hybrids expressing the Cry3Bb protein (MON 853 and MON 863) in preventing damage from three species of corn rootworm larvae. This was accomplished by artificially infesting potted corn plants (treatments consisting of the two transformed hybrid and a non-transformed control hybrid) with eggs from each of three rootworm species. Each plant (in the V2 stage) was infested with approximately 800 eggs (6-8 plants per treatment were used). Root damage was scored using the Iowa Root Damage Rating (RDR) index (1 = no damage, 6 = extensive damage) after 3-4 weeks of larval feeding.

Results from the study showed that both MON 853 and MON 863 experienced significantly less root damage from all three rootworm species than the non-transformed control hybrid. In terms of western and northern corn rootworm damage, MON 863 had significantly less root damage (<2 RDR) than MON 853 (~ 2.3 RDR). For southern corn rootworm, there was no significant difference between MON 853 and MON 863 (RDR ~ 3.5 - 3.8). Southern corn rootworm damage was greater than western or northern corn rootworm damage for all treatments. It is noted that, at present, Monsanto has not proposed to register MON 853.

Efficacy of MON 863 Against Corn Rootworm and Comparison to Insecticide Treatments - Results of Year 2000 Field Trials (MRID # 453613-03)

In this experiment, Monsanto evaluated the relative effectiveness of MON 863 and conventional

pesticide treatments at preventing damage from corn rootworm feeding in field efficacy trials. The pesticides tested (all soil insecticides) included Force 3G (tefluthrin), Counter CR (terbufos), and Lorsban 15G (chlorpyrifos).

The study consisted of three separate field experiments, all of which utilized similar growth stage MON 863 hybrid and a non-transgenic control hybrid (negative MON 863 isoline). In each of the experiments, treatments were deployed using a randomized block design and were scored for root damage in late July. Root damage was assessed using the Iowa Root Damage Rating (RDR) index (1 = no damage, 6 = extensive damage, >3 = economic threshold). For the first experiment (conducted at seven different locations), treatments (MON 863, control, Force 3G, Counter CR, and Lorsban 15G) were deployed as four-row strips (4 replicates per treatment). Each plot was artificially infested with 800 rootworm eggs/foot (species not specified). In the second experiment (conducted at eight different locations), MON 863 was evaluated against Force 3G treatment and an untreated control. Treatments were deployed as single rows and were artificially infested with 1600 rootworm eggs/foot (species not specified). In the third experiment (conducted at nine test sites), treatments (MON 863, control, Force 3G, Counter CR, and Lorsban 15G) were planted in four-row strips in continuous corn acres or a corn/pumpkin trap crop (no artificial rootworm infestation was used). For all tests, RDR damage was analyzed via analysis of variance and t-tests to determine significant differences between treatments. Also, a "consistency rating" was calculated for each experiment by determining the percentage of root damage in a treatment that is below the economic threshold (RDR = 3) when the corresponding control treatment root damage is above the threshold.

The results of the first experiment showed that when summed across all test locations, MON 863 (RDR = 2.02), Force 3G (2.40), Counter CR (2.26), Lorsban 15G (2.40) experienced significantly less root damage than the untreated control (3.91), although there was no significant difference between MON 863 and the insecticide treatments. However, at three of the seven locations, MON 863 had significantly less root damage than all of the other insecticide treatments. For the second experiment, when summed across all eight test sites, MON 863 (RDR = 1.41) and Force 3G treatment (1.91) showed significantly less root damage than the untreated control (3.27). There was no significant difference between MON 863 and Force 3G, although root damage for MON 863 was significantly less than that for Force 3G at five of the test sites. In the third experiment, MON 863 experienced significantly less root damage (RDR = 1.72, summed over all nine locations) than any of the insecticide treatments or the control (all insecticide treatments had significantly less damage than the control). For all three experiments, the "consistency rating" for MON 863 was close to 100%, meaning that damage in MON 863 hybrids was almost always kept below the economic threshold when the control treatment showed damage exceeding the threshold.

Taken together, the results show that MON 863 prevented root damage from rootworm feeding as well or better than rootworm soil insecticides. Root damage ratings for MON 863 were typically between 1.2 and 2.0, a high level of control relative to untreated control hybrids. In addition, the results were generally consistent from location-to-location (test sites included plots

in six separate corn-growing states).

It should be noted that efficacy data such as these (and other studies) will also be reviewed and considered as part of a full benefits assessment for MON 863.

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

JUL 23 2002

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

Subject: Preliminary review of Monsanto's interim insect resistance management plan for *Bacillus thuringiensis* event MON 863 corn rootworm protected field corn. EPA Reg. No. 524-LEI; Chemical No. 006484; Barcode No. D280086; Case No. 069017; Submission No. S607630; MRID Nos. 455770-01, 455382-08 and 451568-05.

From: Robyn Rose, Entomologist Robyn Rose 7-23-02
Biopesticides and Pollution Prevention Division, 7511C

Peer Review: Sharlene Matten, Ph.D, Biologist 7-23-02.
Biopesticides and Pollution Prevention Division, 7511C

Alan Reynolds, Entomologist 7/23/02
Biopesticides and Pollution Prevention Division, 7511C

Classification: It is acceptable to plant MON 863 corn for three years with a 20% non-Bt corn refuge planted within or adjacent to Bt corn fields. Based on the limited acres, use of a refuge and three year time frame, the risk of pest resistance to MON 863 is not a concern.

RECOMMENDATIONS

- Based on current information on CRW biology, MON 863 dose, simulation models, hybrid availability and adoption rate, a 20% refuge should be adequate on an interim basis to produce enough CRW adults to delay resistance. In addition, planting refuges adjacent to or within MON 863 corn fields should allow random mating of CRW even though there is limited movement of larvae and virgin females.

- It is acceptable to plant refuges as continuous blocks or in-field row-strips. Based on the only available currently published data, in-field strips should consist of at least 6 to 12 consecutive rows planted within 9 to 18 m of the center of the transgenic corn field.

- WCRW, NCRW and MCRW only feed on a few weedy species other than corn and the value of these alternate hosts as a refuge is not understood (Meinke *et al.* 2001). Therefore,

Internet Address (URL) • http://www.epa.gov
Recycled/Recyclable • Printed with Vegetable Oil Based Inks on Recycled Paper (Minimum 30% Postconsumer)

alternate hosts should not be considered as refuge for CRW-protected transgenic corn until more information is available on their ability to produce susceptible individuals available to mate with potentially resistance beetles.

- Additional information is needed on various aspects of CRW pest biology as it relates to a long-term IRM strategy. Knowledge of the maximum and average distance an adult CRW moves is limited. Additional research regarding male and female adult and larval WCRW and NCRW dispersal potential is needed to determine placement of non-Bt corn refuges. In addition, more information is needed on mating habits, ovipositional patterns, number of times a female can mate and fecundity.

- Additional research is needed to determine if IRM strategies designed for WCRW and NCRW are appropriate for MCRW and if IRM is needed for SCRW. Since MON 863 expression in corn roots likely declines as the plant matures and MCRW may develop slower, the risk of resistance may differ for MCRW then WCRW and NCRW.

- The mechanism of potential resistance of CRW to MON 863 should be determined to develop an appropriate long-term IRM strategy. Since CRW resistance is necessary to determine the mechanism and genetics of resistance to Cry3Bb1, colonies resistant to Bt should be established and evaluated in the laboratory during the initial three years MON 863 is grown commercially.

- The effect of WCRW ovipositing in soybean prior to overwintering and extended diapause in NCRW on an IRM strategy needs further investigation.

- Based on Monsanto's modified version of Caprio's model, a moderate dose is defined as 30% survival of larvae and a low dose as 50% survival. Data provided by Monsanto shows 17% to 62% survival of larvae, which suggests that there is a low to moderate dose of MON 863 for CRW. "Further research is needed to provide the critical data on the actual dose effects of Bt corn, corn rootworm biology and ecology" (Ostlie 2001).

- Additional knowledge of pest biology, population dynamics and genetics are necessary for the models needed to develop and validate resistance management strategies for CRW-protected corn.

- Resistance management models need further refinement and validation. Monsanto primarily relies upon a modified version of Mike Caprio's model which was originally developed for lepidopteran-active corn (ILSI 1999). Investigation into the validity of this model for coleopteran-protected corn is needed. Additional models should be developed that consider parameters such as seed mixtures, in-field refuge configurations, behavioral avoidance and fitness. Assumptions of simulation models should be tested and validated in the field.

- Grain producers will utilize the option of applying insecticides to treat for CRW adults or larvae on refuge acres because of high economic loss if left untreated . Insecticides are

213

applied to the root zone to control larvae and will not affect CRW out of the treated area (Meinke *et al.* 2001). Therefore, it should be assumed that refuge acres will be treated for CRW larvae with chemical insecticides. However, insecticides should not be used on refuges to control CRW adults. If resistance is confirmed, all acres (Bt fields and non-Bt refuges) should be treated with insecticides targeted at CRW adults as well as larvae.

- A resistance monitoring strategy for CRW-protected 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. Baseline susceptibility studies for WCRW have been initiated from populations collected in 1999 and 2000 by Dr. Blair Siegfried (University of Nebraska), Dr. Larry Chandler (USDA-ARS) and Dr. Wade French (USDA-ARS). These studies should be continued for WCRW and initiated for NCRW and monitoring techniques such as discriminating dose concentration assays need to be thoroughly investigated for their feasibility as resistance monitoring tools.

- There is a concern regarding Monsanto's proposed outline of detecting and confirming resistance. Monsanto suggests that they will initiate mitigation measures when unexpected levels of CRW damage occur. However, Monsanto does not describe what is meant by unexpected levels of damage. Some level of damage is expected since there is not a high dose of MON 863 expressed to control the CRW and research has shown that some level of "grazing" will occur. Since confirmation of resistance is time-consuming, an interim remedial action (mitigation) plan should be implemented as soon as resistance is suspected. Monsanto must identify what unexpected levels of damage are and relay this information to EPA, seed dealers, extension specialists, NCGA and growers. Monsanto should also develop an remedial action plan that is approved by the Agency.

BACKGROUND

Monsanto Co. has submitted an application to EPA for the registration of *Bacillus thuringiensis* (Bt) Cry3Bb1 protein and the genetic material (Vector ZMIR13L) necessary for its production in corn. Corn expressing the Cry3Bb1 protein is intended to provide protection against the corn rootworm (CRW, *Diabrotica* spp.). This product has been designated event MON 863 by Monsanto. Prior to registering Cry3Bb1 corn, an acceptable insect resistance management (IRM) plan is necessary. Monsanto designed a plan intended to be scientifically valid for resistance risk mitigation and feasible for growers to understand, implement and comply with. Since Monsanto acknowledges that a robust and practical IRM plan will require time to develop, they are proposing a three-year interim plan. An interim plan was submitted by Monsanto because they believe growers need to be able to grow MON 863 corn for a period of time so that important information can be generated and growers are provided an understanding of IRM requirements.

Monsanto submitted several documents in support of their proposed IRM plan. An IRM plan for MON 863 corn dated June 20, 2000 was submitted to the Agency (MRID No. 451568-05). This submission included information on dose, CRW biology, simulation models of resistance development, and grower surveys. Research reports and results of grower surveys were also included in the Appendices of the June 2000 submission. An amended IRM plan dated January 8, 2002 was submitted to the Agency for review (MRID No. 455770-01). The amended plan titled "An Interim Insect Resistance Management Plan for Corn Event MON 863: A Transgenic Corn Rootworm Control Product" was intended to supercede MRID No. 451568-05. Therefore, MRID No. 451568-05 was used for additional information and as reference material, but was not formally reviewed. An additional preliminary research report dated February 20, 2001 was submitted to the Agency by Monsanto (MRID No. 453484-01). Although this report is preliminary and was not formally reviewed, information found in MRID No. 453484-01 was considered in evaluating Monsanto's proposed interim IRM plan. A review in the form of a data evaluation report (DER) of Monsanto's interim IRM plan found in MRID No. 455770-01 is attached to this memorandum.

CONCLUSIONS

Currently, there is not enough information available to definitively determine the size and structure of a non-Bt corn refuge for MON 863 needed to appropriately delay the development of CRW resistance. In a memorandum dated April 12, 2002 and e-mailed to the Agency by Russ Schneider (Monsanto Co.), the Canadian Food Inspection Agency (CFIA) states that the current Monsanto IRM plan is based on a high dose product for ECB control; however, MON 863 does not provide a high dose. Therefore, the CFIA concluded that "[i]t is currently uncertain whether requirements for refuge placement and the proportion of field planted to a refuge will be similar or different to that in place for ECB hybrids. The Agency acknowledges that there is a lack of information on dose and pest biology; therefore, a conservative approach to CRW IRM is necessary. Current information suggests that MON 863 provides a low to moderate dose of Cry3Bb1 for CRW.

The Agency agrees with the following recommendations quoted from the CFIA April 12, 2002 memo. "1. The refuge of a non-Bt corn hybrid consists of no less than 20% of the total corn planted in a field. 2. The refuge and Bt planting areas should both have the same crop rotation histories. 3. The refuge is located within a field with a flexible configuration of non-Bt rows planted to convenience the grower and orientation of the field. 4. Where circumstances require that a refuge cannot be planted within a field, the refuge must be planted adjacent to the Bt field. 5. The refuge may be treated with a banded soil insecticide for larval rootworm control."

Based on current information on CRW biology, MON 863 dose, simulation models, hybrid availability and adoption rate, a 20% refuge should be adequate to produce enough CRW adults to delay resistance. In addition, planting refuges adjacent to or within MON 863 corn fields should allow random mating of CRW even though there is limited movement of larvae and virgin females.

Monsanto expects the number of acres of MON 863 corn will be limited in each state during the first three years of registration because of a lack of hybrid availability and slow grower adoption rates as they evaluate the new technology. Therefore, Monsanto suggests that there will potentially be a greater than 20% refuge leading to a decrease in the risk of resistance. Although Monsanto does not plan to plant a refuge for inbred seed increase fields, they will treat with insecticides that will probably control any resistant CRW adults that may emerge. It is, therefore, acceptable to plant inbred seed increase acres without a refuge.

Planting a limited number of acres per state of MON 863 corn with a 20% non-Bt corn refuge planted adjacent to the Bt field or as strips within the field should be adequate to ensure resistance does not develop during the three year interim period requested by Monsanto. Although unmated adult CRW females have been shown to move up to ten rows, it may not be practical for growers to plant ten row strips. Therefore, at least six rows (but preferably 12 rows) of non-Bt corn should be planted when in-field strips are utilized as refuge (Onstad *et al.* 2001). In addition, refuges should be planted with similar hybrids as the MON 863 field and identical agronomic practices should be employed for the transgenic and non-transgenic fields to ensure simultaneous adult emergence.

The NCR-46 committee which consists of research and extension CRW specialists as well as other cooperators commented on Monsanto's interim IRM plan at the request of the BPPD IRM Team. This group is recognized at the national authorities on CRW biology, ecology and management. The NCR-46 agrees that the potential for CRW to develop resistance to MON 863 during a three year interim period with low adoption rates is negligible. Since there is not a high dose of MON 863, there will be a significant number of CRW larvae survival. In addition, evidence of changes in larval feeding behavior on MON 863 corn will lead to increased larval survivorship. This increase in larval survivorship will result in a decreased chance of resistance.

Monsanto claims that MON 863 controls CRW (*Diabrotica* spp.). This implies that all CRW species including WCRW, NCRW, MCRW and SCRW will be controlled by MON 863. However, in their "interim IRM plan", Monsanto does not address the Mexican corn rootworm or southern corn rootworm. Information on pest biology and refuge structure is needed for a general claim of *Diabrotica* spp. control. Additional information is needed on various aspects of CRW pest biology as it relates to an IRM strategy.

Monsanto also does not adequately address grower education or mitigation measures if resistance occurs. A detailed grower education program is necessary to ensure grower compliance, particularly since the IRM requirements may change as more information becomes available. Growers should be required to sign a Technology/Stewardship Agreement each year they intend to plant MON 863 corn to ensure they are aware of the current IRM requirements.

There is also concern regarding Monsanto's outline of detecting and confirming resistance. Monsanto suggests that they will initiate mitigation measures when unexpected levels of CRW damage occur. However, Monsanto does not describe what is meant by unexpected levels of damage. Some level of damage is expected since there is not a high dose of MON 863 expressed

to control the CRW and research has shown that some level of "grazing" will occur. Monsanto must identify what unexpected levels of damage are and relay this information to EPA, seed dealers, extension specialists, NCGA and growers. This may be accomplished in many ways including websites, a 1-800 telephone number and Technology Use Guides. Further investigation into confirming resistance is also needed. Monsanto's time-line includes identifying the insects as resistant, confirming the plant is expressing MON 863 and taking mitigation measures within 90 days. After this length of time, the growing season will likely be over and it will be too late for mitigation measures. It is, therefore, necessary for Monsanto to cease sales in an affected area after a confirmed report of resistance. The area and amount of time (in years) that sales should cease should be determined in consultation with EPA.

In addition, pages 6 and 9 of the IRM submission states that "[h]ybrids containing event MON 863 will be released both as stand-alone products and in combination with the *cry1Ab* gene for lepidopteran control because of the combined insect management needs of many farmers." However, the risk of resistance from a Cry1Ab/Cry3Bb stacked product has not been determined. Prior to the release and distribution of a Cry1Ab/Cry3Bb1 "stacked" product, an acceptable IRM plan specifically designed for a "stacked" product must be submitted to the Agency for review. Although refuges are similar for the lepidopteran protected and coleopteran protected Bt corn, Monsanto should consult with the Agency prior to distributing combination products.

DATA EVALUATION REPORT

REVIEWED BY:	Robyn Rose, Entomologist Biopesticides and Pollution Prevention Division
SECONDARY REVIEWERS:	Sharlene Matten, Ph.D, Biologist Alan Reynolds, Entomologist Biopesticides and Pollution Prevention Division
STUDY TYPE:	Insect Resistance Management Plan
MRID NO.:	455770-01
STUDY NO.:	MSL-17556
SPONSOR:	Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO 63198
TEST MATERIAL:	*Bacillus thuringiensis* (Bt) corn event MON 863
AUTHOR:	Ty Vaughn, Dennis Ward, Jay Pershing, Graham Head, and John McFerson
STUDY COMPLETED:	December 15, 2001
CLASSIFICATION:	No classification since this is a non-guideline study

---

Title: "An Interim Insect Resistance Management Plan for Corn Event MON 863: A Transgenic Corn Rootworm Control Product:

Authors: Ty Vaughn, Dennis Ward, Jay Pershing, Graham Head, and John McFerson

Objective: This plan was designed by Monsanto to ensure that the risk of CRW developing resistance to MON 863 corn is "insignificant" during a three-year interim period.

Summary of the General Elements of Monsanto's Proposed Interim IRM Plan:

This section summarizes Monsanto's discussion of their proposed insect resistance management (IRM) plan summarized in MRID No. 455770-01. Monsanto has developed an IRM plan that they believe will be cost-effective, flexible, easily adopted by growers and compatible with common production practices. They are requesting that this plan be considered an interim IRM plan for *Bacillus thuringiensis* (Bt) event MON 863 corn rootworm-protected corn. Under Monsanto's proposal, this IRM plan would be implemented for three years while additional research is conducted to determine an acceptable long-term strategy to mitigate corn rootworm (CRW; *Diabrotica* spp.) resistance to MON 863 (Table 1). Monsanto intends for this IRM plan to evolve over time as new information becomes available. In their submission, Monsanto suggests planting a 20% non-Bt corn refuge planted within or adjacent to MON 863 fields to encourage random mating of CRW.

Monsanto expects negligible selection pressure for resistance during the first few years after MON 863 is sold commercially because low initial adoption rate is anticipated due to limited hybrid availability and grower characteristics. Availability of MON 863 to growers will be low

initially because of production constraints in converting inbred lines used in the production of commercial hybrids. In addition, grower adoption is expected to be slow while this technology is new and they need to determine how it will fit into their current farming practices. Monsanto also intends to reduce the risk of resistance by encouraging stakeholders such as growers, Extension Agents and crop consultants to promote and practice integrated pest management (IPM) . For instance, scouting fields in the Fall will be encouraged and planting MON 863 on acres with the highest risk of damage will be promoted.

In Monsanto's plan, growers will be encouraged to manage refuges to promote adequate beetle survival and CRW fecundity. For instance, Monsanto suggests that refuges should promote moderate to high CRW populations because decreasing the number of larvae should lead to an increase in the number of adults emerging. This may be accomplished by planting insecticide treated seed or through soil insecticide application. Adult emergence in refuges will be extended by planting a mid to full-season hybrid. Also, beetle survival can be improved in refuges by planting large blocks or adjacent fields prior to egg hatch and away from areas prone to flooding or standing water.

Table 1. List of research currently underway sponsored by Monsanto

Study Title	Author(s)	Relevance to IRM
Post-establishment movement of western corn rootworm larvae - three years of data under Missouri conditions	Bruce E. Hibbard	*Pest Biology & Refuge Placement
Tracking western corn rootworm premating movement	Doug Tallamy	*Pest Biology and Refuge Placement
Efficacy of between field refuges for resistance management of transgenic corn rootworm-insecticidal corn	David Andow & Donald Alstad	*Simulation Models and Refuge Placement
Modeling the dynamics of adaptation to transgenic corn by western corn rootworm	David Onstad***	*Simulation Models
An economic model of CRW protected transgenic corn IRM strategies	Terry Hurley	*Simulation Models
An economic model of CRW protected transgenic corn IRM strategies	John Foster	*Pest Biology
Resistance management for western corn rootworm (Coleoptera: Chrysomelidae) using seed mixtures of transgenic corn expressing the Cry3B protein	Wade French	*Refuge
Resistance management for western corn rootworms (Coleoptera: Chrysomelidae) using seed mixtures of transgenic corn expressing the Cry3B protein	Elson Shields	*Refuge
Resistance management for western corn rootworms (Coleoptera: Chrysomelidae) using seed mixtures of transgenic corn expressing Cry3B protein	Dennis Calvin	*Refuge

219

Study Title	Author(s)	Relevance to IRM
Impact of transgenic corn rootworm protected corn on selected western corn rootworm life history traits	Lance Meinke	*Dose
Flight characteristics of female western corn rootworms exposed to transgenic plants	Jon Tollefson & Ted Wilson	*Pest Biology & Refuge Placement
Efficacy of Monsanto's lead transgenic events resistant to corn rootworm larvae feeding: root damage ratings and adult emergence	Jon Tollefson *et al.*	*Dose
An evaluation of CRW dispersal and mating behavior	Joseph Spencer	*Pest Biology & Refuge Placement
Estimating base line susceptibility in natural population of CRW	Blair Seigfried	*Resistance Monitoring
Estimating the impact of foxtail on CRW populations	Ken Ostlie Mike Gray	*Refuge
Larval feeding behavior of *Diabrotica virgifera virgifera*: progress report	Pete Clark & John Foster	**Pest Biology
An evaluation of Cry3Bb expression and impact on western corn rootworm from transgenic events	Vaughn *et al.*	**Dose
Efficacy of advanced transgenic corn rootworm events against CRW pest species - results of year 2000 & 2001 trials	DeGooyer	**Dose
1999 evaluation of Monsanto transgenic corn germplasm for corn rootworm larval feeding resistance and adult emergence	Hibbard	**Dose
Impact of CRW-protected corn on silk clipping & beetle numbers	Davis, Pilcher & Spangler	**Dose

* Research summarized in MRID 453484-01
** Research summarized in Appendix C of MRID 455770-01
***This paper is now published in Onstad *et al.* 2001

Pest Biology:

Summary of Monsanto's Proposal

Monsanto discusses pest biology in Section IV. Justification of Refuge Configuration in MRID No. 455770-01. Additional research currently underway is summarized in MRID 453484-01 (Table 1). Pest biology is important to refuge placement since the goal is to encourage random mating between pests emerging from the transgenic and non-transgenic corn fields. By placing a non-Bt refuge in close proximity to a Bt corn field, insects that may carry an allele for resistance will potentially encounter and mate with insects that don't have the resistance allele. According to Monsanto, current information indicates that non-Bt refuges should be located within or adjacent to MON 863 corn fields to ensure random mating and dilution of the frequency of resistant alleles. Planting refuges adjacent to or within MON 863 fields is based on potential for and relative distance of adult dispersal.

Several factors will influence CRW fitness and survival including environmental influences, intraspecific and interspecific competition and later adult emergence. Earlier-emerging adults have been found to be more fecund and demonstrate a higher rate of survival then later-emerging adults (Boetel and Fuller 1997). Adult emergence may be delayed by planting later because corn hybrids grow more nodes as they mature which leads to more CRW adults per plant. Time of flowering and temperature also influence when adults emerge (Bergman and Turpin 1984, Naranjo and Sawyer 1988, Elliot *et al.* 1989, Davis *et al.* 1996, Stavisky and Davis 1998). According to Monsanto, studies conducted thus far with MON 863 corn suggest that adult emergence is delayed seven to ten days when developing on Bt then non-Bt hybrids.

Adult WCRW and NCRW feeding and behavior studies were conducted in 1999 to compare silk clipping and within plant spatial distribution in Bt, non-Bt and insecticide treated plots (Appendix C). At the six locations tested, there were few significant differences observed in silk clipping, total number of CRW per plant or adult location within the plant canopy. These results suggest that MON 863 does not repel CRW adults.

According to Monsanto's submission, NCRW and WCRW adults are considered highly mobile insects. Male and female adults are known to fly at different heights within the corn canopy and wind has been shown to deter flight initiation of CRW. Since higher quality food is preferred by adults, CRW fly from more mature corn fields to younger fields, resulting in between field movement. Current unpublished research conducted by Dr. Joseph Spencer (University of Illinois) indicated that millions of male and female WCRW fly over and land in corn fields daily (MRID 453484-01 IRM Trial No. 13). Results of mark-and-recapture studies indicate that male and female adult WCRW will move within and between corn fields (MRID 453484-01 IRM Trial No. 13). NCRW demonstrate more limited movement then WCRW and are not known to migrate long distances. However, NCRW do migrate to new fields particularly if the habitat quality is improved which will ensure the colonization of new fields.

Adult female WCRW are generally believed to mate within the field they emerge from; whereas, adult males and mated females may move between fields. The significant dispersal observed in males can dilute resistance alleles present in a population. Based on the movement of adult CRW, planting refuges adjacent to or within fields will aid in promoting random mating. Experiments conducted by Monsanto suggest that virgin females may fly distances up to ten or more rows and are readily located by males when population densities are high.

Information is limited on CRW dispersal capabilities which is important in the development of an IRM strategy. Dr. Joseph Spencer will continue research on male and female dispersal prior to mating. This research will focus on determining the time males encounter newly emerged females and mark-and-recapture studies will be conducted to evaluate male dispersal capabilities. Additional studies will be conducted with flight mill assays to evaluate unmated female dispersal potential at Iowa State University by Ted Wilson. Another method of studying dispersal and mating behavior of WCRW is being developed by Dr. Doug Tallamy (University of Delaware). Dr Tallamy is breeding a colony with red elytra that will have potential use in field research. WCRW in Illinois, Indiana and Ontario will also be examined by Dr. Art Schaafsma (Ridgetown

College/University of Guelph) for genetic variation between variant and nonvariant populations. This research will provide information on gene flow that can be considered when designing an IRM strategy.

Reviewer's Comments

Monsanto has identified information on CRW biology from published articles as well as current research currently underway that is important to develop a long-term IRM strategy for CRW in MON 863 corn. Additional information is needed on various aspects of CRW pest biology as it relates to an IRM strategy. Knowledge of the maximum and average distance adult and larval CRW move is limited. Additional research regarding male and female (mated and unmated) adult and larval WCRW and NCRW dispersal potential is needed to determine placement of non-Bt corn refuges. In addition, more information is needed on mating habits, ovipositional patterns, number of times a female can mate and fecundity.

There are three *Diabrotica* species found in corn, WCRW (*Diabrotica virgifera virgifera*), NCRW (*Diabrotica barberi*); Mexican corn rootworm (MCRW; *Diabrotica virgifera zea*) and the southern corn rootworm (SCRW; *Diabrotica undecimpunctata howardi*). Knowledge of CRW biology, dispersal characteristics, host range, feeding habits and history of insecticide resistance is important in developing an IRM strategy. The WCRW and NCRW are the most serious insect pests of field corn in the Corn Belt (Levine and Oloumi-Sadeghi 1991). The SCRW (also known as the spotted cucumber beetle) is a polyphagous pest with over 280 host plant species and predominantly feeds on cucurbits (http://ipmwww.ncsu.edu/AG271/corn_sorghum/southern_corn_rootworm.html). The SCRW does not cause serious injury to corn roots in the Corn Belt because of their late arrival. In the U.S., the MCRW is known to occur in Kansas, Oklahoma, and Texas (Levine and Oloumi-Sadeghi 1991). A study conducted by Branson *et al.* (1982) showed no differences in developmental rates of WCRW and MCRW. However, additional studies have shown that the MCRW larvae take longer to develop then WCRW (Jackson and Elliott 1988, Woodson and Chandler 2000). Additional research is needed to determine if IRM strategies designed for WCRW and NCRW are appropriate for MCRW. Since MON 863 expression in corn roots likely declines as the plant matures and MCRW may develop slower the risk of resistance may differ for MCRW then WCRW and NCRW.

Characteristics of pest biology that are relevant to IRM (e.g., movement, feeding habits and ovipositional habits) differ for WCRW and NCRW. WCRW and NCRW adults will feed on corn silks, pollen and young kernels in the ear tip; however, only WCRW feed on leaves. Since NCRW adults don't feed on corn leaves, they leave the field after pollination to find a younger field with pollen available (Branson and Krysan 1981). Young leaves contain the highest level of MON 863 with an average of 81 µg/g, grain has an average of 70 µg/g, root and forage have an average of 39 and 41 µg/g, pollen has and average of 62 µg/g and silks contain the lowest levels with an average of 10 µg/g (MRID No. 451568-02). Since adult and larval CRW feed on various parts of the corn plant, both life stages may be exposed to the Bt protein and extended selection pressure may result (Meinke *et al.* 2001). Severe root damage from larval feeding will

lead to plant lodging and yield losses.

WCRW and NCRW are univoltine, develop through one generation per year, in corn in most of the Corn Belt (Branson and Krysan 1981, Meinke *et al.* 2001). CRW typically oviposit where the adults are feeding which is almost exclusively in corn fields (Branson and Krysan 1981, Levine and Oloumi-Sadeghi 1991). In general, CRW adult emergence varies based on species, geography, weather, management practices such as insecticide use, population density and sex. For instance, males typically emerge before females and emergence, as well as fecundity, longevity and egg viability, are reduced in corn planted later in the season (Boetel and Fuller 1997, Levine and Oloumi-Sadeghi 1991, Meinke *et al.* 2001). It is unknown what effect corn rootworm protected transgenic corn will have on phenology, sex ratio and adult emergence patterns. According to the NCR-46 (a technical committee consisting of research and extension CRW specialists and other cooperators), adult CRW emergence is prolonged four to six weeks in MON 863 corn then non-transgenic corn (Meinke *et al.* 2001). Asynchronous adult emergence for Bt corn fields and non-Bt refuges may lead to nonrandom or assortive mating which may lead to an increase rate in the evolution of resistance. Nonrandom or assortive mating may also occur if Bt corn disrupts the synchrony of male and female CRW adult emergence (Meinke *et al.* 2001). Mating typically occurs within 24 to 48 hours of female adult emergence within the corn fields they emerged from or nearby (Meinke *et al.* 2001).

CRW larval movement is limited particularly in areas with low population densities (Meinke *et al.* 2001). Published and unpublished articles have reported varying distances that CRW larvae move. WCRW larvae may move from 12 to 16 inches and have been found in corn rows planted up to 40 inches apart (Suttle *et al.* 1967, Short and Luedtke 1970, Gray 1999). These studies suggest that CRW larvae hatching from eggs between rows are capable of finding and injuring corn roots regardless of row spacing. Since field corn is typically planted approximately 24 to 30 inches apart, CRW may move between two to three rows. In general, young CRW larvae (e.g., 1st, 2nd and sometimes 3rd instars) tend to move toward actively growing corn roots. Larval tendency toward respiring, growing corn roots is probably because of their ability to detect and move toward CO_2 (Strnad *et al.* 1986, Gray 1999). Young larvae will feed on the distal portion of corn roots and move through the soil to feed on new, short roots as they develop into later instars (Strnad and Bergman 1987, Gray 1999). It is therefore possible that a RS heterozygous larvae with a partially recessive resistance trait will begin feeding on transgenic corn roots and finish its development on adjacent non-transgenic roots which would result in a non-lethal dose of MON 863 and potential resistance. Movement of larvae is deterred by soil that is too wet or too dry and is greater in silty clay or loamy soils then loamy sand soils (Gray 1999).

NCRW and WCRW mated adults may be very mobile and have potentially high dispersal capabilities (Meinke *et al.* 2001). However, local dispersal is more common and involves movement within or among adjacent fields; whereas, migratory dispersal over long distances occurs in a small portion of populations and usually involves females (Meinke *et al.* 2001). Research conducted by Van Woerkom *et al.* (1983) showed that CRW are typically low fliers, wind affects beetle mobility and deters flight initiation, females fly higher than males and are found in greater numbers above the crop canopy. Dispersal capabilities of the WCRW are

greater than the NCRW. The WCRW is also a greater competitor and displaced the NCRW in Nebraska by 1980 (Hill and Mayo 1980). WCRW post-mating dispersal may be local or migratory.

Coates *et al.* (1986) used mated WCRW females collected from the field in a flight mill to determine duration and length of flights. Trivial flights (<30 min.) lasted from one to 17 minutes and sustained flights (≥30 min.) for females less than nine days old occurred up to four hours. Based on this data, Coates *et al.* (1986) speculated that preovipositional WCRW females capable of unassisted flights could move an average of 35 km per day (200 km overall) for the first six days after emergence. This data suggests that some females may leave the field after mating to oviposit elsewhere. While sustained flights by mated female CRW are possible, movement by unmated females is limited. Knowledge of the maximum and average distance an adult CRW moves is limited. Additional research regarding adult and larval WCRW and NCRW dispersal potential is needed to determine placement of non-Bt corn refuges.

CRW Adaptive Strategies:

Summary of Monsanto's Proposal

CRW have developed several adaptions to control methods including crop rotation and insecticide resistance. A common agronomic practice used to control CRW involves rotating corn with soybeans. Rotating soybeans after corn decreases the need for CRW-targeted insecticide applications. However, WCRW has developed an adaptation to resist the corn/soybean rotation in Illinois and Indiana (Levine and Oloumi-Sadeghi (1996). WCRW have been found to lay their eggs and overwinter in soybeans allowing them to emerge in corn fields the following spring. NCRW populations have also developed resistance to the corn/soybean rotation in Minnesota, Iowa, and South Dakota (Gray *et al.* 1998). NCRW have developed the ability for extended diapause resulting in a significant proportion of their eggs hatching after two winters.

The WCRW has also developed resistance to soil-applied organochlorines as well as organophosphate and carbamate sprays in Nebraska (Ball and Weekman 1963, Metcalf 1983). Organochlorine insecticides were used as pre-plant treatments in the 1940's and resistance occurred by the 1950's. Cyclodiene insecticides including aldrin, chlordane and heptachlor were used in the 1950's to control root feeding damage; resistance to these chemicals was observed after 12 years and led to widespread control failures within 20 years. Organophosphate and Carbamate insecticides were introduced to replace Cyclodienes. However, Meinke *et al.* (1998) found adult populations resistant to organophosphates and carbamates in Nebraska and also documented control failures of methyl parathion in the early 1990s.

Instances of CRW resistance to crop rotation and/or insecticide use typically develop on a local scale which is probably due to limited adult movement before and after mating. In these cases, resistance took at least ten and usually more than 15 years to develop with out implementing IRM strategies. Research is currently underway at the University of Nebraska and USDA-ARS

in North Dakota to detemine the genetics of esterase-mediated insecticide resistance in WCRW populations. Results of this research are intended to provide knowledge on localized selection and migration that may aid in refining future IRM strategies.

Reviewer's Comments

WCRW has developed resistance to certain pesticides and crop rotation practices. For instance, WCRW was four times less susceptible to aldrin in 1981 than when it began to be used 20 years earlier in Nebraska and most corn acres were affected by 1980 (Ball 1983, Metcalf 1986, Gray 1999). Additional WCRW resistance to methyl parathion and carbaryl was documented by Meinke *et al.* (1998) in Nebraska and heritability of the resistant trait was shown from F_1 generation individuals. A 16.4-fold resistance to methyl parathion and 9.4-fold increase in resistance to carbaryl was determined (Meinke *et al.* 1998). There has been some research conducted to determine the mechanism of insecticide resistance in CRW populations. Studies were conducted by Miota *et al.* (1998) to examine two WCRW populations to determine the mechanism of methyl parathion and ethyl parathion. Resistance was due to a combination of metabolic detoxificatiion and target site insensitivity; however, the mechanism of resistance differed among the observed populations. Miota *et al.* (1998) concluded that the mechanism of resistance for one of the observed populations was due to increased metabolic detoxification by NADPH-dependent monooxygenases and general esterases and another population was due to and interaction of acetylcholinesterase sensitivity and hydrolytic metabolism.

It is likely that the mechanism of CRW resistance differs between insecticides and Cry3Bb1. The mechanism and genetics (e.g., one gene, multiple genes) of potential resistance of CRW to MON 863 needs to be determined to develop an appropriate IRM strategy. Since CRW resistance to Cry3Bb1 under field conditions is necessary to determine the mechanism and genetics of resistance, colonies resistant to Bt should be established and evaluated in the laboratory during the initial three years MON 863 is grown commercially.

Since CRW predominantly oviposit in corn fields, rotating corn with small grains, hay, clover or alfalfa has been utilized as a control method (Levine and Oloumi-Sadeghi 1991). CRW have also been controlled by planting soybean after corn since CRW cannot survive on soybean. However, in areas such as east-central Illinois and northern Indiana, the WCRW has been found to have the ability to lay eggs in soybean, overwinter and hatch the following year in corn (Levine and Oloumi-Sadeghi 1991, Levine and Oloumi-Sadeghi 1996, O'Neal *et al.* 1999, Isard *et al.* 1999, Isard *et al.* 2000). NCRW has developed a prolonged or extended diapause adaptation to rotating corn with crops such as soybean. Prolonged diapause of NCRW involves eggs that remain viable for two winters and hatch two seasons after being laid. Extended diapause has been verified in the laboratory from NCRW eggs collected from South Dakota, Minnesota, Illinois and Michigan (Krysan *et al.* 1984, Krysan *et al* 1986, Levine and Oloumi-Sadeghi 1991 Levine *et al.* 1992a, Levine *et al.* 1992b). Field studies conducted by Tollefson (1988) in northwestern Iowa corn fields suggests that extended diapause occurs throughout NCRW distribution in rotated fields. Another study conducted by Levine and Oloumi-Sadeghi (1996) suggests that the WCRW does not demonstrate extended diapause. The effect of WCRW

ovipositing in soybean prior to overwintering and extended diapause in NCRW on an IRM strategy needs further investigation.

Dose:

Summary of Monsanto's Proposal

Determining the level of dose is crucial to what size and structure of a refuge is needed to delay CRW resistance to Cry3Bb proteins. Monsanto identifies a high dose and low dose strategy for delaying the development of resistance. A high dose strategy would involve killing a high proportion of the target pest population. As part of the IRM strategy, the high dose would be used in conjunction with a structured refuge designed to produce a large number of susceptible pests. The high dose/refuge strategy is currently in use to delay European corn borer (ECB; *Ostrinia nubilalis*) resistance to lepidopteran-active Bt crops.

A low dose strategy identified by Monsanto is employed for products that have limited impact on the fitness, survival and selection pressure of the target pest. Products with a low dose may not require a refuge and may have a longer period of durability. In general, refuge size for a low dose product is not well understood. With a low dose strategy it may be difficult to sufficiently control the pest while limiting effect on its fitness. Since CRW feed on corn roots and some level of this damage may be tolerated by the plant, Monsanto proposes that a low dose strategy may be appropriate.

According to Monsanto, research on CRW feeding behavior and survival and root expression data will help identify the dose of MON 863. Studies recently conducted by Monsanto as well as Dr. John Foster and Mr. Pete Clark (University of Nebraska - Lincoln) show that CRW larvae that feed on MON 863 corn begin feeding on growing root tips, but don't penetrate into the root (Appendix C). CRW larvae feeding on corn roots were observed to graze around the exterior of the root without penetrating, thus slowing their development. Larval feeding behavior observed on MON 863 corn roots may be due to an adverse reaction of CRW taste receptors or a physiological effect leading to a change in feeding behavior. A number of roots evaluated in the field by Root Damage Rating (RDR) had channeling on the outside of roots and no penetration. The RDR is based on a 1-6 rating scale and is considered the most sensitive and consistent method of evaluating CRW damage (Branson *et al.* 1980, Knutson *et al.* 1999). Lower values on the root rating scale are considered to have a lower level of damage. A reduction in CRW feeding on MON 863 was demonstrated by a screening trial conducted by Monsanto that showed an average damage rating for MON 863 to be 1.85 on the RDR scale compared to a non-Bt check which had a 4.44 RDR rating (MRID 451568-05, Table 3 on pg 61).

The dose level of MON 863 against WCRW larvae was determined by root expression assays and CRW bioassays conducted by Dudin *et al.* (2001). Results of this study determined the Cry3Bb1 expression in corn roots is 58 ppm which is slightly lower than the LC_{50} of 75 ppm determined from laboratory colonies (see Monitoring section for LC_{50} information). Cry3Bb1 protein expression is thought to be high in the root tips where neonate larvae initiate feeding.

Neonates that avoid toxic doses of MON 863 because of the grazing behavior observed on the exterior of roots may tolerate the Bt toxin during the remainder of their development. Therefore, Monsanto concluded that a high dose strategy is not appropriate for MON 863 expressing corn based on the LC_{50} determined from root feeding and a reduction in susceptibility of second and third instar larvae observed in laboratory bioassays. Monsanto also suggests that efficacy studies indicate that MON 863 provides improved and more consistent protection of corn roots then insecticides (MRID 453613-03). In corn with the same CRW larval pressure, an average root rating of 2 has been determined for MON 863, whereas an average root rating of 3 has been determined for insecticide treated corn. This indicates a higher level of protection provided by MON 863 then currently registered larval insecticides.

A high dose can be defined as <0.001% survival of larvae (FIFRA SAP 1998); therefore, larval survival is another technique that may be utilized to determine dose. Survival of CRW larvae on MON 863 expressing roots has been estimated to range from 17% to 62% (Appendix C). Gray *et al.* (1992) found insecticide control of CRW larvae is highly variable. This variability may be due to a lack of root availability leading to density-dependent mortality, soil moisture and rainfall, characteristics of insecticides, application rate and methods (Branson and Sutter 1985, Elliott *et al.* 1989, Levine and Oloumi-Sadeghi 1991). These same variables that affect efficacy of insecticides will likely affect effectiveness of MON 863.

Data from additional research being conducted will help identify MON 863 dose and effective refuge size. Dr. Lance Meinke (University of Nebraska) is studying fitness of WCRW feeding on MON 863 including fecundity, longevity, developmental time, size and sexual competitiveness of males feeding on MON 863 root tissue in the greenhouse.

Reviewer's Comments

For lepidopteran-active Bt corn products, a high dose is recommended to delay resistance in ECB. A high dose for lepidopteran-active Bt proteins is defined as 25 times the amount of Bt delta-endotoxin necessary to kill susceptible individuals (SAP 1998). Bt plant incorporated protectants (PIPs) could be considered to provide a high dose for ECB if verified by at least two of the following five approaches: 1) Serial dilution bioassay with artificial diet containing lyophilized tissues of *Bt* plants using tissues from non-*Bt* plants as controls; 2) Bioassays using plant lines with expression levels approximately 25-fold lower than the commercial cultivar determined by quantitative ELISA or some more reliable technique; 3) Survey large numbers of commercial plants in the field to make sure that the cultivar is at the $LD_{99.9}$ or higher to assure that 95% of heterozygotes would be killed (see Andow and Hutchison, 1998); 4) Similar to #3 above, but would use controlled infestation with a laboratory strain of the pest that had an LD_{50} value similar to field strains; and 5) Determine if a later larval instar of the targeted pest could be found with an LD_{50} that was about 25-fold higher than that of the neonate larvae. If so, the stage could be tested on the *Bt* crop plants to determine if 95% or more of the later stage larvae were killed (SAP 1998). It has not been determined if this definition and verification of a high dose is appropriate for coleopteran-active Bt corn hybrids nor has it been concluded that a high dose is necessary to mitigate resistance.

For a high dose strategy, a resistance (*R*) allele frequency should be <.001 (Andow and Alstad 2002). According to Monsanto, a high dose strategy for coleopteran protected corn would involve killing a high proportion of the target pest population. However, according to the definition of a high dose (SAP 1998) all susceptible insects would be killed. Monsanto provided data that suggest 17-62% of CRW larvae may survive when feeding on MON 863. Therefore, it can be concluded that MON 863 does not have a high dose for control of CRW.

A moderate dose was defined by Monsanto in their use of Caprio's model as 30% survival of larvae and a low dose as 50% survival. Since data suggests that there is >17% survival of CRW larvae feeding on MON 863 corn roots, it can be concluded from the modified Caprio model that there is a low to moderate dose of MON 863 for CRW.

"Further research is needed to provide the critical data on the actual dose effects of Bt corn, corn rootworm biology and ecology" (Ostlie. 2001). Ongoing research is being conducted by Monsanto. Although adult CRW will feed on corn tissue expressing the Cry 3Bb1 protein, larval feeding on corn roots is the predominant mode of exposure. Therefore, expression of MON 863 in roots is particularly important to determining the level of MON 863 CRW are exposed to. First and second instar WCRW larvae have been observed to feed on the meristematic region of the root or tip (MRID No. 455770-01 Appendix C). Less older instars have been found on MON 863 roots then non-transgenic corn roots when feeding during the V4 and V6 growth stages; however, older instars did not seem to be affected by the V8 growth stage (MRID No. 455770-01 Appendix D). Preliminary results of Monsanto's root expression study can be found on pg. 99, Figure 4a-h of MRID No. 455770-01 and an explanation of acronyms is found of pg.95. A final report of these studies should be submitted to the Agency for review upon completion. Research is being conducted in Wharlon, TX to evaluate the efficacy of transgenic coleopteran-protected Bt corn against MCRW (http://usda-apmru.tamu.edu/WCH/evaluation_of_genetically_modifedi.htm).

Simulation Models of Resistance:

Summary of Monsanto's Proposal

Simulation models are used to evaluate the importance of IRM and appropriate strategies to delay resistance. Assumptions in resistance models are based on aspects of pest biology including CRW survival and fitness. A conservative model developed by Dr. Michael Caprio (Mississippi State University) was modified and used by Monsanto to predict the risk of CRW developing resistance to MON 863 corn (Tables 2 and 3). Caprio's model assumes 100% market penetration, no alternate host crops, no fitness costs in resistant CRW and that resistance is due to a single gene. Resistance due to multiple genes would probably occur slower or at the same rate as single gene influences. Based on results of this model, Monsanto concluded that a 20% non-Bt refuge planted within or adjacent to MON 863 fields would delay resistance for 11 years regardless of dose. Since there will not be a 100% initial adoption of MON 863 corn, Monsanto suggests that the "effective refuge" will be much greater then 20%.

Two models, one developed by Dr. David Andow (University of Minnesota) and another developed by Dr. David Onstad and Mr. Charles Guse (University of Illinois), will also be used by Monsanto to develop their long-term IRM plan (Table 4). The Onstad and Guse model considers four crops (continuous corn, first year corn, soybean and a non-corn or cucurbitaceous crop) and six fields (up to four corn fields). Their model suggest that planting refuges as blocks would delay CRW resistance longer than in-field strips (Onstad *et al.*2001). According to Monsanto, Onstad and Guse's model as well as the Monsanto modified Caprio model indicate that product durability is not greatly affected by refuge size in low and moderate dose CRW protected Bt corn. See Figure 1 on page 26 of MRID 455770-01 for a graphic comparison of refuge size and dose on the number of years to development of resistance shown by Caprio's model. Andow's model considers typical midwestern planting practices including continuous and rotated Bt or non-Bt corn and soybean. Therefore, Andow's model (as well as Onstad's model) accounts for CRW populations ability to adapt to crop rotation.

Assumptions of models created thus far have not been tested or validated. Field testing and validation of these models will be continued during the three year interim period requested by Monsanto. Additional models will also consider the potential of seed mixtures, dose and behavioral avoidance.

Table 2. Input values for MON 863 performance parameters used in Caprio simulation. These values are applicable for low to moderate dose product.

Parameter	Input Value
Survival of SS genotype	0.1 - 0.5
Survival of SR genotype	1
Survival of RR genotype	1
Refuge size (%)	0 - 60
Initial resistance allele frequency, q (t=0)	0.001
Move outside habitat before mating (R)	1
Move outside habitat before ovipositing (M)	1

Table copied from MRID 455770-01 page 25 Table 3.

Table 3. Caprio model predictions of product durability, expressed in years to resistance as a function of refuge size and dose (*i.e.*, survival rate). Input values for the low and SS genotype was set at 0.01 and survival of he SR genotype was set at 0.1.

Survival Rate	Refuge Size (%)			
	0	20	40	60
0.01 (high dose)	4	19	42	86
0.3 (moderate dose)	8	11	16	26
0.5 (low dose)	13	17	24	27

Table copied from MRID 455770-01 page 25 Table 4.

Table 4. Monsanto's comparison of model structure, parameters, and assumptions for Andow and Onstad's models

Parameter or Description	Andow Model	Illinois Models
Model type	stochastic	stochastic
Model structure	5-patch w/populations in +/- CRW cont. corn, +/- CRW rotated corn, soybean	4 to 6 patch w/populations in +/- CRW cont. corn, +/- CRW rotated corn, soybean, & non-crop
Proportion of crop types	variable, tested 100% & 40% continuous corn	variable, tested 100% continuous corn
Time Step	yearly	daily, day 187-292
Overwintering egg survival	combined with fecundity, default: 50 viable overwintering eggs/female	50%
Density-dependent larval survival	Hassel function w/parameters fit to survival data from artificial infestations or field sampling	exponential function fit to combined data from artificial and field sampling
Larval survival on transgenics	variable (0.001-0.5), imposed prior to density dependent mortality	variable (0.001-0.5), imposed prior to density-dependent mortality
Beetle emergence	–	37 day period, mean emergence: day 207 - males, day 215 - females
Mating pattern	females mate 1 time	females mate up to 2 times, males mate once every 2 days and search 100 m^2 per day
Premating dispersal	random in field, no between field movement	teneral females do not move, random within field
Post mating dispersal	proportional to patch area	mated females distributed proportional to patch size, between fields: 0.02 mated females/day, 0.005 males/day
Oviposition/fecundity	distributed proportional to crop area and crop preference, 50 overwintering eggs/female	random within field, oviposition period: 60 (1st) & 40 (2nd) days, 440 eggs/female, viability declines with beetle age
Beetle survival	–	influenced by frost (day 292), toxicity of transgenic to adults, nutritional status of other crops, and beetle age
CRW Genotypes	SS, SR, RR	SS, SR, RR
Dominance	variable (0.05-1)	variable (0, 0.5, 1)
Fitness cost of R genes	none	none
Initial resistance gene frequency	variable (10^{-3} to 10^{-5})	variable (default 10^{-4})

230

Parameter or Description	Andow Model	Illinois Models
Dominance	variable (0.05-1)	variable (0, 0.5, 1)
Fitness cost of R genes	none	none
Initial resistance gene frequency	variable (10^{-3} to 10^{-5})	variable (default 10^{-4})
Definition of resistance	gene frequency >0.5	gene frequency >0.3
Refuge placement	separate fields	separate fields that remain in same location over time or row strips within field
Treatment of refuge	no	default: no

This table is copied from page 26 Table 1 of MRID 451568-05

Reviewer's Comments

EPA has used predictive models to compare IRM strategies for Bt crops. Because models cannot be validated without actual field resistance and other data, models have limitations and the information gained from the use of models is only a part of the weight of evidence used by EPA in assessing the risks of resistance development. It was the consensus of the 2000 FIFRA SAP Subpanel that models were an important tool in determining appropriate Bt crop IRM strategies. They agreed that models were "the only scientifically rigorous way to integrate all of the biological information available, and that without these models, the Agency would have little scientific basis for choosing among alternative resistance management options." They also recommended that models must have an agreed upon time frame for resistance protection. For example, conventional growers may desire a maximum planning horizon of five years, while organic growers may desire an indefinite planning horizon. The Subpanel recommended that model design should be peer reviewed and parameters validated. Models should also include such factors as level of Bt crop adoption, level of compliance, economics, fitness costs of resistance, alternate hosts, spatial components, stochasticity, and pest population dynamics.

Simulation models are an important tool used to predict possible strategies (e.g., refuge size and structure) to delay insect resistance. Knowledge of pest biology is necessary to creating useful models. Since CRW adults and larvae feed on corn tissue and MON 863 is expressed throughout the plant, models should consider the exposure to all life stages (Gray 1999). Information is still needed to develop appropriate simulation models of resistance for CRW-protected corn. For instance, the initial resistance (*R*) allele frequency in CRW for Cry3Bb proteins is unknown.

A limited number of models have been developed to address IRM strategies for CRW protected corn. Monsanto modified a model developed by Caprio (1998) for cotton bollworm (*Helicoverpa zea*; CBW) to address CRW biology and IRM. Additional models developed for WCRW by Andow and Alstad (2002) and Onstad *et al.* (2001) are summarized below. These models assume 100% grower adoption of Bt corn.

Caprio Model (ILSI-HESI 1998)

A conservative model originally developed by Dr. Michael Caprio (Mississippi State University) for cotton bollworm (*Helicoverpa zea*; CBW) resistance in cotton was modified by Monsanto to predict the risk of CRW developing resistance to MON 863 corn. MON 863 performance parameters were used in Caprio's deterministic nonrandom mating model described in the International Life Sciences Institute Health and Environmental Sciences Institute (ILSI-HESI) report (1998). This model was designed to run with sprayed and unsprayed refuge options. Initially, the entire population in this model is divided into two habitats including transgenic fields and refuge fields. The population is subjected to user-defined parameters of selection pressure to determine what level of the surviving population is SS (homozygous susceptible), SR (heterozygous), and RR (homozygous resistant) genotypes. Input values used by Monsanto for low and moderate dose included: 1) Survival of SS genotype = 0.1 - 0.5 ; 2) Survival of SR genotype = 1; 3) Survival of RR genotype = 1. A high dose simulation of survival was run with input values of 0.01 for the SS genotype and 0.1 for the SR genotype. However, it has not been determined if these values are appropriate for a simulation model addressing CRW resistance management.

Application of non-selective insecticides to refuges is also considered. Insecticide application is assumed to reduce the population size without changing relative allele frequencies. There is a portion of the population assumed to disperse prior to mating and a part that mates before dispersing resulting in random and non-random mating patterns (ILSI-HESI 1998). Monsanto used an input value of 1 to identify the portion that move outside the habitat before mating (R) and the portion that move outside the habitat before ovipositing (M). However, female CRW typically mate within the field they emerge from. Since females do not leave the field prior to mating, an input value of 1 for R is probably inappropriate for CRW IRM models. Postmating, preoviposition dispersal is assumed in this model and individuals that mate randomly will oviposit at random and these eggs are divided between transgenic fields and refuges. Eggs produced from non-random mating are assumed to be laid in the field (Bt or non-Bt) where mating occurred (ILSI-HESI 1998). However, it is likely that mated CRW females leave the field to oviposit so this parameter of the model may not apply to MON 863. The resistance allele frequency Monsanto used in this model was 0.001 and a 0 - 60% refuge size was considered.

According to the results of this model, a 20% refuge for a high dose product (0.01survival rate) would delay resistance for 19 years, a moderate dose (0.3 survival rate) would delay resistance for 11 years, and a low dose product (0.5 survival rate) would delay resistance for 17 years. These results suggest that a 20% refuge in a low dose product would delay resistance 30% longer (17 years vs 13 years) than no refuge and 27% (11 years vs 8 years) for a moderate dose product. However, further investigation into the validity of this model for the CRW is needed. Assuming MON 863 confers a low to moderate dose, delaying resistance for 11 years may not be an adequate duration. This model also does not use a stochastic simulation nor does it consider spatial factors involved in the evolution of resistance. Further refinement of this model is needed.

Andow and Alstad Model (Andow and Alstad 2002)

Drs. David Andow and David Alstad (University of Minnesota) developed a deterministic model for resistance evolution of WCRW using between field refuges. This patch model addresses WCRW ability to oviposit in soybeans or corn and accounts for a corn-soybean or corn-alfalfa cropping system. Areas with 100% continuous corn and areas with 40% continuous corn and 60% crop rotation for a total of 70% potential corn acreage (40% continuous + (60/2)% rotated) are considered in this model. WCRW ability to oviposit in soybeans at the end of the season and emerge in corn fields the following spring is included in this model. A 5-50% refuge is assumed for continuous and rotated corn and refuges are planted in alternating blocks with transgenic corn. High risk areas with considerable levels of damage to first year corn are addressed in this model. Based on CRW biology, this model assumes pre-mating dispersal is negligible, random mating within fields and a high rate of post mating dispersal which is appropriate based on CRW biology. Five types of patches are included and post mating dispersal among patches is random. It is assumed that there is no pest management on the refuges, however, application of soil applied insecticides are expected to be used on refuge acres. According to Andow, the fundamental questions addressed in this model are "(a) how does the rate of evolution of resistance depend on the relative areas of the different crops, especially the proportion of non-transgenic refuge, and (b) how sensitive are these rates to variation in the many parameters. Specifically, we evaluate the effects of SS survival (μ), dominance (ϵ), initial R allele frequency (p_0), fecundity (fec), preference for ovipositing on soybean (ρ), and density dependence ($\propto$ and β)."

There was no difference in the number of WCRW generations needed to exceed a 0.5 resistance (R) allele frequency for continuous versus rotated corn (40% continuous) with a 20% refuge in first year and continuous corn, when oviposition is allowed in soybean and p_0=0.00001 (a very low resistance allele frequency). Results of this model indicated that there was virtually no difference between 100% continuous corn and 40% continuous corn simulations in the number of generations needed for the R allele frequency to exceed 0.5. Survival of susceptible homozygotes (SS) and heterozygote dominance was varied in the model over a wide range. Results indicated that susceptible homozygotes would remain susceptible to transgenic corn for < 15 generations. This is probably because local mating is leading to assortive mating among dominant resistant (RR) genotypes. If SS survival is large enough and dominance is small enough, then > 15 generations may occur before 0.5 R allele frequency is reached and a low dose IRM strategy may be effective. A high dose strategy is only effective under a narrow range of parameter values (SS survival <0.01 and dominance <0.05).

Another set of scenarios (e.g., 40% continuous corn, 20% refuge, potential oviposition in soybean, varied survival of homozygotes and heterozygous dominance) examined the effect of the initial R allele frequency on resistance management strategies. The initial R resistance allele frequencies varied from 0.00001(best case scenario), to 0.0001 and 0.001 (worst case scenario). Results of this model predict that resistance could not be delayed for > 15 years with a high dose strategy for WCRW and a 20% refuge planted between Bt fields when SS survival was less than

or equal to 0.02. Simulations suggest that resistance may be delayed for > 15 years with a low dose strategy if the *SS* survival is ≥0.2 and heterozygote dominance is ≤0.17. If MON 863 only produces a low dose rather than a moderate dose for control of WCRW, this model predicts that resistance may be delayed > 15 years in areas of high adoption of continuos corn.

This model was also run to evaluate the sensitivity of fecundity (varied from 20-220 viable eggs/female) and percent refuge (varied from 5-50%) in 40% continuous corn, p_0=0.00001, oviposition in soybean allowed, and susceptible homozygote survival and heterozygote dominance fixed for a high or low dose. Results of this run indicated that fecundity and variation in refuge size did not affect durability of resistance for high or low dose simulations. The lack of effect refuge has on the evolution of resistance is probably due to local mating which results in reduced dilution on homozygous recessive genotypes and susceptible genotypes.

Another simulation was run that considered a preference to oviposit in soybean, a 5-50% refuge, 40% continuous corn, p_0=0.00001, fecundity of 50 viable eggs/female and susceptible homozygote survival and heterozygote dominance fixed for a high or low dose. Risk of resistance was not affected by a preference for WCRW to oviposit in soybean versus corn which implies that ovipositional preference is probably not affecting the outcome of these models nor durability of resistance.

A simulation was also run to evaluate variance in density dependent values and percent refuge (5-50%), using parameter values of 40% continuous corn, p_0=0.00001, oviposition in soybean allowed and susceptible homozygote survival and heterozygote dominance fixed for a high or low dose. Density dependent factors considered were larval density at which strong density dependence occurs ($\propto$) and the level at which density dependent mortality intensifies with increasing density (β). There is uncertainty in the estimation of $\propto$ and β.

Results of the simulations indicate that these parameters probably do not affect the durability of resistance nor conclusions based on the model. Andow and Alstad's model suggest that between field refuges for resistance management of high dose (μ=0.01, ϵ=0.05) transgenic CRW protected Bt corn are likely to be ineffective. However, a low dose (μ=0.2, ϵ=0.2) strategy with a 20% between field refuge would probably delay resistance for >15 generations because selection pressure in favor of resistance is weak. Details of this model can be found in MRID 455770-01 Appendix H. Further refinement of this model is needed.

Onstad Model (Onstad *et al.* 2001)

Onstad's patch model is based on the biology, population dynamics and genetics of the WCRW and was designed to address corn (continuous or rotated), soybean and other crops (e.g., oats or alfalfa). This model assumes resistance is due to two loci and two alleles per locus because resistance to both crop rotation and transgenic corn are considered. It is also allows for the possibility of incorporating the use of soil insecticides or seed treatments and assumes the refuge is maintained in the same place each year. An initial R allele frequency is assumed to be 0.0001 in continuos corn and the simulation measured the time to reach R=0.03.

Allele expression in the CRW and dose were the two most important factors affecting the outcome of the model. A dominant resistance allele lead to the quick evolution of resistance to CRW protected Bt corn; whereas, a recessive allele delayed resistance for more than 99 years. In other words, when resistance is recessive, the resistance allele frequency did not exceed 3% for the 99-year period simulated. However, when resistance is dominant, the resistance allele frequency rapidly exceeded 3% within two to nine years as refuge size ranged from 5 to 30% for all doses of the toxin greater than 0.001.

Genotype, field and age are distinguished for adult males and unmated females; whereas, mated females are distinguished by genotype, field, age and genotype of mate. Aspects of corn phenology (period between anthesis to maturation is considered) and pest biology including phenology adult dispersal, sexual activity, oviposition, sex ratio and survival of immature beetles are considered in this model. However, it is unclear why this model considers "remating" of female.

Results of this model account for delayed emergence by susceptible adult CRW and refuge structure such as planting as row strips or blocks. Row strips were shown to reach a resistance allele frequency of 0.3 much more quickly then a block configuration. These results showed that high dose products will delay resistance (time required to reach 0.03 resistance allele frequency) for 13 years to more than 99 years when the refuge size is varied from 5 to 30%. A block refuge size was not affected when a high dose (*SS* survival ≤0.001) was considered an a 0.03% resistance allele frequency was not reached in > 99 years. For a high dose product, a 5% refuge planted as strips reached a 0.03 resistance allele frequency in 13 years and a 30% refuge will delay resistance for 34 years. A lower dose product (SS survival ≥0.05 and ≤0.2) with 5-30% block refuge was shown to delay resistance (time to a 0.03 allele frequency) for five to nine years respectively.

Lower dose products with a 5-30% refuge planted as row strips delayed resistance two to six years respectively. Resistance evolved faster in row strips than in a between field block refuge. Onstad *et al* (2001) indicate that the faster evolution of resistance is due to greater selection pressure with row strips because of the greater likelihood of oviposition in the transgenic strips compared with separate blocks. Sensitivity analyses indicated that changes in parameter values had little effect on the time to resistance when resistance genes A and B are additive and a block configuration is used.

Onstad *et al.* (2001)simulate dose of toxin expression in the plant measured as the survival of susceptibles at 0, 0.001, 0.05, 0.1 and 0.2. MON 863 appears to have a low to moderate dose because data show 17% to 62% larval survival. What the dose MON 863 is actually producing is critical to the assumptions of this model (or any genetic model). If lower doses > 0.2 (survival of susceptibles) were used in the model, it is likely that the predicted years to resistance (R=0.3) would increase to greater than 10 years. This conclusion is the same whether the refuge is in-field strips or between field blocks. Further refinement of this model is needed.

Refuge:

Summary of Monsanto's Proposal

According to Monsanto's submission, a refuge will be required to be planted with MON 863 corn hybrids that will produce CRW susceptible to the Cry3Bb1 protein. Refuges will be allowed to be planted as continuous blocks adjacent to the MON 863 fields, perimeter strips or non-transgenic strips planted within transgenic fields. Since field surveys conducted in 1999 showed that adult CRW are not repelled by MON 863 (Appendix C), planting a refuge within or adjacent to the transgenic field will not deter adults from migrating into the refuge. Seed mixtures of transgenic and non-transgenic corn will not be allowed until information on its adequacy is gathered.

Growers will be required to plant one acre of non-Bt corn for every four acres of MON 863 corn planted. This is equivalent to planting a 20% non-Bt corn refuge and is based on the current understanding of CRW biology, Cry3Bb1 effective dose, preliminary modeling results and agronomic considerations. Monsanto expects a "*de facto*" refuge to exist on every farm because a limited availability of MON 863 hybrids are expected during the first three years of the commercial registration. Seed and granular insecticide treatments to control CRW larvae will be allowed on refuge acres. Treating refuges with larval insecticides are needed because of the potential for severe damage and economic impact. However, treating refuges for adult CRW control will be prohibited because adult treatments may diminish the effectiveness of the refuge. If growers spray their corn fields with insecticides to control pests other than CRW, then all acres (Bt and non-Bt) should be treated identically.

Bt fields and the non-Bt refuge acres should be treated with identical agronomic practices such as irrigating all corn (Bt and non-Bt) at the same time. To ensure the production of similar numbers of CRW, Bt and non-Bt corn should be planted in fields with similar backgrounds. For example, if MON 863 hybrids are planted on continuous corn fields then the non-Bt refuge should be planted on continuous corn fields or both should be planted on first-year corn acres. Non-Bt refuges should not be planted on first year corn fields if the MON 863 hybrids are planted on rotated fields.

This interim IRM plan is not intended for fields planted to increase inbred seed since these fields need to be isolated from external corn pollen sources. An in-field or adjacent non-Bt corn refuge would be inconsistent with inbred seed production practices. Monsanto does not believe that planting these fields without an associated refuge will increase the risk of CRW resistance to Cry3Bb because these fields will be planted after soybean and soil insecticides will be applied at planting.

Reviewer's Comments

The 1998 Science Advisory Panel Subpanel concluded that an appropriate resistance management strategy is necessary to mitigate the development of insect resistance to Bt proteins

expressed in transgenic crop plants. The 1998 Subpanel recognized that resistance management programs for ECB should be based on the use of both a high dose of Bt and structured refuges designed to provide sufficient numbers of susceptible adult insects. The high dose/refuge strategy assumes that resistance to Bt is recessive and is conferred by a single locus with two alleles resulting in three genotypes: susceptible homozygotes (SS), heterozygotes (RS), and resistant homozygotes (RR). It also assumes that there will be a low initial resistance allele frequency and that there will be extensive random mating between resistant and susceptible adults. Under ideal circumstances, only rare RR individuals will survive a high dose produced by the Bt crop. Both SS and RS individuals will be susceptible to the Bt toxin. A structured refuge is a non-Bt portion of a grower's field or set of fields that provides for the production of susceptible (SS) insects that may randomly mate with rare resistant (RR) insects surviving the Bt crop to produce susceptible RS heterozygotes that will be killed by the Bt crop. This will remove resistant (R) alleles from the insect populations and delay the evolution of resistance. However, it is currently unknown if one or more loci are involved with CRW resistance to Cry proteins or what the initial resistance allele frequency is for these beetles. This information is necessary to determine what dose is needed and what size and structure of refuge is required to delay resistance.

Based on CRW biology, current information provided from simulation models and expected low initial adoption rates (Monsanto suggests <5% in the 1st year, <20% in 2nd year and <40% the third year) and hybrid availability, a 20% non-Bt corn refuge planted with or adjacent to MON 863 corn fields is expected to delay the risk of CRW developing resistance to Cry 3Bb1. Considering the limited movement of CRW larvae, planting refuges close to transgenic fields in large blocks is preferred to narrow strips (Gray 1999, Meinke *et al.* 2001). If a 20% refuge is planted as row strips within a corn field, then at least 6 to 12 consecutive rows should be planted 9 to 18 m from the center of the transgenic corn (Onstad *et al.* 2001). Based on the models discussed above, the "lower" dose of MON 863 seemingly supports a 20% refuge.

WCRW, NCRW and MCRW only feed on a few weedy species other than corn and the value of these alternate hosts as a refuge is not understood (Meinke *et al.* 2001). Therefore, alternate hosts should not be considered as refuge for CRW-protected transgenic corn until more information is available on their effectiveness.

Farmers will expect the option of applying insecticides to treat for CRW adults or larvae on refuge acres because of potential economic loss due to factors such as plant lodging. Insecticides are applied to the root zone to control larvae and will not affect CRW out of the treated area (Meinke *et al.* 2001). Therefore, it should be assumed that refuge acres will be treated for CRW larvae with chemical insecticides. However, refuge acres should not be treated with insecticides to control CRW adults.

Monitoring:

Summary of Monsanto's Proposal

Changes in susceptibility of target pests to Cry3Bb1 insecticidal protein will be monitored. Baseline susceptibility studies WCRW have been initiated from populations collected in 1999 and 2000 by Dr. Blair Siegfried (University of Nebraska), Dr. Larry Chandler (USDA-ARS) and Dr. Wade French (USDA-ARS). This baseline monitoring was concentrated in areas that are expected to have the greatest risk of resistance, particularly in areas known to have high CRW populations and are expected to have high adoption of MON 863 corn. Populations of WCRW and NCRW were also be collected in 2001 and will be collected in 2002 to determine baseline susceptibility.

Laboratory bioassays using nondiapausing, laboratory-reared WCRW larvae resulted in a estimated LC_{50} for MON 863 of 75 ppm. Ten populations of field collected WCRW larvae were assayed by Dr. Blair Siegfried (University of Nebraska) and demonstrated an average of twice the susceptibility to MON 863 then the laboratory colonies. Additional WCRW and NCRW larvae collected from the field during 2001 will be assayed in addition to populations that will be collected during the 2002 growing season. Results from the assays will be used to develop baseline susceptibility data.

Reviewer's Comments

CRW management practices can be determined by scouting fields the previous year to estimate damage that will occur the following year (O'Neal *et al.* 2001, Meinke *et a;*. 2001). Therefore, sampling fields such as soybean the year before growing corn may provide an indication if CRW control will be needed (O'Neal *et al.* 2001). Insecticide treatments are not typically applied for adult CRW in the eastern Corn Belt; however, broadcast insecticides are used in the western Corn Belt, particularly Nebraska, to control WCRW females and prevent them from laying eggs (Gray 2000). Threshold levels recommended for insecticide applications vary according to region, expert opinion and rotation practices (e.g., first year corn vs continuous corn) (Gray 2000). According to the NCR-46, "[s]couting techniques and thresholds are available for continuous corn, extended diapause of northern corn rootworms, and western corn rootworm oviposition in soybean. These methodologies would enable the use of transgenic varieties in a prescriptive, pest management approach" (Meinke *et al.* 2001). Application of insecticides have been recommended when 0.75 CRW are found per corn plant and 10% of those beetles are gravid females. Monitoring should continue weekly and insecticides should be reapplied if 0.5 beetles per plant are found in the field. Scouting results from mid-July to early September can be used to determine potential larval damage and the need for treatment the following year (Gray and Steffey 1999). Rotating a non-host crop the year following corn is recommended when 0.5 to1 beetle per plant is found during peak pest population density. The use of root ratings may has also been recommended to predict economic injury levels of CRW. On a scale of 1-6, a mean root rating of >2.5 is expected to lead to economic loss (Levine and Oloumi-Sadeghi 1991). However, studies have shown that root damage ratings should not be used to determine

insecticide efficacy or predict yield because results are inconsistent (Spike and Tollefson 1989, Sutter *et al.* 1990, Levine and Oloumi-Sadeghi 1991).

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. Monitoring techniques such as discriminating dose concentration assays need to be thoroughly investigated for *Diabrotica* spp. for their feasibility as resistance monitoring tools.

Grower participation (e.g., reports of unexpected damage) is an important first step for resistance monitoring. Resistance monitoring is also important because it provides validation of biological parameters used in models. However, resistance detection/monitoring is a difficult and imprecise task. It requires both high sensitivity and accuracy. Good resistance monitoring should have well-established baseline susceptibility data prior to widespread introduction of Bt crops. Although baseline susceptibility data is not completed at this time, research is being conducted to develop the baseline susceptibility of WCRW and NCRW to MON 863. These data are also needed for MCRW.

A comprehensive monitoring plan that targets the CRW and addresses when and where monitoring will occur is needed and should be developed within two years of commercialization. Monitoring will become more important after the accrual of multiple growing seasons of exposure and grower adoption increases. In addition to baseline susceptibility data, information is needed to determine how many individuals need to be sampled and in how many locations. The chances of finding a resistant larvae in a Bt crop depend on the level of pest pressure, the frequency of resistant individuals, the location and number of samples that are collected, and the sensitivity of the detection technique. Therefore, as the frequency of resistant individuals or the number of collected samples increases, the likelihood of locating a resistant individual increases (Roush and Miller 1986). If the phenotypic frequency of resistance is one in 1,000, then more than 3,000 individuals must be sampled to have a 95% probability of one resistant individual (Roush and Miller 1986). Current sampling strategies for ECB have a target of 100 to 200 individuals per location. Previous experience with conventional insecticides has shown than once resistant phenotypes are detected at a frequency >10%, control or crop failures are common (Roush & Miller 1986). Because of sampling limitations and monitoring technique sensitivity, resistance could develop to Bt toxins prior to it being easily detected in the field.

Interim Mitigation/Remedial Action:

Summary of Monsanto's Proposal

Since this IRM plan is limited to three years, Monsanto believes that the risk of resistance is negligible, particularly considering the fact that CRW has one generation per year. If unexpected levels of CRW damage occur during this interim period, growers and/or seed dealers will be

instructed to contact Monsanto or an authorized distributor. Upon reports of unexpected CRW damage in MON 863 corn hybrids, Monsanto will sample local pest populations and conduct necessary *in vitro* and *in planta* assays. If presence of the Cry3Bb1 protein is confirmed, then studies will be conducted to determine if the level of susceptibility of the field collected insects differs from the baseline level. Discriminating concentration bioassays will be used when possible to determine is levels of susceptibility have increased.

If discriminating concentration bioassays are unavailable, insect resistance will be determined from neonate bioassays of progeny of the sampled pest population. In the absence of the discriminating concentration bioassay, Monsanto will determine if CRW susceptibility to MON 863 is increased if neonate larvae exhibit both of the following two criteria. 1. "An LC_{50} in a standard diet bioassay (incorporating the Cry3Bb protein) that exceeds the upper limit of the 95% confidence interval of the mean historical LC_{50} for susceptible pest populations, as established by the baseline measurements." 2. "Over 50% of Cry3Bb-expressing plants with one or more root nodes destroyed under controlled laboratory conditions." The definition of confirmed resistance may evolve and change as new information becomes available.

Any instance of confirmed CRW resistance will be reported to the Agency within 30 days and mitigation measures will be reported within 90 days. Mitigation measures will include: 1. Informing growers and extension specialists in the affected area of the confirmed report of CRW resistance. 2. Sales in the affected area will be terminated immediately. 3. Alternate CRW control measures will be recommended to growers and extension specialists in the affected areas. Monsanto will also notify the Agency of a long term IRM strategy for an affected area within 90 days of confirmed resistance and work closely with the Agency to mitigate resistance.

As part of a mitigation action plan, Monsanto will initiate some or all of the following actions. Growers and extension agents in the affected areas will be informed of the resistance incidence and monitoring will be increased in that area. Alternative CRW control measures will be recommended and intensified IRM measures will be instituted. If these measures do not mitigate resistance, Monsanto will cease sales in the affected area until the Agency agrees that an effective IRM plan has been implemented.

Reviewer's Comments

There is a concern regarding Monsanto's outline of detecting and confirming resistance. Monsanto suggests that they will initiate mitigation measures when unexpected levels of CRW damage occur. However, Monsanto does not describe what is meant by unexpected levels of damage. Some level of damage is expected since there is not a high dose of MON 863 expressed to control the CRW and research has shown that some level of "grazing" will occur. Monsanto must identify what unexpected levels of damage are and relay this information to EPA, seed dealers, extension specialists, NCGA and growers. This may be accomplished in many ways including websites and Technology Use Guides.

Confirmed reports of pest resistance should be reported to the Agency as soon as possible but

must be within 30 days. Once resistance has been confirmed, alternative control measures to reduce or control the local target pest population should be recommended to customers, extension agents, consultants, university cooperators, seed distributors, processors, state regulatory authorities, EPA regional and national authorities, and any other pertinent personnel of the incidence(s) of resistance in the affected area. Where appropriate, customers and extension agents in the affected area should apply insecticides and/or crop rotation practices to control any potentially resistant individuals.

As soon as possible following confirmation of resistance, but must be within 90 days of a confirmed instance of pest resistance, as defined above, Monsanto should notify the Agency of the immediate mitigation measures that were implemented and submit a proposed long-term resistance management action plan for the affected area. Monsanto should work closely with the Agency in assuring that an appropriate long-term resistance management action plan for the affected area is implemented. An action plan that is approved by EPA should be implemented that consists of some or all the following elements, as warranted: 1) Inform customers and extension agents in the affected area of pest resistance; 2) Increase monitoring in the affected area, and ensuring that local target pest populations are sampled on an annual basis; 3) Recommend alternative measures to reduce or control target pest populations in the affected area; 4) Implement intensified local IRM measures in the affected area based on the latest research results. The implementation of such measures will be coordinated by the Agency with other registrants; and 5) Monsanto should cease sales of all MON 863 Bt corn hybrids until resistance has been shown to have been abated. During the sales suspension period, Monsanto 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 should be coordinated with the Agency.

For the growing season(s) following a confirmed resistance incident(s), Monsanto should maintain the sales and distribution suspension of all MON 863 hybrids potentially affected by the resistant pest populations or areas in which resistance is considered to be serious. This must be done within the affected region or if undetermined, the affected county(ies) and proximate surrounding counties. This sales suspension should remain in place until resistance has been determined to have subsided (within 5 to 10% or one standard deviation of baseline levels). In addition, Monsanto should develop, recommend, and implement alternative resistance management strategies for controlling the resistant pest(s) on corn with all necessary personnel (e.g. growers, extension agents, consultants, seed distributors, processors, university cooperators, and state/federal officials) in the affected region/county(ies) and surrounding counties of the resistance situation. All necessary personnel (e.g. growers, consultants, extension agents, seed distributors, processors, university cooperators, and state/federal authorities) in the affected region/county(ies) and surrounding counties of the resistance situation should be informed. Monitoring and surveillance in the affected area(s) for resistance and define the boundaries of the affected region should be intensified and studies on the rate of decline of resistance in the field should be conducted. Monsanto should continue to work with the Agency, states, grower groups, extension agents, consultants, university cooperators, or other expert personnel and other stakeholders to insure the implementation and development of appropriate mitigation measures for resistance in the affected areas.

If EPA agrees that an effective local resistance management plan has been implemented which mitigates resistance, the registrants can resume sales in the affected county(ies).

Grower Adoption and Hybrid Availability:

Summary of Monsanto's Proposal

Monsanto anticipates that initial adoption rates of MON 863 corn will be low relative to the potential market share at full maturity. With any new technology, initial grower adoption rates are initially low and increase over time (Grieshop *et al.* 1988). Effectiveness of the technology, information dissemination, hybrid availability and grower characteristics influence the growers level of awareness, interest, evaluation, trial and eventual adoption of MON 863 corn. Growers will also probably utilize other CRW control techniques that will limit adoption of MON 863 over time particularly as seed treatments and other transgenic products become available. Adoption rates will be further limited by the lack of approval for MON 863 corn in the European Union, thus restricting sales to domestic and other approved outlets. The European Union will probably not approve the sale of MON 863 corn during the interim three years requested by Monsanto.

Growers do not typically plant a single corn hybrid. Doane (2000) showed that an average of 3.4 hybrids purchased from 1.6 suppliers are planted by growers. In addition, research and seed company experience indicate that growers do not commit to large volumes of a particular hybrid until the test it first. Currently registered transgenic crops including Roundup ready soybeans, Roundup Ready corn and Yieldgard corn were initially adopted at low rates and incrementally increased over time. In general, growers will chose their seed hybrids based on confidence in performance and yield.

MON 863 will be sold in Monsanto's Asgrow and DEKALB corn hybrids in addition to being licensed to other seed companies. Monsanto expects MON 863 to be available from less then 50% of the total market share of seed companies. Seed availability and number of corn hybrids containing MON 863 will be limited initially due to breeding and manufacturing limitations and will gradually increase during the first three years of registration. According to Monsanto, four to five years are needed for a company to be able to offer all hybrids as transgenic corn.

Reviewer's Comments

Although Monsanto anticipates grower adoption rates to be slow initially (<5% the 1st year, <20% the 2nd year and <40% the 3rd year), the Agency acknowledges that there is no guarantee of how quickly growers will adopt CRW-protected transgenic corn. According to Dr. Michael Gray (University of Illinois), adoption of CRW protected transgenic corn is expected be occur much more quickly then the lepidopteran-active transgenic corn was adopted by growers. Gray suggests that growers are likely to accept the costs of growing transgenic corn cultivars to control CRW in order to reduce the costs associated with soil insecticide applications (Gray 1999).

242

Grower Education:

Summary of Monsanto's Proposal

Growers will be required to sign a legal contract in which they agree to adhere to IRM requirements. An education program will be initiated by Monsanto to inform growers of IRM requirements, why they are important and what individual grower responsibilities are. Information will be available to growers in various ways, but primarily from the Technology Use Guide. The Technology/Stewardship Agreement signed by growers will inform them of IRM requirements outlined in the Technology Use Guide. Signed Technology Agreements will inform growers that access to MON 863 will be lost if IRM requirements are not adhered to. Additional information to the Technology Use Guide will be mailed to growers as new information is available.

Monsanto also intends to develop a comprehensive stewardship training course that will provide growers planting MON 863 corn with information on IRM requirements and grower responsibilities. In addition, seed sales representatives will be trained to address IRM requirements as part of their selling endeavors. This approach to grower education should be useful since many growers have indicated that they receive most of their IRM information from seed dealers. Monsanto will also work with the NCR-46, American Crop Protection Association (ACPA), Biotechnology Industry Organization (BIO), the National Alliance of Independent Crop Consultants, extension specialists, United States Department of Agriculture (USDA), EPA, National Corn Growers Association (NCGA) as well as state and county corn growers associations and other relevant groups.

A survey will be sponsored by Monsanto and conducted by an independent firm to determine if growers are receiving the necessary IRM information and implementing IRM. This survey should aid in improving education and compliance efforts. A coalition of stakeholders (e.g., NCGA and extension specialists) will be formed by Monsanto to address CRW IRM issues including efforts in grower education.

Reviewer's Comments

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. The 2000 SAP also suggested that a comprehensive education program may help increase IRM compliance (SAP 2001). Ideally, the educational messages presented to growers should be consistent (among different registrants if applicable for CRW) and reflect the most current resistance management guidelines. 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.

Grower Compliance:

Summary of Monsanto's Proposal

Adhering to IRM requirements economically impacts growers based on monetary and time costs. According to Monsanto, if IRM requirements are too complex or time consuming to initiate, growers may avoid planting Bt corn. The rate CRW resistance to MON 863 develops may be impacted by growers farming and Agronomic practices. The 1998 Scientific Advisory Panel (SAP) on IRM for Bt crops acknowledged the importance of creating an IRM plan that is feasible for growers to institute. In addition, ILSI-HESI concluded that "the IRM plan should be cost-effective, flexible, easily adopted, and compatible with common production practices." Based on conclusions of these expert Panels as well as grower surveys, Monsanto acknowledges a need for multiple IRM strategy options available for different types of fields and farming practices.

According to Monsanto, refuge size should be based on the goals of IRM as well as grower economic considerations. Due to the potential economic losses, it is important for growers to be allowed to spray their refuges with insecticides when economic thresholds of CRW larvae occur. Although growers may spray their refuges, insecticide use will still be significantly reduced in areas CRW protected corn is grown. Grower surveys conducted by independent groups and sponsored by Monsanto between the years of 1997 and 2000 indicate that growers need a variety of options to implement IRM requirements regardless of farm size. There is also information that suggests that a grower may implement more than one design when planting a refuge. If refuge designs are too restrictive, surveys indicate that growers will be less likely to plant CRW protected Bt corn. In general, refuge requirements need to be kept simple, practical and flexible. A survey conducted by Monsanto in 2000 concluded that growers considered a 20% non-Bt refuge as generally acceptable, but many expressed a concern that a 30% refuge may not be feasible. Growers also preferred to plant their refuge as a seed mix; however, a seed mix is not being recommended by Monsanto at this time.

There are several reasons identified by Monsanto that explain why restricting refuges for MON 863 corn to in-field strips and limiting adjacent blocks as an option may be too difficult or unmanageable for many growers. Most fields are managed as whole production units that are rotated annually. Production practices are dependent on unpredictable impacts such as weather so flexibility and the ability to modify planting schemes are necessary. As farming efficiency has improved, the use of bulk seed handling and planting systems are being increasingly adopted, thus making it difficult for growers to micromanage a field. A quick and simple planting operation is needed by growers since there is a short time-period in the Spring when fields are planted.

Reviewer's Comments

Evaluation of grower compliance is largely based on results of grower surveys. Surveys indicate that 100% compliance is not likely and that some level of non-compliance must be expected. An expectation of 30% (or greater) non-compliance may be reasonable, given survey results for

244

lepidopteran protected corn. However, the 2000 SAP indicated that while surveys such as these are useful for tracking grower attitudes, they are not reliable for determining actual grower compliance (SAP 2001). The format of the surveys (mail or phone interviews) may encourage non-compliant growers to misrepresent their actions or "cheat" in their responses. Without confirmatory visits to individual farms (i.e., audits), it may be impossible to verify the accuracy of grower responses. The end result could be increased "false-positives," which may artificially inflate estimates of grower compliance. As such, actual non-compliance may be significantly higher than the survey results would suggest. To resolve this problem, the 2000 SAP suggested utilizing surveys created and conducted by independent parties to assess grower practices (SAP 2001). In addition to this recommendation, it may be useful to conduct some on-farm visits for firsthand verification of compliance. Such visits could be performed as part of a survey process, to evaluate the accuracy of grower survey responses.

Grower compliance is a greater concern because Monsanto is proposing an interim IRM plan which may be amended as new information becomes available. If the IRM requirements change based on additional information gotten during the interim period, it may be problematic to get the new information to growers and grower compliance with any new requirements may decline. Therefore, growers should sign agreements every year they grow MON 863 and growers should be required to read the Technology Use Guide prior to signing agreements.

The following requirements should be adopted to help ensure a high level of grower compliance with IRM requirements. To avoid confusing or discouraging growers, new IRM programs should be kept simple and consistent with existing programs so that growers will not be discourage from properly implement IRM or will not grow transgenic crops. Growers should be required to sign a technology use agreement that outlines IRM requirements and acknowledges the growers responsibility to comply with them on an annual basis. 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. An annual industry-supported survey conducted by a third party should be submitted to the Agency as a tool to monitor grower compliance. Additional education efforts should target non-compliant growers and access to the technology will be limited for growers found to be non-compliant.

Monsanto's Conclusions:

The use of MON 863 corn to control CRW larvae will be easier to use and safer then chemical inecticide control. Both growers and consumers will benefit from the reduction in insecticide applications. An IRM plan should not limit the use of MON 863 corn; rather, an IRM plan should be flexible, not overly encumber growers and fit into differing field situations and agronomic practices. It is also important for the plan to allow for the utilization of IPM practices. Monsanto suggests that refuge acres should be planted where CRW-infestations are low and insecticide use will be low. Planting MON 863 corn in areas with lower CRW larval infestations should lead to an increase in adult productivity and survival. Aspects of pest biology, grower needs, market penetration and impacts on growers were all considered in Monsanto's interim IRM plan.

Monsanto will continue to spousor research intended to be used to develop a long-term IRM plan (Table 1). In populations and dispersal. Feasibility and grower compliance of IRM strategies for MON 863 corn will also be evaluated during the interim registration period. An interim period is necessary to allow planting of MON 863 corn, conduct research and evaluate IRM strategies.

Reviewer's Conclusions:

Currently, there is not enough information available to definitively determine the size and structure of a non-Bt corn refuge for MON 863 needed to appropriately delay the development of CRW resistance. In a memorandum dated April 12, 2002 and e-mailed to the Agency by Russ Schneider (Monsanto Co.), the Canadian Food Inspection Agency (CFIA) states that the current Monsanto IRM plan is based on a high dose product for ECB control; however, MON 863 does not provide a high dose. Therefore, the CFIA concluded that "[i]t is currently uncertain whether requirements for refuge placement and the proportion of field planted to a refuge will be similar or different to that in place for ECB hybrids. The Agency acknowledges that there is a lack of information on dose and pest biology; therefore, a conservative approach to CRW IRM is necessary. Current information suggests that MON 863 provides a low to moderate dose of Cry3Bb1 for CRW.

The Agency agrees with the following recommendations quoted from the CFIA April 12, 2002 memo. "1. The refuge of a non-Bt corn hybrid consists of no less than 20% of the total corn planted in a field. 2. The refuge and Bt planting areas should both have the same crop rotation histories. 3. The refuge is located within a field with a flexible configuration of non-Bt rows planted to convenience the grower and orientation of the field. 4. Where circumstances require that a refuge cannot be planted within a field, the refuge must be planted adjacent to the Bt field. 5. The refuge may be treated with a banded soil insecticide for larval rootworm control."

Based on current information on CRW biology, MON 863 dose, simulation models, hybrid availability and adoption rate, a 20% refuge should be adequate to produce enough CRW adults to delay resistance. In addition, planting refuges adjacent to or within MON 863 corn fields should allow random mating of CRW even though there is limited movement of larvae and virgin females.

Monsanto expects the number of acres of MON 863 corn will be limited in each state during the first three years of registration because of a lack of hybrid availability and slow grower adoption rates as they evaluate the new technology. Therefore, Monsanto suggests that there will potentially be a greater than 20% refuge leading to a decrease in the risk of resistance. Although Monsanto does not plan to plant a refuge for inbred seed increase fields, they will treat with insecticides that will probably control any resistant CRW adults that may emerge. It is, therefore, acceptable to plant inbred seed increase acres without a refuge.

Planting a limited number of acres per state of MON 863 corn with a 20% non-Bt corn refuge planted adjacent to the Bt field or as strips within the field should be adequate to ensure resistance does not develop during the three year interim period requested by Monsanto.

246

Although unmated adult CRW females have been shown to move up to ten rows, it may not be practical for growers to plant ten row strips. Therefore, at least six rows (but preferably 12 rows) of non-Bt corn should be planted when in-field strips are utilized as refuge (Onstad *et al.* 2001). In addition, refuges should be planted with similar hybrids as the MON 863 field and identical agronomic practices should be employed for the transgenic and non-transgenic fields to ensure simultaneous adult emergence.

The NCR-46 committee which consists of research and extension CRW specialists as well as other cooperators commented on Monsanto's interim IRM plan at the request of the BPPD IRM Team. This group is recognized at the national authorities on CRW biology, ecology and management. The NCR-46 agrees that the potential for CRW to develop resistance to MON 863 during a three year interim period with low adoption rates is negligible. Since there is not a high dose of MON 863, there will be a significant number of CRW larvae survival. In addition, evidence of changes in larval feeding behavior on MON 863 corn will lead to increased larval survivorship. This increase in larval survivorship will result in a decreased chance of resistance.

Monsanto claims that MON 863 controls CRW (*Diabrotica* spp.). This implies that all CRW species including WCRW, NCRW, MCRW and SCRW will be controlled by MON 863. However, in their "interim IRM plan", Monsanto does not address the Mexican corn rootworm or southern corn rootworm. Information on pest biology and refuge structure is needed for a general claim of *Diabrotica* spp. control. Additional information is needed on various aspects of CRW pest biology as it relates to an IRM strategy.

Monsanto also does not adequately address grower education or mitigation measures if resistance occurs. A detailed grower education program is necessary to ensure grower compliance, particularly since the IRM requirements may change as more information becomes available. Growers should be required to sign a Technology/Stewardship Agreement each year they intend to plant MON 863 corn to ensure they are aware of the current IRM requirements.

There is also concern regarding Monsanto's outline of detecting and confirming resistance. Monsanto suggests that they will initiate mitigation measures when unexpected levels of CRW damage occur. However, Monsanto does not describe what is meant by unexpected levels of damage. Some level of damage is expected since there is not a high dose of MON 863 expressed to control the CRW and research has shown that some level of "grazing" will occur. Monsanto must identify what unexpected levels of damage are and relay this information to EPA, seed dealers, extension specialists, NCGA and growers. This may be accomplished in many ways including websites, a 1-800 telephone number and Technology Use Guides. Further investigation into confirming resistance is also needed. Monsanto's time-line includes identifying the insects as resistant, confirming the plant is expressing MON 863 and taking mitigation measures within 90 days. After this length of time, the growing season will likely be over and it will be too late for mitigation measures. It is, therefore, necessary for Monsanto to cease sales in an affected area after a confirmed report of resistance. The area and amount of time (in years) that sales should cease should be determined in consultation with EPA.

In addition, pages 6 and 9 of the IRM submission states that "[h]ybrids containing event MON 863 will be released both as stand-alone products and in combination with the *cry1Ab* gene for lepidopteran control because of the combined insect management needs of many farmers." However, the risk of resistance from a Cry1Ab/Cry3Bb stacked product has not been determined. Prior to the release and distribution of a Cry1Ab/Cry3Bb1 "stacked" product, an acceptable IRM plan specifically designed for a "stacked" product must be submitted to the Agency for review. Although refuges are similar for the lepidopteran protected and coleopteran protected Bt corn, Monsanto should consult with the Agency prior to distributing combination products.

<u>References</u>:

Andow, D. A. and D. N. Alstad. 2002. Preliminary draft: efficacy of between field refuges for resistance management of transgenic corn rootworm insecticidal corn: model results. MRID 455770-01

Ball, H. J. and G. T. Weekman. 1963. Differential resistance of corn rootworms to insecticides in Nebraska and adjoining states. J. Econ. Entomol. 56: 553-555.

Ball, H. J. 1983. Aldrin and dieldrin residues in Nebraska (USA) soils as related to decreasing LD_{50} values for the adult western corn rootworm (*Diabrotica virgifera*), 1962-1981. J. Environ. Sci. Heal. Part B Pestic. Food Contam. And Agri. Wastes. 18: 735-744.

Bergman, M. K. and F. T. Turpin. 1984. Impact of corn planting date on the population dynamics of corn rootworm (Coleoptera: Chrysomelidae). Environ. Entomol. 13: 898-901.

Boetel, M. A. and B. W. Fuller. 1997. Seasonal emergence-time effects on adult longevity, fecundity, and egg viability of northern and western corn rootworms (Coleoptera: Chrysomelidae). Environ. Entomol. 26: 1208-1212.

Branson *et al.* 1980

Branson, T. F. and J. L. Krysan. 1981. Feeding and oviposition behavior and life cycle of *Diabrotica*: an evolutionary view with implications for pest management. Environ. Entomol. 10(6): 826-831.

Branson, T. F., J. R. Reyes, and H. M. Valdes. 1982. Field biology of Mexican corn rootworm, *Diabrotica virgifera zeae* (Coleoptera: Chrysomelidae), in central Mexico. Environ. Entomol. 11: 1078-1083.

Branson, T. F. and G. R. Sutter. 1985. Influence of population density of immatures on size, longevity, and fecundity of adult *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). Environ. Entomol. 14: 687-690.

Coates, S. A., J. J. Tollefson and J. A. Mutchmor. 1986. Study of migratory flight in the

western corn rootworm (Coleoptera: Chrysomelidae). Environ. Entomol. 15: 1-6.

Davis, P. M., N. Brenes and L. L. Allee. 1996. Temperature dependent models to predict regional difference in corn rootworm (Coleoptera: Chrysomelidae) phenology. Environ. Entomol. 25: 767-775.

Doane 2000

Dudin, Y. A., B-P. Tonnu, L. D. Albee and R. P. Lirette. 2001. Amended report for MSL 16559: *B.t.* Cry3Bb1.11098 and NPTII protein levels in tissue samples collected from corn event MON 863 grown in 1999 field trials. Report No. MSL-17181; an unpublished study conducted by Monsanto Company. Submitted to EPA on May 21, 2001, MRID 454240-01.

Elliott, N. C., G. R. Sutter, T. F. Branson and J. R. Fisher. 1989. Effect of population density of immatures on survival and development of the western corn rootworm (Coleoptera: Chrysomelidae). J. Entomol. Sci. 24: 209-213.

Elliot, N. C. and G. L. Hein. 1991. Population dynamics of the western corn rootworm: formulation, validation, and analysis of a simulation model. Ecol. Modeling. 59: 93-122.

FIFRA Scientific Advisory Panel (SAP). 1998. Subpanel on *Bacillus thuringiensis* (*B.t.*) Plant-Pesticides and Resistance Management. A report prepared for the Environmental Protection Agency, dated February 9-10, 1998. (Docket No.: OPP 00231).

Gray, M. E. 1999. Transgenic insecticidal cultivars for corn rootworms: resistance management considerations. *In* Proceedings of the Crop Protection Technology Conference. University of Illinois, Urbana-Champaign. pp 50-55.

Gray, M. E. 2000. Prescriptive use of transgenic hybrids for corn rootworms: an ominous cloud on the horizon? *In.* Proceedings of the Crop Protection Technology Conference. University of Illinois, Urbana-Champaign. pp. 97-103.

Gray, M. E., E. Levine and H. Oloumi-Sadeghi. 1998. Adaptation to crop rotation: Western and northern corn rootworms respond uniquely to a cultural practice. Recent Res. Devel. Entomol. 2: 19-31.

Gray, M. E. and K. L. Steffey. 1999. Insect pest management for field and forage crops. *In* Illinois Agricultural Pest Management Handbook, University of Illinois, Urbana-Champaign.

Gustin, R. D. and T. E. Schumacher. 1989. Relationship of some soil pore paramenters to movement of first-instar western corn rootworm (Coleoptera: Chrysomelidae). Environ. Entomol. 18: 343-346.

Hill, R. E. and Z. B. Mayo. 1980. Distribution and abundance of corn rootworm species as

influenced by topography and crop rotation in eastern Nebraska, USA. Environ. Entomol. 9: 122-127.

International Life Science Institute Health and Environmental Sciences Institute (ILSI-HESI) 1999. An evaluation of insect resistance management in Bt field corn: a science-based framework for risk assessment and risk management. Report of an expert panel. November 23, 1998.

Isard, S. A., M. A. Nasser, J. L. Spencer and E. Levine. 1999. The influence of weather on western corn rootworm flight activity at the borders of a soybean field in east central Illinois. Aerobiologia. 15: 95-104.

Isard, S. A., J. L. Spencer, M. A. Nasser and E. Levine. 2000. Aerial movement of western corn rootworm (Coleoptera: Chrysomelidae) : diel periodicity of flight activity in soybean fields. Environ. Entomol. 29(2): 226-234.

Jackson, J. J. and N. E. Elliott. 1988. Temperature-dependent development of immature stages of the western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae): description, distribution, and sexual compatibility. Ann. Enomol. Soc. Amer. 73: 123-130.

Krysan, J. L., J. J. Jackson and A. C. Lew. 1984. Field termination of egg diapause in *Diabrotica* with new evidence of extended diapause in *D. barberi* (Coleoptera: Chrysomelidae). Environ. Entomol. 13: 1237-1240.

Krysan, J. L. , D. E. Foster, T. F. Branson, K. R. Ostlie, and W.S. Cranshaw. 1986. Two years before the hatch: rootworms adapt to crop rotation. Bull. Entomol. Soc. Amer. 32: 250-253.

Knutson, J. L., B. E. Hibbard, B. D. Barry, V. A. Smith and L. L. Darrah. 1999. Comparison of screening techniques for western corn rootworm (Coleoptera: Chrysomelidae) host plant resistance. J. Econ. Entomol. 92: 714-722.

Levine, E. and H. Oloumi-Sadeghi. 1991. Management of Diabroticite rootworms in corn. Annu. Rev. Entomol. 36: 229-255.

Levine, E., H. Oloumi-Sadeghi and J. R. Fisher. 1992a. Discovery of multiyear diapause in Illinois and South Dakota northern corn rootworm (Coleoptera: Chrysomelidae) eggs and incidence of the prolonged diapause trait in Illinois. J. Econ. Entomol. 85(1): 262-267.

Levine, E., H. Oloumi-Sadeghi and C. R. Ellis. 1992b. Thermal requirements, hatching patterns, and prolonged diapause in western corn rootworm (Coleoptera: Chrysomelidae) eggs. J. Econ. Entomol. 85(6): 2425-2432.

Levine, E. and H. Oloumi-Sadeghi. 1996. Western corn rootworm (Coleoptera: Chrysomelidae) larval injury to corn for seed production following soybean grown for seed

production. J. Econ. Entomol. 89: 1010-1016.

Meinke, L. J., B. D. Siegfried, R. J. Wright and L. D. Chandler. 1998. Adult susceptibility of Nebraska western corn rootworm populations to selected insecticides. J. Econ. Entomol. 91: 594-600.

Metcalf, R.L. 1983. Implications and prognosis of resistance to insecticides. *In* Pest Resistance to Pesticides. G. P. Georghiou and T. Sato [eds.]. pp. 703-733. Plenum Press, New York.

Metcalf, R. L. 1986. Foreword. *In* Methods for the study of pests *Diabrotica*, pp. Vii-xv. J. L. Krysan and T. A. Miller (eds.). New York, Springer-Verlag. 260 pp.

Miota, F. M. E. Scharf, M. Ono, P. Marcon, L. J. Meinke, R. J. Wright, L. D. Chandler and B. D. Siegfried. 1998. Mechanism of methyl and ethyl parathion resistance in western corn rootworm (Coleoptera: Chrysomelidae). Pest. Biochem. Physiol. 61: 39-52.

O'Neal, M. E., M. E. Gray and C. A. Smyth. 1999. Population characteristics of a western corn rootworm (Coleoptera: Chrysomelidae) strain in east-central Illinois corn and soybean fields. J. Econ. Entomol. 92(6): 1301-1310.

O'Neal, M. E., M. E. Gray, S. Ratcliffe and K. L. Steffey. 2001. Predicting western corn rootworm (Coleoptera: Chrysomelidae) larval injury to rotated corn with Pherocon AM Traps in soybean. J. Econ. Entomol. 94(1): 98-105.

Onstad, D. W., C. A. Guse, J. L. Spencer, E. Levine and M. E. Gray. 2001. Modeling the dynamics of adaptation to transgenic corn by western corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 94(2): 529-540.

Ostlie, K. 2001. Crafting crop resistance to corn rootworms. Nat. Biotech. 19: 624-625.

Naranjo, S. E. and A. Sawyer. 1988. Impact of host plant phenology on the population dynamics and oviposition of northern corn rootworm. *Diabrotica barberi* (Coleoptera: Chrysomelidae), in field corn. Environ. Entomol. 17: 508-521.

Roush, R. T., and G. L. Miller, 1986. Considerations for design of insecticide resistance monitoring programs. J. Econ. Entom. 79: 293-298.

Short, D. E. and R. J. Luedtke. 1970. Larval migration of the western corn rootworm. J. Econ. Entomol. 63: 325-326.

Spike, B. P. and J. J. Tollefson. 1989. Relationship of root ratings, root size, and root regrowth to yield of corn injured by western corn rootworm (Coloeoptera: Chrysomelidae). 82(6): 1760-1763.

Stavisky, J. and P. M. Davis. 1998. The effects of corn maturity class on western corn rootworm (Coleoptera: Chrysomelidae) phenology. J. Kans. Entomol. Soc. 70: 261-271.

Strnad, S. P., M. K. Bergman and W. C. Fulton. 1986. First-instar western corn rootworm (Coleoptera: Chrysomelidae) response to carbon dioxide. Environ. Entomol. 15: 839-842.

Strnad, S. P. and M. K. Bergman. 1987. Movement of first-instar western corn rootworms (Coleoptera: Chrysomelidae) larvae in corn roots. Environ. Entomol. 16: 1193-1198.

Strnad, S. P. and M. K. Bergman. 1987. Distribution and orientation of western corn rootworm (Coleoptera: Chrysomelidae) larvae in corn roots. Environ. Entomol. 16: 1193-1198.

Sutter, G. R., J. R. Fisher, N. C. Elliott and T. F. Branson. 1990. Effect of insecticide treatments on root lodging and yields maize in controlled infestations of western corn rootworms (Coleoptera: Chrysomelidae). J. Econ Entomol. 83(6): 2414-2420.

Suttle, P. J., G. J. Musick and M. L. Fairchild. 1967. Study of larval migration of the western corn rootworm. J. Econ. Entomol. 60: 1226-1228.

Tollefson, J. J. 1988. A pest adapts to the cultural control of crop rotation. Proc. Brit. Crop Prot. Conf. 3: 1029-1033.

Van Woerkom, G. J., F. T. Turpin and J. R. Barrett, Jr. 1983. Wind effect on western corn rootworm (Coleoptera: Chrysomelidae) flight behavior. Environ. Entomol. 12: 196-200.

Woodson, W. D. and L. D. Chandler. 2000. Effects on development of immature Mexican corn rootworm (Coleoptera: Chrysomelidae). Ann. Entomol. Soc. Amer. 93(1): 55-58.

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

FEB 20 2001

MEMORANDUM

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

TO: Michael Mendelsohn, Regulatory Action Leader
Biopesticides and Pollution Prevention Division, 7511C

FROM: Robyn Rose, Entomologist Robyn Rose 2/20/01
Biopesticides and Pollution Prevention Division, 7511C

PEER REVIEW: Alan Reynolds, Entomologist 2/20/01
Biopesticides and Pollution Prevention Division, 7511C

and

Sharlene Matten, Ph.D., Biologist 2/20/01
Biopesticides and Pollution Prevention Division, 7511C

SUBJECT: Review of Insect Resistance Management (IRM) for Monsanto's request for a seed increase for *Bacillus thuringiensis* Event MON 863: Corn Rootworm Protected Corn (Vector ZMIR13L). EPA Reg. No. 524-LEI; Barcode No. D271626; Case No. 069017; Submission No. S589326; Chemical No. 006484.

CLASSIFICATION: Acceptable

LABEL STATEMENTS PROPOSED BY MONSANTO:

The following is a list of statements found on the label that are relevant to insect resistance management (IRM). Each grower will receive a copy of the label.

- "This corn has been genetically improved to produce a *Bacillus thuringiensis* Cry3Bb protein that controls corn rootworm larvae, *Diabrotica spp.*"
- Directions for Use: "This seed may only be planted for inbred seed propagation or hybrid seed production purposes. All *MaxGard rootworm protected corn* seed used for seed propagation and hybrid production by Monsanto and its seed partners must be accompanied by a copy of this label. Prior to planting, growers are instructed to read the terms of their seed production contract and their regulatory compliance packets for information on agronomic procedures, restrictions, and record keeping requirements."
- "This seed and any plant materials produced from it may not be released into commerce or used for food or feed until all federal regulatory approvals have been granted."
- Containment:
  - "*MaxGard* corn seed production fields should be spatially isolated from other

253

corn plants by a least 660 feet; (and) or

- *MaxGard* corn should be reproductively isolated from other corn by detasseling or otherwise prevented from setting pollen"

- Options for monitoring for volunteer Cry3Bb corn the season following harvest:
  - "Production fields may be left fallow, monitored and all corn volunteers destroyed"
  - "Production fields may be planted to a rotational crop that allows for clear identification and destruction of all corn volunteers:
  - "Production fields may be replanted to *MaxGard* rootworm protected corn"
  - "Production fields may be planted to a herbicide tolerant corn variety, for example *Roundup Ready® Corn*, and treated with that herbicide to destroy *MaxGard* corn volunteers"
  - At continuous nursery locations, harvest must be followed by a 60-day irrigated fallow period during which corn volunteers are destroyed"

BPPD CONCLUSIONS:

Due to the limited number of acres of Cry3Bb MON 863 corn that will be planted, short duration of the program and containment process, it is acceptable for Monsanto to plant the 22,875 acres they have requested. Monsanto will be planting 4,000 acres under their experimental use permit (Permit No. 525-EUP-93) and 22,875 acres for precommercial inbred seed propagation and hybrid seed production. A total of 26,875 acres of MON 863 field corn will be grown in 2001. This request for a conditional registration extends through one growing season. Containment of each test plot will be ensured through isolation from other crops by 660 feet and all volunteer plants will be destroyed for one year following harvest.

Since small plots will be planted under this EUP, insect resistance is of limited concern. A formal insect resistance management (IRM) strategy is, therefore, not required for this conditional registration. However, to ensure resistance to Cry3Bb does not occur, after test plots are harvested, all plant material must be destroyed and fields should not be planted with corn for at least one full year. The latter recommendation is based upon an e-mail sent on 2/16/01 from Dr. Jon Tollefson (Iowa State University) on behalf of the NC-46 to Dr. Sharlene Matten. The e-mail from Dr. Tollefson stated: "Given the conditions described under Monsanto's request for increased acreage for seed production, as given to us by the EPA on 1/31/01, NCR-46 does not have concerns about insect resistance management by allowing a section 3 seed increase of MON 863 in 2001, provided that corn will not be planted in the following year in a field used for seed increase."

Monsanto must address IRM specifically for *Diabrotica* spp. in Cry3Bb Bt corn in their Section 3 Registration submission for full commercial use. The BPPD IRM Team's recommendations can be found in the December 10, 1999 memorandum to Michael Mendelsohn from Robyn Rose and Alan Reynolds (peer review by Sharlene Matten) that is attached. In particular, the BPPD IRM Team recommends studies be conducted regarding CRW adult and larval movement, mating, and high dose verification (e-mail from Jay Pershing to Robyn Rose on 2/15/01).

BACKGROUND:
Monsanto's corn rootworm insect protected corn (*MaxGard®* rootworm protected corn) expresses *Bacillus thuringiensis* subspecies *kumamotoensis* Cry3Bb protein. This field corn has been genetically modified with vector ZMIR13L to produce Event MON 863. *MaxGard* is toxic to certain coleopterans including corn rootworm (*Diabrotica spp.*). Event MON 863 corn has previously been evaluated under experimental use permit number 524-EUP-93. Monsanto has submitted an additional request for an EUP extension to plant an additional 4,000 acres of Event MON 863 corn during the 2001 growing season.

This review addresses Monsanto's application to amend their previously submitted application for registration of Event MON 863: Corn Rootworm Protected Corn (Vector ZMIR13L). A FIFRA Section 3(c)(5) application for registration of Event MON 863 was submitted to the Agency on June 23, 2000. On November 22, 2000, Monsanto submitted an amendment to that application. This amendment requests a one year conditional registration, under FIFRA Section 3(c)7, of Event MON 863 for precommercial inbred seed propagation and hybrid seed production. Under this conditional registration, 22,875 acres of Event MON 863 would be planted among 12 states including California, Hawaii, Iowa, Illinois, Indiana, Kansas, Michigan, Nebraska, South Dakota, Texas, Wisconsin, and the territory of Puerto Rico.

According to Monsanto, less than 500 acres will be planted to produce traditional inbred seed. These inbred corn plants will be homozygous for the Cry3Bb gene (personal communication between Robyn Rose (BPPD), Dennis Ward (Monsanto), and Jay Pershing (Monsanto) on February 15, 2001). Otherwise, fields of Cry3Bb hybrids will be planted. Every fifth row of the hybrid fields will be planted as male plants that are non-transgenic for breeding purposes. Therefore, approximately 20% of the field will be non-Bt corn. Most of the fields will be planted on first year ground, but not entirely, thus, few, if any CRW larvae are expected to be present. In addition, fields will be sprayed with insecticides to control secondary pests that will also reduce the numbers of CRW in the fields.

REGISTRATION REQUEST:
The 22,875 acres of Event MON 863 will be grown in California (280 acres), Hawaii (310 acres) Iowa (6,550 acres), Illinois (3,640 acres), Indiana (2,000 acres), Kansas (300 acres), Michigan (1,900 acres), Nebraska (4,864 acres), Puerto Rico (300 acres), South Dakota (300 acres), Texas (1,000 acres), and Wisconsin (1,431 acres). The state, release county, acres, participant, participant address, and participant phone number of the Cooperators in this seed production program have been provided to the Agency as confidential business information (CBI).

Event MON 863 inbred seed increase fields range in size from 0.125 to 250 acres and MON 863 hybrid production fields will be 5 to 300 acres in size. Each of these fields will be spatially and/or reproductively isolated from other corn fields by at least 660 feet. This 660 foot buffer along with the containment requirements listed on the label are consistent with USDA Performance Standards for regulated plants. These requirements should ensure that environmental dispersion of the *cry3Bb* gene will not occur and the genetic quality of the seed

will be preserved.

Inbred field corn seed will be derived from an individual parental ear. These fields will be used to obtain hand pollinated and open pollinated seed. Harvested hand pollinated seed will be stored until it is used for new production initiatives. Seed increases will be obtained from open pollinated corn that is classified as "Foundation Seed" and "Certified Seed" will be obtained from the second generation of this seed.

Hybrid production fields will include pollen (male) and fertile (female) parents typically planted in a ratio of 1 pollen:4 fertile seeds. The fertile parent will typically contain the *cry3Bb* gene. Pollen parent seed will be planted around the perimeter of the fields to avoid contamination from external pollen. To ensure fertilization of the female parent by the male, fertile plants will be detasseled prior to the onset of anthesis and silk emergence. After anthesis, the male pollen parent rows will be destroyed leaving only the female parent plants in the field. In some instances, male plants may be left in the field and destroyed after female plants are harvested.

To maintain seed quality, foliar insecticides and fungicides may be applied during these trials. Therefore, no corn rootworms are expected to be found in the production fields. In addition, a significant number of non-transgenic corn plants will be planted in these fields to produce hybrids.

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

FEB 24 2003

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: Amended EPA Public Interest Finding and Review of the Benefits for Monsanto Company's Corn Rootworm-Protected Field Corn Product (Event MON 863) for the Section 3(c)(7)(C) Full Commercial Registration [Reg. No. 524-LEI; Chemical No. 006484; DP Barcodes: D275903, D280086, D282927, D263097; Submissions: S574896, S599542, S615309; MRID#s: 453613-03, 455382--08, 450297-01, 456530-01-03, 456923-01, 455382-08]

TO: Mike Mendelsohn (PM-90)
Regulatory Action Leader
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511C)

FROM: Edward B. Brandt, M.A., Economist; Edward Brandt
Sharlene R. Matten, Ph.D., Biologist; Alan Reynolds for
Alan H. Reynolds, M.S., Entomologist; Alan Reynolds
Biopesticides and
Pollution Prevention Division (7511C)

ACTION REQUESTED: To review the public interest documents and other benefits information submitted by Monsanto to support a public interest finding and an overall benefits assessment for Section 3(c)(7)(C) conditional full commercial registration of Event MON 863 Corn rootworm-protected field corn products [Monsanto Company's application 524-LEI]

CONCLUSIONS:

1) Public interest finding. To grant a conditional registration under Section 3(c)(7)(C) of FIFRA, EPA must determine that such conditional registration will, *inter alia,* be in the public interest. EPA determines whether conditional registration of a pesticide is in the public interest in accordance with the criteria set forth at 51 Fed. Reg. 7628 (*Conditional Registration of New Pesticides* (Mar. 5, 1986)). On the basis of analysis utilizing these criteria, EPA concludes that

257

the use of MON 863 CRW-protected corn (EPA Reg. No.524-LEI) is in the public interest and supports the conditional registration of this pesticide under FIFRA section 3(c)(7)(C). EPA determines that use of MON 863 CRW-protected corn will be in the public interest because it will replace or reduce the use of a number of higher risk pesticides for CRW control that are of Agency concern (e.g., terbufos, chlorpyrifos, phorate, carbofuran).

2) Infested acreage. At 80 million planted acres, corn is the largest crop grown in the U.S. and accounts for 20% of total agricultural cropland. Over the past 5 years, conventional insecticides have been applied to between 14 to 18 million acres for control of CRW. This single corn pest accounts for 1 out of every 7 insecticide applications to agricultural crops. While CRW is the single largest insect pest treated by conventional insecticides, only one half of the infested acres are treated in any given year. To justify the cost of treatment, the expected loss due to rootworm damage must exceed the cost of pesticide applications. The historically low corn prices over the past 5 years have reduced returns on pest control and increased the economic threshold for rootworm control. Infested acreage is increasing due to extended diapause and behavior modification as CRW lays it's eggs in soybean fields on rotational corn. Acreage infested with CRW is expected to grow 2.6% per year and, by 2013, to total 39 million acres.

3) MON863 efficacy studies and yield benefits. Efficacy studies using MON 863 and non-transgenic corn showed that MON 863 experienced significantly less root damage from southern, northern, and western corn rootworm than a non-transformed control hybrid. Root damage was assessed using the Iowa Root Damage Rating (RDR) index (1 = no damage, 6 = extensive damage, >3 = economic threshold). The average root rating for MON 863 was < 2 RDR for western and northern CRW, significantly less that the average root rating for the non-transgenic hybrids. In all treatments, damage from southern corn rootworm exceeded that from northern and western rootworms.

In separate comparative efficacy studies with registered insecticides, MON 863 prevented root damage from rootworm feeding as well or better than commonly used corn rootworm soil insecticides including Force 3G (tefluthrin), Counter CR (terbufos), and Lorsban 15G (chlorpyrifos). Root damage ratings for MON 863 were typically between 1.2 and 2.0, a high level of control relative to untreated control hybrids (RDR 3.3 to 3.9). The root damage ratings for the soil insecticides were typically between 1.9 and 2.4.

MON 863 corn is as effective or more effective than chemical insecticides in protecting corn roots from CRW larval feeding damage, based on the review of the submitted field efficacy studies. Monsanto compared the relative efficacy of two transformed corn hybrids expressing the Cry3Bb protein (MON 853 and MON 863) in preventing damage from three species of corn rootworm larvae. Studies using a composed error model for insect damage functions indicate that MON 863 corn hybrids have a yield benefit of 1.5 to 4.5% relative to control with a soil insecticide. Without MON 863 or conventional insecticides, CRW can reduce yields from up to 9 to 28%. Since these studies are all based on infestation levels obtained by artificial innoculation, the benefits assessment bases these yield improvements as reflective of conditions of maximum levels of pest infestation.

4) Practical benefits. MON 863 corn offers many practical advantages to corn growers than the current alternatives. It can be planted early for a longer growing season and potentially higher yield, while ensuring adequate CRW protection throughout the growing season. In addition, growers should be able to plant their crop more quickly because they won't have to continually have to stop and refill the insecticide boxes. MON 863 seeds can also have seed treatments that will allow even greater control of other associated pests such as wireworm, grub, maggots, and cutworms. Thus, growers will have multi-pest protection while carrying out insect control in essentially a single step at planting. All of these advantages to planting MON 863 corn are practical, easier, and safer for the grower. Planting MON 863 corn will save the grower money in application, insecticide, labor, fuel, equipment, storage and disposal costs (since there will be no insecticide containers needed for CRW control). Plus, MON 863 is labeled for general use and will replace current reliance on restricted use products. MON 863 will provide the grower and other occupational workers greater safety, protect water bodies from run-off, and mitigate spray-drift and potential impacts on local non target organisms, such as bird populations.

5) Comparative human health acute risks. Based on the Agency's review of the submitted studies, the 50-year history of safe use of Cry proteins in U.S. agriculture, and comparable results to studies of other proteins in the Cry3 class, EPA determines that there is a reasonable certainty of no harm resulting from exposure to this protein. MON 863 corn presents no unreasonable risks to humans during any stage of its life cycle, from production, handling, storage, ingestion, to disposal. Cry3Bb1protein has no toxic effects on mammals, and is not likely to induce allergic or hypersensitive responses based on results in all appropriate tests. Of the commonly used insecticides that MON 863 will replace, about one third are labeled as danger, the highest category for acute toxicity. MON 863 is less toxic than all of the major insecticides currently used to control CRW damage.

6) Comparative environmental risks. All of the major chemicals used for CRW control can cause adverse environmental effects under conditions of normal use. These products are formulated with the following active ingredients: chlorethoxyfos, phorate, terbufos, tefluthrin, methyl parathion, carbofuran, fipronil, bifenthrin, cyfluthrin, esfenvalerate, permethrin, diazinon, chlorpyrifos, dimethoate, methomyl, ethoprop, carbaryl, lambda-cyhalothrin, and the commercial combination of tebupirimfos and cyfluthrin (Aztec). Fifteen products are labeled as "toxic," 6 as "highly toxic," 1 as "very highly toxic," and 14 as "extremely toxic" to birds, fish and other wildlife. EPA has identified 10 insecticides used in agriculture as the most toxic to birds and 3 are currently used to control the corn rootworm (carbofuran, phorate and methyl parathion). In contrast, the Cry3Bb1 protein has no toxic effects on non-target organisms based on results in all appropriate tests. In addition, Cry3Bb1 is degraded rapidly in the soil (reducing non-target exposure). The Cry3Bb1 protein is expressed by the corn plant; thus, reducing the exposure to non-target organisms. In addition, Cry3Bb1 has a narrow target range. Monsanto has performed dietary bioassays to determine the insecticidal spectrum of the Cry3Bb1 protein. The protein is effective at killing only beetles of the family Chrysomelidae, specifically CRW and Colorado potato beetle (*Leptinotarsa decemlineata (Say)*). The family Chrysomelidae contains no known endangered species. Receptor binding is a critical step in the mechanism of action of the Cry proteins because without a binding site there is no toxic effect exerted. To date, there have been no functional receptors for Cry proteins on intestinal cells of fish, birds, or mammals. As

labeled, MON 863 corn poses less risk to the environment than the registered alternatives.

7) **Grower benefits.** Cumulative grower benefits are projected to be $49.2 million for the three year period 2003 to 2005. The time necessary to develop corn hybrids containing Cry3Bb1 and the time necessary to obtain full European Union approval are key factors affecting the growth rate in benefits. At full commercial maturity, MON 863 adoption rates are projected to be 43% of infested acreage resulting in annual grower benefits of $110 million per year. Grower benefits are defined as the difference between the value of MON 863 and it's cost. The value is based on expected yield improvements, reduced costs for insecticides, and practical benefits related to a more flexible and safer product for growers to use than the alternatives. Average grower benefits are estimated to be $6.56/acre which is about 2% of gross income. Crop budgets suggest a net return per acre for corn (not including land charges) of around $60 per acre so MON 863 has the potential to increase profits by 10%.

The benefit estimates include all economic costs, such as out of pocket plus opportunity costs. This includes an estimated MON 863 technology fee as well as costs of market acceptance and refuge requirements unique to genetically-modified organisms (GMO's). The effective cost of export market acceptance are quantified by including the per cent of growers who would not accept GMO's in the construction of a demand curve for MON 863. A recent survey from ACGA reports that sixteen percent (16%) of respondents stated they would not be willing to grow more non-GMO corn varieties (ACGA 2002). The EPA estimate of grower benefits is based on 9% of growers that would not purchase MON 863 at technology fee of zero. A 16% removal from the target market would reduce adoption rates from 43% to 35% which would translate to lower grower benefits and less use reduction.

Any added cost of insect resistant management requirements unique to genetically-modified crops are minor in relation to the export issues. Refuge requirements have been accepted by growers to conserve the technology and benefits for future use. EPA's estimate of total grower benefit are 20% to 40% lower than comparable benefit estimates in Alston et al. (2002) and Gray (2000).

8) **Estimation of pesticide use reduction.** Pesticide use reduction projections indicate that as MON 863 CRW-protected corn adoption increases in the next five years, acre treatments will be reduced extensively for all chemical insecticides used currently to control CRW. The greatest use reductions are seen in both the organophosphate and synthetic pyrethroid classes. By 2005, approximately 1.5 million acre treatments of organophosphate insecticides, 1.9 million acre treatments of synthetic pyrethroid insecticides, 0.1 million acre treatments of carbamate insecticides, and 0.5 million acre treatments of other chemical insecticides including members of the phenyl pyrazole class (e.g., fipronil) will be reduced based on current projections. By 2007, the extent of insecticide use reduction will be even greater, approximately 2.5 million acre treatments of organophosphate insecticides, 3.5 million acre treatments of synthetic pyrethroid insecticides, 0.1 million acre treatments of carbamate insecticides, and 0.9 million acre treatments of other chemical insecticides are expected to be reduced.

9) Suggested measures to monitor environmental and grower benefits

Assessing the changes in the following variable will help to measure the environmental and grower benefits of MON 863 corn:

- infested acreage
- cost and adopted acreage for MON 863
- cost and adopted acreage for conventional insecticides
- CRW resistance monitoring
- frequency of complaints and related compliance inspections
- incidence of adverse reports under Section 6(a)(2)
- water quality monitoring for insecticides (NAWQA)
- environmental indicators, e.g., trends avian species found in and around corn fields
- environmental outcomes, e.g., trends in invasive, non native, nuisance or species of management concern

Background

Corn is the largest cultivated crop grown in the U.S. in terms of acreage planted and net value. The crop was planted on 79.5 million acres in the year 2000, yielding 10 billion bushels with a net value of $18.4 billion. There are nearly 410 million acres of agricultural land being used to grow crops nationwide, including Conservation Reserve Program lands (USDA/NRI). Corn rootworm (CRW, *Diabrotica* spp.) is one of a spectrum of insect pests that a farmer may choose to control. Other insect pests include cutworms, wireworms, white grubs, flea beetles, seedcorn maggots, black cutworms, and cornborer species. However, CRW is one of the most damaging insect pests of corn and is responsible for economic damages (costs associated with insecticides and crop losses) totaling nearly $1 billion (Gray, 2000). The three CRW control methods have been: use of crop rotation, soil-applied insecticides, and limited use of rescue-treatments for CRW adult beetles.

Event MON 863 corn rootworm-protected corn (MON 863 corn) (vector ZMIR13L) contains the *cry3Bb1* gene that produces the insecticidal crystal protein, Cry3Bb1, and a *nptII* marker gene that encodes neomycyin phosphotransferase II. MON 863 corn is targeted against the corn rootworm (CRW) complex (*Diabrotica* spp.), comprised primarily of the northern corn rootworm (NCRW, *Diabrotica barberi*, Smith and Lawrence), the western corn rootworm (WCRW, *D. virgifera virgifera*, LeConte), the Mexican corn rootworm (MCRW, *D. virgifera zeae*, Krysan and Smith). One additional *Diabrotica* species, the southern corn rootworm (SCRW, *D. undecimpunctata howardi*, Barber) is considered a relatively minor pest of corn that inhabits the southeastern coastal regions of the U.S. CRW (*Diabrotica* spp.) accounts for more chemical pesticide usage on corn than does any other pest. Approximately 28 million acres of corn are infested with CRW. In the year 2000, approximately 8 million pounds of insecticidal active ingredient costing $172 million were applied to 14 million acres of corn targeted to reduce CRW damage. There were approximately 24 million acres of corn treated with insecticides for CRW and other pests (e.g., grubs, maggots, cutworms, wireworms). USDA/NASS figures from 2001 indicate that 9.8 million pounds of insecticide active ingredients registered for CRW control were applied on more than 31% of the planted acres. Left untreated, CRW can cause severe yield loss, typically in the range from 8 to 16 percent, although reductions in yield may be as high as 28%.

Monsanto's public interest documents (Ward 2002 (MRID# 456539-01), Miller 2000 (450297-01)) provide a summary of information about corn rootworm as a pest in corn, current control measures, and the benefits of MON 863 corn. EPA has used Monsanto's submissions, public comments, syndicated marketing research studies and the published literature to perform its public interest finding and benefits assessment of MON 863. Corn insecticide use and current CRW control practices will be discussed in more detail in EPA's review below. Crop rotation, primarily corn rotated with soybean, has been used effectively, in many areas for many years, to help manage CRW damage (Levine and Sadeghi, 1991). However, the emergence of two different behavior changes in NCRW (extended diapause) and WCRW (soybean rotation) have reduced the effectiveness of the traditional corn-soybean rotation. Crop rotation with soybeans is less effective because some CRW can now infest soybeans (WCRW soybean rotational variant) (Levine et al., 1992a) or overwinter for 2 years until corn is planted again (NCRW

extended-diapause variant) (Levine et al., 1992b). The spread of the NCRW extended diapause variant and the WCRW soybean variant have increased the number of first year corn acres needing an insecticide treatment.

Resistance has been reported to some CRW adulticides (e.g., methyl parathion and carbaryl) (Meinke et al., 1998; Scharf et al., 1999; Zhu et al., 2001). Adulticides account for about 9% of the use (see Table 4). In addition, use of chemical insecticides against CRW appears to be increasing as other control measures become less effective. Increased use of chemical pesticides, because of CRW adaptation and resistance results in corresponding increases in risk and cost of using the pesticides. Development of resistance also requires a constant search for new control means, including new chemicals. In contrast, there is no known field resistance to Cry3Bb1 protein or any other Cry 3 class proteins. Adoption of insect resistance management strategies designed for MON 863 will also lessen the likelihood of CRW resistance developing to the Cry3Bb1 protein.

All CRW insecticides display a broad spectrum of activity and operate via a neurotoxic mechanism of action. The majority (68%) of these products labeled for CRW control are classified as "Restricted Use." These include chloroethoxyfos, phorate, terbufos, tefluthrin, and the commercial combination of tebupirimfos and cyfluthrin (Aztec). Most are highly toxic to fish, birds, and other wildlife species. Each year there are confirmed reports of human illness, as well as fish and bird poisonings, associated with the registered chemical insecticide alternatives. Several of the current CRW insecticides are in Agency Special Review (i.e., dimethoate, phorate, and terbufos).

There are no registered microbial Bt pesticide products for the control of corn rootworms. There are microbial Bt pesticides that use Cry3 Bt proteins to control Colorado potato beetle, but Colorado potato beetle is not a pest of corn (not a host). Corn rootworm is not a pest of potatoes (not a host). Ward (2002) notes that Cry3Bb1 shares approximately 67% amino acid sequence homology with the Cry3Aa4 protein (GenBank Accession No. M30503) which has been commercialized in the U.S. and other countries for the control of Colorado potato beetle (Perlak et al., 1993).

MON 863 corn represents a new method for controlling CRW. MON 863 corn was developed using recombinant DNA techniques, so that the corn tissues produce the Cry3Bb1 protein that is specifically toxic to CRW. In reviewing the available information, EPA considered in its review the following categories of potential benefits of a MON 863 corn based control strategy for CRW infestations.

- Safer for handlers, applicators, farmers, and the public than chemical alternatives in current use,
- Safer for the environment than current chemical alternatives
- Practical–easier and safer for growers to use than current chemical alternatives
- Comparable or greater efficacy than current chemical alternatives
- Yield benefits
- Reduced use of higher risk chemical alternatives

- Economic benefits to growers

PUBLIC INTEREST FINDING (MRID#s 456530-01 and 450297-01)

The criteria for a determination as to whether registration of a pesticide chemical is in the public interest are set forth in a 1986 Federal Register Notice entitled *Conditional Registration of New Pesticides*, 51 Fed. Reg. 7628 (Mar. 5, 1986). In accordance with this FR Notice, there is a presumption that conditional registration of a pesticide is in the public interest if one of the following criteria is met: (i) the use is for a minor crop; (ii) the use is a replacement for another pesticide that is of continuing concern to the Agency; (iii) the use is one for which an emergency exemption under FIFRA Section 18 has been granted for lack of an alternative pest control method, or (iv) the use is against a pest of public health significance. (Section IV.A. of the FR Notice). Notwithstanding whether a registration of a pesticide chemical may be presumed to be in the public interest, EPA may determine that such a registration is in the public interest on the basis of the following criteria: (i) there is a need for the new chemical that is not being met by currently registered pesticides; (ii) the new pesticide is comparatively less risky to health or the environment than currently registered pesticides; or (iii) the benefits (including economic benefits) from the use of the new active ingredient exceed those of alternative registered pesticides and other available non-chemical techniques. (Section IV.B. of the FR Notice). EPA determines that conditional registration of MON 863 is in the public interest currently used for CRW control that are of continuing concern to the Agency as indicated by the following factors.

1. **Special review.** Certain pesticides that are used for CRW control have been reviewed under EPA Special Review as described at 40 CFR section 154 because the use of these pesticides may result in unreasonable adverse effects to humans or the environment. The pesticides in this category are: dimethoate, phorate, and terbufos. Terbofos and phorate are being evaluated for reregistration.

2. **Acute avian risk from granular pesticides.** EPA issued in 1992 the "Comparative Analysis of Acute Avian Risk from Granular Pesticides"(EPA, 1992). This analysis indentifed 14 granular pesticides that the Agency believed posed potentially higher risk of killing birds due to the higher acute toxicity and availability in the environment. Among these granular pesticides are several that are still in use for CRW control: carbofuran (no longer used as a granular, but still a pesticide of Agency concern), phorate, terbufos, and chlorpyrifos. The results of the Agency's initiative to reduce exposure to these highly toxic granular pesticides is presented in the 1994 EPA report "Avian Granular Risk Reduction Initiative" (EPA, 1994).

3. **Restricted use.** Many of the granular pesticides used for CRW control have been classified as restricted use due to adverse environmental effects under use practices (40 CFR 152.171(a)) that limits the use of these chemicals to certified pesticide applicators. These include chloroethoxyfos, phorate, terbufos, tefluthrin, and the commercial combination of tebupirimfos and cyfluthrin (Aztec).

4. **Food Quality Protection Act of 1996.** EPA must reassess all existing tolerances to

be sure that they meet the standard of "reasonable certainty of no harm." The EPA is required to consider first those pesticides that pose the highest risk to humans. EPA is reviewing the organophosphate and carbamate pesticides because of their known risk of acute and chronic toxicity to humans as well as wildlife. The organophosphates and carbamates share the same mode of action. The organophosphate insecticides used for CRW control include: chlorpyrifos, terbufos, phorate, chloroethoxyfos, dimethoate, and tebupirimphos. Carbofuran is used for CRW control and is in the carbamate family.

In addition, EPA also determines, in accordance with the criteria set forth in section IV.B. of the FR Notice, that MON 863 corn qualifies for a positive public interest finding. To qualify under part IV.B, the product must demonstrate advantages in terms of the need for the chemical, its comparative benefits, risks, and costs. Monsanto has submitted two public interest documents (PIDs) and other supporting documents that present the potential benefits of the MON 863 corn product: MRID#s: 450297-01 and 456530-01 – both public interest documents; MRID# 456530-02, Yield Benefit of Corn Event MON 863; MRID# 456430-03, Efficacy of MON 863 Against Corn Rootworm and Comparison to Insecticide Treatments - Results of Year 2000 Field Trials; MRID # 455382-08, Comparing the Efficacy of MON 853 and MON 863 to Three Corn Rootworm Species, Northern Corn Rootworm (*Diabrotica barberi*), Southern Corn Rootworm (*D. undecimpunctata howardi*), and Western Corn Rootworm (*D. virgifera virgifera*); MRID# 456923-01, An Ex Ante Analysis of the Benefits from the Adoption of Monsanto's Corn Rootworm Resistant Varietal Technology – YieldGard® Rootworm. Additional information related to grower adoption of MON 863 corn is presented in An Interim Insect Resistance Management Plan for Corn Event MON 863: A Transgenic Corn Rootworm Control Product, MRID# 455770-01. EPA has reviewed the submitted documents, public comments, syndicated marketing research studies and published information. The potential benefits have been identified and evaluated.

The major proposed benefits of MON 863 corn for CRW control are:

- Safer for handlers, applicators, growers, and the public than current chemical alternatives
- Safer for the environment than use of available chemical pesticides
- Easier and less time consuming for farmers to use than current control options
- Comparable or improved efficacy relative to the current chemical alternatives
- Yield benefits
- Reduced use of current higher risk chemical alternatives
- Economic benefits to farmers from increased yields and decreased cost of rootworm control as compared with conventional control

Based upon this review, the use of MON 863 CRW-protected corn is presumed to be in the public interest under section IV.A. of 52 FR 7628 because it will replace or reduce the use of a number of higher risk pesticides for CRW control that are of Agency concern as discussed above (e.g., terbufos, chlorpyrifos, phorate). It also has clearly identified benefits and meets the criteria in part IV.B of the FR notice. Therefore, EPA concludes that the use of MON 863 CRW-protected corn is in the public interest and supports the conditional registration under FIFRA section 3(c)(7)(C).

EPA REVIEW OF THE BENEFITS OF MON 863

1. Characterization and use of chemical insecticides to control Corn Rootworm (CRW)

Three CRW control methods have been used for decades: crop rotation (typically with soybeans), soil-applied insecticides to control larvae (approximately 90% of the total CRW treated acres), and use of adulticides to control CRW adult beetles (approximately 10% of the total CRW treated acres) (Levine and Oloumi-Sadeghi, 1991). Greater than 90% of the growers use soil-applied insecticides applied at planting to control larvae due to greater efficacy and ease of application. Historically, crop rotation has been the primary method of controlling CRW (Levine and Oloumi-Sadeghi, 1991). However, crop rotation is now far less effective because of the existence of a WCRW soybean rotational variant primarily in Eastern Illinois and Western Indiana, that colonizes soybeans (Levine and Oloumi-Sadeghi, 1991; Levine et al., 1992a) and of a NCRW extended diapause (2 year extended) variant, primarily in parts of Minnesota, Iowa, and South Dakota (Krysan et al., 1996; Levine et al., 1992b). In addition, CRW has developed resistance to methyl parathion and carbaryl, both adulticides used in rescue treatments (Meinke et al., 1998). Therefore, growers have become increasingly dependent on chemical pesticides to limit CRW losses. EPA has registered 36 insecticide products for control of CRW. These are listed in Table 1. The insecticides used to control corn rootworm in conventionally grown (non-Bt) corn consist mainly of organophosphates (9), carbamates (3), synthetic pyrethroids (6), and phenyl pyrazole (1) classes of chemistry. Twenty-five products are classified as "restricted use." All 36 insecticides are toxic to extremely toxic to fish, aquatic invertebrates, bees, and/or wildlife. Three products have been involved in the Agency's Special Review Process: terbufos, dimethoate, and terbufos. Twenty-six of these products contain active ingredients either from the organophosphate or carbamate classes, both of which are considered to be top priorities under FQPA and tolerance reassessment. Terbufos, phorate, chlorpyrifos, diazinon, ethoprop, carbofuran, and methomyl have presented varying levels of concern regarding avian, aquatic, and mammalian risk. EPA has specified specific risk mitigation measures for all of these chemicals and, in many cases, certain uses have been eliminated because of either human or environmental risk concerns.

266

Table 1. Insecticide end-use products registered by EPA for use on corn for control of corn rootworm species (Reprinted from p. 18-21, Ward 2002, MRID# 456530-01 and verified by EPA).

Product	Active Ingredients	Type [a]	Use Rate [b]	Use	Classification [c]
Ambush® Insecticide - Syngenta	permethrin – 25.6%	SP	0.2 lb/ac	Adult control	**WARNING. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees
Asana® XL Insecticide 0.66 Emulsifiable Concentrate - DuPont	esfenvalerate – 8.4%	SP	0.05 lb/ac	Adult control	**WARNING. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees
Aztec® 2.1% Granular Insecticide – Bayer Corp.	tebupirimfos – 2.0% cyfluthrin – 0.1%	OP SP	0.15 lb/ac 0.01 lb/ac	Larval control	**WARNING. Restricted Use**; toxic to fish and wildlife
Baythroid® 2 Emulsifiable Pyrethroid Insecticide - Bayer	cyfluthrin – 25%	SP	0.04 lb/ac	Adult control	**DANGER. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees, may cause allergic skin reactions
Capture® 2EC Insecticide/Miticide – FMC Corp.	bifenthrin – 25.1%	SP	0.3 lb/ac	Larval control	**WARNING. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees
Chlorfos® 15G Insecticide Granular – Griffin LLC	chlorpyrifos – 15%	OP	2.02 lb/ac	Larval control	**CAUTION.** Toxic to birds and wildlife, extremely toxic to fish and aquatic organisms
Chlorfos® 4E Insecticide – Griffin LLC	chlorpyrifos – 42%	OP	2.52 lb/ac	Adult & Larval control	**WARNING.** Toxic to birds and wildlife, extremely toxic to fish and aquatic organisms
Counter® CR Systemic Insecticide-Nematicide – American Cyanamid Company	terbufos – 20%	OP	1.30 lb/ac	Larval control	**DANGER. Restricted Use**; fatal if swallowed, inhaled or absorbed through skin, extremely toxic to fish and wildlife
D-z-n® diazinon AG500 Insecticide - Syngenta	diazinon – 48%	OP	0.48 lb/ac	Adult control	**CAUTION. Restricted Use**; highly toxic to birds, fish and other wildlife, highly toxic to bees
D-z-n® diazinon AG600 WBC Insecticide - Syngenta	diazinon - 56%	OP	0.45 lb/ac	Adult control	**CAUTION. Restricted Use**; highly toxic to birds, fish and other wildlife, highly toxic to bees

247

Product	Active Ingredients	Type [a]	Use Rate [b]	Use	Classification [c]
Declare® Emulsifiable Insecticide Concentrate – Griffin LLC	methyl parathion – 45.11%	OP	0.22 lb/ac	Adult control	**DANGER. Restricted Use:** fatal if swallowed, inhaled or absorbed through skin, highly toxic to aquatic invertebrates and wildlife, highly toxic to bees
Diazinon 500-AG Organophosphate Insecticide – UAP	diazinon – 48%	OP	0.48 lb/ac	Adult control	**CAUTION. Restricted Use;** highly toxic to birds, fish and other wildlife, highly toxic to bees
Dimethoate 4 EC Systemic Insecticide - Helena	dimethoate – 44.8%	OP	0.45 lb/ac	Adult control	**WARNING.** Toxic to wildlife and aquatic invertebrates, highly toxic to bees
Dimethoate 400 Systemic Insecticide-Miticide - UAP	dimethoate – 43.5%	OP	0.44 lb/ac	Adult control	**WARNING.** Toxic to wildlife and aquatic invertebrates, highly toxic to bees
5 lb. Dimethoate Systemic Insecticide - Helena	dimethoate – 57%	OP	0.46 lb/ac	Adult control	**DANGER.** Toxic to wildlife and aquatic invertebrates, highly toxic to bees
Force® 3G Insecticide - Syngenta	tefluthrin – 3%	SP	0.17 lb/ac	Larval control	**CAUTION. Restricted Use;** very highly toxic to freshwater and estuarine fish and invertebrates
Fortress® 2.5G granular insecticide - DuPont	chlorethoxyfos – 2.5%	OP	0.16 lb/ac	Larval control	**DANGER. Restricted Use;** toxic to wild mammals, birds, fish and aquatic invertebrates
Fortress® 5G granular insecticide – DuPont	chlorethoxyfos – 5%	OP	0.16 lb/ac	Larval control	**DANGER. Restricted Use;** toxic to wild mammals, birds, fish and aquatic invertebrates

228

Product	Active Ingredients	Type [a]	Use Rate [b]	Use	Classification [c]
Furadan® 4F insecticide/ nematicide – FMC Corp.	carbofuran – 44%	C	0.88 lb/ac	Adult & larval control	**DANGER.** Restricted Use; poisonous if swallowed or inhaled, toxic to fish, birds and other wildlife, highly toxic to bees, can seep or leach through soil and can contaminate groundwater
Lannate® LV insecticide – DuPont	methomyl – 29%	C	0.65 lb/ac	Adult control	**DANGER.** Restricted Use; fatal if swallowed, toxic to fish, aquatic invertebrates and mammals, highly toxic to bees, known to leach through soil into groundwater
Lannate® SP insecticide - DuPont	methomyl – 90%	C	0.45 lb/ac	Adult control	**DANGER.** Restricted Use; fatal if swallowed, may cause blindness, toxic to fish, aquatic invertebrates and mammals, highly toxic to bees, known to leach through soil into groundwater
Lorsban® 15G Granular Insecticide – Dow Agrosciences	chlorpyrifos – 15%	OP	2.03 lb/ac	Larval control	**CAUTION.** Toxic to birds and wildlife, extremely toxic to fish and aquatic organisms
Lorsban® -4E Insecticide– Dow Agrosciences	chlorpyrifos – 44.9%	OP	2.69 lb/ac	Adult & larval control	**WARNING.** Toxic to birds and wildlife, extremely toxic to fish and aquatic organisms
Mocap® *10% Granular Nematicide Insecticide* – Aventis CropScience	ethoprop – 10%	OP	3.53 lb/ac	Larval control	**WARNING.** Toxic to aquatic organisms and wildlife

209

Product	Active Ingredients	Type [a]	Use Rate [b]	Use	Classification [c]
Mocap® *EC Nematicide-Insecticide* – Aventis CropScience	ethoprop – 69.6%	OP	3.34 lb/ac	Larval control	**DANGER. Restricted Use**; toxic to aquatic organisms and extremely toxic to birds
Penncap-M® Microencapsulated Insecticide – Elf Atochem	methyl parathion – 22%	OP	0.44 lb/ac	Adult control	**WARNING. Restricted Use**; highly toxic to aquatic invertebrates and wildlife
Phorate 20 G Organophosphate Insecticide - UAP	phorate – 20%	OP	1.3 lb/ac	Adult & larval control	**DANGER. Restricted Use**; extremely toxic to fish and wildlife
Pounce® WSB Insecticide – FMC Corporation	permethrin - 24.7%	SP	0.2 lb/ac	Adult control	**WARNING. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees
Pounce® 3.2 EC Insecticide – FMC Corporation	permethrin – 38.4%	SP	0.2 lb/ac	Adult control	**CAUTION. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees
Pounce® 25 WP Insecticide – FMC Corporation	permethrin – 25%	SP	0.2 lb/ac	Adult control	**WARNING. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees
Regent® 4 SC Insecticide – Aventis CropScience	fipronil – 39.4%	PP	0.13 lb/ac	Larval control	**WARNING. Restricted Use**; toxic to birds, fish and aquatic invertebrates
Sevin® brand 80S Carbaryl Insecticide – Aventis CropScience	carbaryl – 80%	C	2.0 lb/ac	Adult control	**WARNING.** Extremely toxic to aquatic and estuarine invertebrates, highly toxic to bees
Sevin® brand XLR PLUS Carbaryl Insecticide – Aventis CropScience	carbaryl – 44.1%	C	1.76 lb/ac	Adult control	**CAUTION.** Extremely toxic to aquatic and estuarine invertebrates, highly toxic to bees

010

Product	Active Ingredients	Type [a]	Use Rate [b]	Use	Classification [c]
Thimet® 20-G Soil and Systemic Insecticide – American Cyanamid	phorate – 20%	OP	1.3 lb/ac	Larval control	**DANGER. Restricted Use**; extremely toxic to fish and wildlife
Thimet® 20-G Soil and Systemic Insecticide – American Cyanamid	phorate – 20%	OP	1.3 lb/ac	Larval control	**DANGER. Restricted Use**; extremely toxic to fish and wildlife
Warrior® Insecticide with Zeon Technology - Syngenta	lambda-cyhalothrin – 11.4%	SP	0.03 lb/ac	Adult control	**WARNING. Restricted Use**; extremely toxic to fish and aquatic organisms and toxic to wildlife, highly toxic to bees

a - OP: organophosphate; SP: synthetic pyrethroid; C: carbamate; PP: phenyl pyrazole
b – maximum labeled use rate expressed in pounds of active ingredient per acre (assume that 1 liq pt ≅ 1 lb)
c – precautionary language as stated on label

In the year 2000, approximately 8 million pounds of insecticidal active ingredient costing $172 million were applied to 14 million acres of corn targeted to reduce CRW damage (see Table 2 below). These data were independently verified by EPA. There were approximately 24 million acres of corn treated with insecticides. Growers indicated that CRW was the sole target pest on approximately 7 million acres and one of a number of pests on the remaining treated acres. CRW-targeted acres (14 million) that received an insecticide treatment represented 18% of the total acres of corn planted in the continental U.S. and 59% of the total acres receiving any insecticide treatment in 2000. Continuous corn and first year corn acres (rotated acres) received 58% and 42% of the CRW-target insecticide applications, respectively. Continuous corn use areas include western Iowa, Nebraska, eastern Colorado, eastern South Dakota, the panhandle areas of Texas and Oklahoma, northeastern New Mexico, and southern Minnesota. Rotational acres are located predominantly in eastern Iowa, most of Minnesota, Wisconsin, Missouri, Illinois, Indiana, Michigan and other areas of the Eastern corn belt.

The 14 million acre treatments does not include acreage where the expected level of infestation is below the economic threshold, that is, where the expected loss is less than the $15.00 cost of treatment. National estimates of infested acreage are not published (unlike the Cotton Council for example that publishes estimates of infested acreage). Market analyses estimate all infested acreage with CRW to be around 28 million acres. This estimate is reasonable with what could be expected on the basis of yield losses without treatment and prices in year 2000. At depressed corn prices of $2 per bushel, yields at 175 bu/acre, a maximum yield loss of 8.5% would result in a $30 loss. Half of infested acres would be below the $15.00 cost of treatment. If corn prices rise or the cost of control decreases, the percent of infested acres that is treated would likely increase.

The infested acreage is expected to grow by 2.6% per year which implies that the 28 million in year 2000 will grow to 39 million acres by the year 2013. These factors needs to be considered when projecting the amount of chemical acre treatments and adopted acres of MON 863.

Table 2. Insecticide usage on corn in year 2000 reprinted from Ward 2002, MRID# 456530-01, p. 22

Parameter	Continuous Corn	First Year Corn	All Corn
Acres planted (x1,000)	22,269	57,310	79,579
Total insecticide treated acres (x1,000)	11,590	12,518	24,108
Total insecticide a.i. applied (lb x1,000)	6,332	6,011	12,343
CRW-targeted acres (x1,000)	**8,271**	**5,926**	**14,197**
a.i. applied to CRW-targeted acres (lb x1,000)	4,699	3,137	7,836
Average a.i. rate applied (lb/ac)	0.568	0.529	0.552
Average cost per acre	$11.95	$12.27	$12.08
Total cost of CRW insecticide purchased (x1,000)	$98,811	$72,699	$171,510

Only a few of the 36 products listed in Table 1 that are registered for control of CRW dominate the market. Table 3 below shows that five active ingredients, tefluthrin, chlorpyrifos, terbufos,

fipronil, cyfluthrin/tebupirimfos, are applied to 86% of the acres treated. Three of these active ingredients are organophosphates and three are classified as restricted use. In terms of pounds of insecticide applied, chlorpyrifos and terbufos (both organophosphates), account for 77% of the total insecticide products (almost 6 million pounds) applied to CRW-targeted acres. These 11 active ingredients (in Table 3) accounted for 98.3% of the total quantity of insecticide applied to CRW-target acres in 2000.

Table 3. Insecticide active ingredients applied to corn rootworm-targeted acres in year 2000 reprinted from p. 24, Ward 2002, MRID# 456530-01

Active Ingredient	Acres Treated (X 1,000)	Pounds Applied (Formulated Product)
Carbofuran	342	242,379
Chlorethoxyfos	361	55,485
Chlorpyrifos	**3,557**	**3,765,310**
Cyfluthrin/Tebupirimfos	1,326	179,527
Fipronil	1,498	158,141
Lambda-cyhalothrin	179	3,846
Methyl parathion	367	142,011
Permethrin	246	24,344
Phorate	508	588,380
Tefluthrin	3,570	400,339
Terbufos	2,044	2,146,761
Total	13,998	7,706,523

Table 4 provides a listing of the 13 major end-use products that are applied to CRW-targeted areas (Table 1 lists end-use products that are labeled for CRW control). The use of five products, Aztec 2.1% Granular Insecticide, Counter CR Systemic Insecticide-Nematicide, Force 3G Insecticide, Lorsban 15G Granular Insecticide, and Regent 4 SC Insecticide accounted for applications to 83% of the CRW-targeted areas. These products are all for larvicide rather than adulticide use. All of these products are restricted use except Lorsban 15G Granular Insecticide. A small number of seed-applied insecticides have been recently approved for use: Gaucho seed-applied insecticide (Gustafson, LLC), Prescribe seed-applied insecticide (Gustafson, LLC), and Force ST seed-applied insecticide (Syngenta). Imidacloprid is the active ingredient in Gaucho and Prescribe. Tefluthrin is the active ingredient in Prescribe. Ward (2002) describes that the performance of these seed-applied insecticides is inconsistent and weak under conditions of high CRW pressure and that these products do not perform as well as most soil-applied insecticides.

Table 4. Insecticide end-use products used for control of corn rootworm in year 2000 reprinted from p. 25, Ward 2002, MRID# 456530-01

Product[a]	Average Cost ($/A)	Adult (A)/Larval (L) Control	EPA Classification	Acres Treated (X 1,000)
Aztec 2.1% Granular Insecticide (tebupirimfos/cyfluthrin)	$13.05	L	Restricted	1327
Counter CR Systemic Insecticide-Nematicide (terbufos)	$13.10-$13.50	L	Restricted	2,044
Force 3G Insecticide (tefluthrin)	$14.48	L	Restricted	3,570
Fortress 5G granular insecticide (chlorethoxyfor)	$14.65	L	Restricted	361
Furadan 4F insecticide/ nematicide (carbofuran)	$11.74	L	Restricted	342
Lorsban 15G Granular Insecticide (chlorpyrifos)	$11.79	L	Unrestricted	3,165
Lorsban 4E Insecticide (chlorpyrifos)	$10.52	A	Unrestricted	374
Penncap-M Microencapsulated Insecticide (methyl parathion)	$6.79	A	Restricted	330
Pounce 3.2 EC Insecticide (permethrin)	$4.42	A	Restricted	224

Product[a]	Average Cost ($/A)	Adult (A)/Larval (L) Control	EPA Classification	Acres Treated (X 1,000)
Regent 4 SC Insecticide (fipronil)	$14.65	L	Restricted	1,392
Regent 80 WG Insecticide (fipronil)	$8.57	L	Restricted	106
Thimet 20-G Soil and Systemic Insecticide (phorate)	$10.90-$12.74	L	Restricted	508
Warrior Insecticide with Zeon Technology (Lambda-cyhalothrin)	$7.37	A	Restricted	173
Total				
Adult Control	$4.42 – $10.52			8%
Larval Control	$8.57 - $14.65			92%
Restricted Use				73%
Unrestricted				27%

a - active ingredient(s) stated in parentheses.

2. Comparative toxicity to humans (MRID#s 456530-01 and 450297-01)

MON 863 CRW-protected corn is safer for handlers, applicators, farmers, and the public than chemical pesticides in current use. Adoption of MON 863 corn hybrids will reduce the occupational, farmer, and public risks associated with the manufacture, transportation, storage, handling, application, and disposal of conventional insecticides. Many comments were received concerned with the potential contact to growers, their families and communities with the application, drift and on-farm storage of toxic materials. At product maturity, MON 863 hybrids have the potential to reduce insecticide applications by millions of pounds. This reduction of insecticide use will lead to both reduced human and environmental risks. The potential insecticide use reduction caused by adoption of MON 863 corn hybrids is discussed in section 7)

below.

Virtually all of the registered conventional insecticides used to control CRW are of special concern to EPA because of risks to humans (see Table 1 and discussion on Public Interest Finding above). Of the 36 registered insecticides for CRW control listed in Table 1, 25 are classified as "Restricted Use" and 12 have the "Danger" label classification. These include products formulated with the following active ingredients: chloroethoxyfos, phorate, terbufos, tefluthrin, and the commercial combination of tebupirimfos and cyfluthrin (Aztec). Each year there are confirmed reports of human illness associated with the registered chemical insecticide alternatives (See Agency's Incident Data Base, http://www.opp.gov/pesticides). Several of the current CRW insecticides are in Agency Special Review (i.e., dimethoate, phorate, and terbufos). Twenty-six of the 36 products contain either organophosphate and carbamate active ingredients that are listed as top priorities for tolerance reassessment under FQPA because of their high risk to humans and the environment. Because of EPA's concern with the conventional insecticide alternatives for CRW control, special precautions are required during all stages of their life cycle, including manufacture, transportation, storage, use, and disposal.

By contrast, MON 863 corn presents minimal or no risks to humans during any stage of its life cycle, from production to ingestion to disposal. Cry3Bb1protein has no toxic effects on mammals, and is not likely to induce allergic or hypersensitive responses based on results in all appropriate tests. Use of this new pesticide could potentially reduce use of CRW chemical pesticides by millions of pounds per year.

All of the conventional insecticide alternatives require a tolerance (maximum allowable level of pesticide residue in food). In contrast, the Agency has exempted the Cry3Bb1 protein and the genetic material necessary for its production in corn from the requirement for a tolerance, based on its human and animal safety (EPA, 2001). The Agency determined that there is a reasonable certainty that no harm will result from the aggregate exposure of Cry3Bb1 proteins (and the genetic material necessary for its production) to the U.S. population, including infants and children. As part of the rationale for the tolerance exemption, the Agency stated that "the lack of mammalian toxicity at high levels of exposure to the Cry3Bb1 [...] proteins demonstrate the safety of the product at levels well above maximum exposure levels anticipated in the crop." The calculated margin of exposure (MOE) for the Cry3Bb1 protein is 1.9×10^5 and was computed based on estimates of corn dietary intake (Ward 2002). The MOE is defined as the ratio of an appropriate NOEL (no observable effects level) to an estimate of human daily dietary exposure (DDE). An MOE of greater than 100 is considered as being protective of human health. The large MOE for the Cry3Bb1 protein ensures that there is a reasonable certainty of no harm for humans and other mammals exposed to this product. The estimate of daily human corn consumption is 0.2 g corn per kg body weight and the highest level of Cry3Bb1 found in samples of corn grain is 0.086 mg protein/g of grain.

The mammalian safety studies submitted by Monsanto (MRID#s 455382-02, -03; 454240-06, -08) have been reviewed by the Agency (C. Wozniak memo to M. Mendelsohn, 3/29/02). The rodent acute oral NOEL for the Cry3Bb1 protein (MON 863) is ≥3200 mg/kg (the highest dose tested). It has been previously determined by the Agency that oral ingestion is the only

meaningful route of exposure to humans to Cry proteins produced by plant-incorporated protectants (PIPs). Sjoblad *et al.* (1992) stated that when proteins are toxic that they act via acute mechanisms and at very low doses. Therefore, the Agency uses high dose acute exposure studies for assessing the potential toxicity of Cry proteins to mammals. In addition, the Agency requires in vitro digestibility studies, heat stability studies, and amino acid sequence comparisons with known allergens and toxins to examine the potential of Cry proteins for allergenicity. The results of the in vitro digestive fate studies indicate that Cry3Bb1 proteins degrade to nondetectable levels in simulated gastric fluid in less than one minute. Cry3Bb1 was not detectable in MON 863 grain following baking at typical grain processing temperatures. A comparison of amino acid sequences of known allergens and toxins revealed no relevant structural similarities to the Cry3Bb1 protein produced in MON 863 corn. Based on the Agency's review of the submitted studies, the 50-year history of safe use of Cry proteins in the U.S. agriculture, and comparable results to studies of other proteins in the Cry3 class, there is a reasonable certainty of no harm for this protein. MON 863 corn presents no unreasonable risks to humans during any stage of its life cycle, from production, handling, storage, ingestion, to disposal. Cry3Bb1protein has no toxic effects on mammals, and is not likely to induce allergic or hypersensitive responses based on results in all appropriate tests. In addition, use of this new pesticide could potentially reduce the use of CRW chemical pesticides by millions of pounds per year and substantially reduce mammalian risk.

3. Comparative toxicity and potential for adverse environmental effects (MRID#s 456530-01 and 450297-01)

All of the major chemicals used for CRW control can cause major adverse environmental effects under conditions of normal use (see Table 1 and the discussion in the Public Interest Finding section above). These products are formulated with the following active ingredients: chlorethoxyfos, phorate, terbufos, tefluthrin, methyl parathion, carbofuran, fipronil, bifenthrin, cyfluthrin, esfenvalerate, permethrin, diazanon, chlorpyrifos, dimethoate, methomyl, ethoprop, carbaryl, lambda-cyhalothrin, and the commercial combination of tebupirimfos and cyfluthrin (Aztec). Fifteen products are labeled as "toxic," 6 as "highly toxic," 1 as "very highly toxic," and 14 as "extremely toxic" to birds, fish and other wildlife. Each year there are confirmed reports of fish and bird poisonings associated with the registered chemical insecticide alternatives. Environmental effects from CRW chemical pesticides include toxicity and mortality in fish, birds, terrestrial mammals, aquatic invertebrates, and non-target insects. These chemicals can also spread via spray drift and runoff, thus contaminating both land and water bodies and impacting non-target organisms. Of the 36 registered insecticides for CRW control listed in Table 1, 25 are classified as "Restricted Use" and 12 have the "Danger" label classification. Table 5 below, compared the ecological risk for selected endpoints for the three top CRW insecticides: terbufos, chlorpyrifos, and tefluthrin. Together these three insecticides account for 63% of the acres treated (see Table 4 above). Tefluthrin poses lower risk than either chlorpyrifos or terbufos (see Table 5 below).

Table 5. Comparison of ecological risks associated with terbufos, chlorpyrifos, and tefluthrin.

Endpoint	Terbufos[a]	Chlorpyrifos[a]	Tefluthrin[a]
Mammalian Acute RQ	50	1	0.008
Avian Acute RQ	0.27	0.55	0.0001
Fish acute RQ	11	2	0.77
Freshwater invertebrates RQ	50	20	0.77
Marine/Estuarine Invertebrates RQ	53	162	0.87

[a]Risk is defined as the risk quotient (RQ)> level of concern (LOC). RQ = Toxicity/Exposure. LOC = 1

Potential adverse effects on non-target organisms resulting from the exposure to Cry3Bb1 have been evaluated in a series of studies with representative avian, aquatic, and terrestrial beneficial invertebrate species (see Agency review of ecological risk, Z. Vaituzis memo to M.. Mendelsohn, 2/12/03 and summarized in Ward 2002). These non-target organisms were exposed to high doses of leaf tissue, grain, or pollen containing a plant-produced Cry3Bb1 variant or to an artificial diet containing a *Bt*-produced Cry3Bb1 variant. In each case, a No Observable Effect Concentration (NOEC) for Cry3Bb1 was established. Results indicate that the Cry3Bb1 protein poses no significant risk to the test organisms. The Cry3Bb1 protein is essentially non-toxic to non-target species. The NOECs are all at least 4-fold to 141-fold higher than the protein concentrations measured in different plant tissues of MON 863 corn, indicating an adequate margin of safety. The results of these laboratory assays are summarized in Table 6 below and have been verified in the Agency review cited above. Preliminary results from a two-year field monitoring study collaborate these laboratory findings. That is, the abundance of prominent beneficial non-target invertebrate species was found to be comparable in conventional and MON 863 corn fields, and in some cases, the beneficial organisms were higher in MON 863 corn fields.

Table 6. Summary of results from ecological effects tests with Cry3Bb1 proteins. (Reprinted from p. 35, Ward, 2002 (MRID#456530-01)) Note: risk conclusions are based on protein concentrations measured in plant tissues of corn event MON 863 (Dudin et al., 2001 and verified by EPA).

Test Organism	Test Substance	Results [a]	Conclusions [b]	Reference
Cladoceran (*Daphnia magna*)	Pollen	NOEC ≥ 2.26 μg/l	NOEC ≥141x surface water MEEC	Drottar and Krueger, 1999
Collembola (*Folsomia candida*)	Leaf	NOEC ≥ 872.5 μg/g	NOEC ≥66x soil MEEC	Teixeira, 1999
Channel Catfish (*Ictalurus punctatus*)	Grain	No effect on growth or survival at 35% of diet	No significant risk	Li and Robinson, 1999
Bobwhite Quail (*Colinus virginianus*)	Grain	No effect on growth or survival at 10% of diet	No significant risk	Gallagher *et al.*, 1999
Adult Honey Bee (*Apis mellifera*)	Purified protein	NOEC ≥360 μg/ml	NOEC ≥3.8x maximum pollen level	Maggi, 1999a
Larval Honey Bee (*Apis mellifera*)	Purified protein	NOEC ≥1790 μg/ml as a single dose	NOEC ≥19x maximum pollen level	Maggi, 1999b
Adult Ladybird Beetle (*Hippodamia convergens*)	Purified protein	NOEC ≥8000 μg/g	NOEC ≥86x maximum pollen level	Palmer and Krueger, 1999c
Adult Ladybird Beetle (*Hippodamia convergens*)	Pollen	No effect on growth or behavior at 50% of diet	No significant risk	Bryan *et al.*, 2001

Test Organism	Test Substance	Results [a]	Conclusions [b]	Reference
Larval Ladybird Beetle (*Coleomegilla maculata*)	Pollen	No effect on growth or survival at 50% of diet	No significant risk	Duan *et al.*, 2001a
Adult Ladybird Beetle (*Coleomegilla maculata*)	Pollen	No effect on survival at 50% of diet	No significant risk	Duan *et al.*, 2001b
Monarch Butterfly Larvae (*Danus plexippus*)	Pollen	No effect on growth or survival	No significant risk	Sears and Mattila, 2001
Green Lacewing Larvae (*Chrysoperla carnea*)	Purified protein	NOEC ≥ 8000 μg/g	NOEC ≥ 86x maximum pollen level	Palmer and Krueger, 1999a
Parasitic Hymenoptera (*Nasonia vitripennis*)	Purified protein	NOEC = 400 μg/ml	NOEC ≥ 4.3x maximum pollen level	Palmer and Krueger, 1999b
Earthworm (*Eisenia fetida*)	Purified protein	NOEC = 57 mg/kg	NOEC ≥ 4.3x MEEC in soil	Hoxter *et al.*, 1999

[a] NOEC- No Observable Effect Concentration
[b] MEEC- Maximum Expected Environmental Concentration

Results of the environmental fate studies indicate that the Cry3Bb1 protein does not accumulate in the environment (e.g., air, soil, water), or in animal tissues (summarized in Ward, 2002 and reviewed in (EPA memo Z. Vaituzis to M. Mendelsohn, 2/12/03). Tests of aerobic soil degradation, using both an insect bioassay and an ELISA assay, resulted in DT50 estimates of 2.4 and 2.8 days respectively, and DT90 estimates of 7.9 and 9.2 days respectively, indicating rapid natural breakdown. Therefore, non-target soil organisms will be minimally exposed to the Cry3Bb1 protein based on the rapid degradation of Cry3Bb1 in soil.

In summary, Cry3Bb1 poses less risk to the environment than tefluthrin, terbufos, chlorpyrifos, fipronil or any other conventional insecticide labeled for CRW control. MON 863 corn poses minimal risk to non-target organisms. The Cry3Bb1 protein has no toxic effects on non-target organisms based on results in all appropriate tests. In addition, Cry3Bb1 is degraded rapidly in the soil (reducing non-target exposure). The Cry3Bb1 protein is expressed by the corn plant; thus, reducing the exposure to non-target organisms. In addition, Cry3Bb1 has a narrow target range. Monsanto has performed dietary bioassays to determine the insecticidal spectrum of the Cry3Bb1 protein. The protein is effective at killing only beetles of the family Chrysomelidae, specifically CRW and Colorado potato beetle (*Leptinotarsa decemlineata (Say)*). The family Chrysomelidae contains no known endangered species. Receptor binding is a critical step in the mechanism of action of the Cry proteins because without a binding site there is no toxic effect exerted. To date, there have been no functional receptors for Cry proteins on intestinal cells of fish, birds, or mammals (Ward, 2002). Use of this new pesticide could potentially reduce the use of CRW chemical pesticides by millions of pounds per year and substantially reduce non-target organism risk.

4. Practical–easier and safer for growers to use than current alternatives (MRID#s 456530-01 and 450297-01)

MON 863 corn offers many practical advantages to corn growers than the current alternatives. It can be planted early for a longer growing season and potentially higher yield, while ensuring adequate CRW protection throughout the growing season. Early planting corn is desirable to boost yield, but it can also reduce insecticide performance because of chemical dissipation prior to larval hatch. With MON 863 corn, the grower can plant early and not have to worry about timing or chemical dissipation. In addition, growers should be able to plant their crop more quickly because they won't have to continually have to stop and refill the insecticide boxes. MON 863 seeds can also have seed treatments that will allow even greater control of other associated pests such as wireworm, grub, maggots, and cutworms. Thus, growers will have multi-pest protection while carrying out insect control in essentially a single step at planting. All of these advantages to planting MON 863 corn are practical, easier, and safer for the grower. Planting MON 863 corn will save the grower money in application, insecticide, labor, fuel, equipment, storage and disposal costs (since there will be no insecticide containers needed for CRW control). Plus, it will provide the grower and other occupational workers greater safety, protect water bodies from run-off, and mitigate spray-drift and non-target effects. Grower interest in MON 863 is high, approximately 70% of growers survived were either "very interested" or "somewhat interested" in the new CRW trait hybrids (Vaughn et al., 2002, p. 48).

The three CRW control methods have been use of crop rotation and soil-applied insecticides, with limited use of rescue-treatments for CRW adult beetles. Historically, crop rotation has been the primary method of controlling CRW (Levine and Oloumi-Sadeghi, 1991). However, crop rotation is now far less effective because of the existence of a WCRW soybean rotational variant primarily in Eastern Illinois and Western Indiana, that oviposits in soybean fields soybeans (Levine and Oloumi-Sadeghi, 1991) and of a NCRW extended diapause (2 year extended) variant, primarily in parts of Minnesota, Iowa, and South Dakota (Krysan et al., 1996). In addition, CRW has developed resistance to methyl parathion and carbaryl, both adulticides used in rescue treatments (Meinke et al., 1998). Therefore, growers have become increasingly dependent on chemical pesticides to limit CRW losses. MON 863 CRW-protected corn offers a way to potentially control CRW behavioral variants and insecticide-resistant populations more effectively than through the use of chemical pesticides for CRW and still utilize effective corn-soybean (or other crop) rotations. MON 863 corn will likely reduce or eliminate the use of certain CRW insecticides (see discussion in section 7) below).

MON 863 gives growers equal or higher yields than use of chemical pesticides, while requiring less input of time and other resources. Preliminary results put this yield benefits at 1.5-4.5% (Mitchell, 2002). For a reasonable range of prices and yields, the value of this yield benefit to growers is $4-$12/A relative to the use of a soil-applied insecticides and depending on the CRW pressure.

Farmers were surveyed (Alston et al., 2002) to determine major factors that would be important to them in deciding whether to plant a transgenic corn with CRW resistance, such as MON 863, in place of their current corn. In addition to economic considerations, the farmers indicated the following non-monetary benefits would also be important.

- Safety of not handling a toxic insecticide
- Easy to use and handle
- All-in-one-product insect control
- Saving time and labor
- Better pest control

Farmers were especially interested in minimizing health and environmental effects of the pesticides they use and prefer a general use product over a restricted use product if cost and performance are comparable. Again, the survey in Alston et al (2002) indicates that farmers will favor the pesticide that minimizes adverse effects on the environment.

5. Efficacy of Event MON 863 (MRID#s 453613-03 and 455382-07)

Based on the review of the submitted field efficacy studies, MON 863 corn is as effective or more effective than chemical insecticides in protecting corn roots from CRW larval feeding damage. Chemical pesticides for CRW are usually applied to the soil at the time of planting. However, the pesticide may dissipate and no longer be effective by the time the larvae hatch. Timing is not a problem with MON 863 corn because the pesticide is incorporated within the corn roots and is produced at a relatively constant rate in growing corn. Weather is unlikely to

282

effect the efficacy of MON 863 corn as much as it might decrease the effectiveness of the chemical insecticides. Based on the results discussed below, the extent of root damage sustained by MON 863 was less than that seen in the control corn, and less than or equal to the damage in corn treated with any of the other chemical pesticides used in the comparative analysis.

5.1. Comparing the Efficacy of MON 853 and MON 863 to Three Corn Rootworm Species, Northern Corn Rootworm (*Diabrotica barberi*), Southern Corn Rootworm (*D. undecimpunctata howardi*), and Western Corn Rootworm (*D. virgifera virgifera*) (MRID # 455382-08)

In this experiment, Monsanto compared the relative efficacy of two transformed corn hybrids expressing the Cry3Bb protein (MON 853 and MON 863) in preventing damage from three species of corn rootworm larvae. This was accomplished by artificially infesting potted corn plants (treatments consisting of the two transformed hybrid and a non-transformed control hybrid) with eggs from each of three rootworm species. Each plant (in the V2 stage) was infested with approximately 800 eggs (6-8 plants per treatment were used). Root damage was scored using the Iowa Root Damage Rating (RDR) index (1 = no damage, 6 = extensive damage) after 3-4 weeks of larval feeding.

Results from the study showed that both MON 853 and MON 863 experienced significantly less root damage from all three rootworm species than the non-transformed control hybrid. In terms of western and northern corn rootworm damage, MON 863 had significantly less root damage (<2 RDR) than MON 853 (~ 2.3 RDR). For southern corn rootworm, there was no significant difference between MON 853 and MON 863 (RDR ~ 3.5-3.8). Southern corn rootworm damage was greater than western or northern corn rootworm damage for all treatments. It is noted that Monsanto is only commercializing Event MON 863.

5.2 Efficacy of MON 863 Against Corn Rootworm and Comparison to Insecticide Treatments - Results of Year 2000 Field Trials (MRID # 453613-03)

In this experiment, Monsanto evaluated the relative effectiveness of MON 863 and conventional pesticide treatments at preventing damage from corn rootworm feeding in field efficacy trials. The pesticides tested (all soil insecticides) included Force 3G (tefluthrin), Counter CR (terbufos), and Lorsban 15G (chlorpyrifos).

The study consisted of three separate field experiments, all of which utilized similar growth stage MON 863 hybrid and a non-transgenic control hybrid (negative MON 863 isoline). In each of the experiments, treatments were deployed using a randomized block design and were scored for root damage in late July. Root damage was assessed using the Iowa Root Damage Rating (RDR) index (1 = no damage, 6 = extensive damage, >3 = economic threshold). For the first experiment (conducted at seven different locations), treatments (MON 863, control, Force 3G, Counter CR, and Lorsban 15G) were deployed as four-row strips (4 replicates per treatment). Each plot was artificially infested with 800 rootworm eggs/foot (species not specified). In the second experiment (conducted at eight different locations), MON 863 was evaluated against Force 3G treatment and an untreated control. Treatments were deployed as single rows and were

artificially infested with 1600 rootworm eggs/foot (species not specified). In the third experiment (conducted at nine test sites), treatments (MON 863, control, Force 3G, Counter CR, and Lorsban 15G) were planted in four-row strips in continuous corn acres or a corn/pumpkin trap crop (no artificial rootworm infestation was used). For all tests, RDR damage was analyzed via analysis of variance and t-tests to determine significant differences between treatments. Also, a "consistency rating" was calculated for each experiment by determining the percentage of root damage in a treatment that is below the economic threshold (RDR = 3) when the corresponding control treatment root damage is above the threshold.

The results of the first experiment showed that when summed across all test locations, MON 863 (RDR = 2.02), Force 3G (2.40), Counter CR (2.26), Lorsban 15G (2.40) experienced significantly less root damage than the untreated control (3.91), although there was no significant difference between MON 863 and the insecticide treatments. However, at three of the seven locations, MON 863 had significantly less root damage than all of the other insecticide treatments. For the second experiment, when summed across all eight test sites, MON 863 (RDR = 1.41) and Force 3G treatment (1.91) showed significantly less root damage than the untreated control (3.27). There was no significant difference between MON 863 and Force 3G, although root damage for MON 863 was significantly less than that for Force 3G at five of the test sites. In the third experiment, MON 863 experienced significantly less root damage (RDR = 1.72, summed over all nine locations) than any of the insecticide treatments or the control (all insecticide treatments had significantly less damage than the control). For all three experiments, the "consistency rating" for MON 863 was close to 100%, meaning that damage in MON 863 hybrids was almost always kept below the economic threshold when the control treatment showed damage exceeding the threshold.

Taken together, the results show that MON 863 prevented root damage from rootworm feeding as well or better than rootworm soil insecticides. Root damage ratings for MON 863 were typically between 1.2 and 2.0, a high level of control relative to untreated control hybrids. In addition, the results were generally consistent from location-to-location (test sites included plots in six separate corn-growing states).

6. Yield benefits (MRID# 456530-02)

The field efficacy data discussed above were used to estimate the yield benefit of MON 863 corn hybrids relative to nontransgenic corn hybrids without corn rootworm control and with a soil insecticide for corn rootworm control (Mitchell, 2002). Field data were collected to estimate the proportional yield loss as a function of the root rating difference (1-6 root rating scale of Hills and Peters). Three years of data (1994-1996) in 2 locations in Illinois (near Urbana and DeKalb) were used for the analysis. Data from efficacy experiments conducted in 1999 and 2000 in several locations were used to estimate the impact of event MON 863 on the root rating. Preliminary estimates using a composed error model for insect damage functions indicate that MON 863 corn hybrids have a yield benefit of 1.5 to 4.5% relative to control with a soil insecticide and 9 to 28% relative to no control. The value of these benefits is estimated to be $4-$12/acre relative to control with a soil insecticide, depending on the corn rootworm pressure and $25-$75/acre relative to no control. Because there is a low correlation between root rating

difference and yield loss, there is uncertainty in the realized yield benefit. This uncertainty is not due to MON 863 *per se*, but to the numerous environmental and agronomic factors determining a corn plant's yield and yield response to corn rootworm larval damage.

7. Grower benefits (MRID#s 456923-01, 456530-01-03, 450297-01, 455770-01)

Monsanto submitted a study entitled "An Ex Ante Analysis of the Benefits from the Adoption of Monsanto's Rootworm Resistant Varietal Technology - YieldGard® Rootworm" by Alston et al. (2002). Alston et al. (2002) examined the potential economic impacts in the U.S. of the commercial adoption of MON 863 corn (YieldGard® Rootworm technology). The model estimates the economic impacts if MON 863 corn had been available and was priced such that the technology fee per acre would be the same as for a representative conventional (non-Bt) CRW control technology. Alston et al (2002) used data from the year 2000 and made certain assumptions where necessary. For the year 2000, almost 8 million pounds of CRW insecticide costing $172 million were applied to 14 million acres (i.e., 17% of total corn acres planted). For a reasonable range of prices and yields, benefit to growers was estimated at $4 to $12/acre, depending on root cornworm pressure. They estimated one-year total benefits (in the year 2000) with 100 percent adoption of MON 863 corn in year 2000 of $460 million. This benefit includes $171 million to Monsanto and other seed companies and $231 million to farmers from yield gains, and $58 million to farmers from reduced risk and time savings, and other benefits associated with the reduced use of insecticides.

7.1. EPA projections of grower benefits and environmental benefits

Grower benefits are a theoretical construct that cannot be directly measured or monitored. They are defined as the premium a grower would pay for MON 863 or the difference between the value of MON863 and its' costs. Grower benefits can be depicted in a graph as the area above the technology fee and below the demand curve. This is where product value as measured by willingness to pay exceeds the technology fee. Grower benefit projections are best confirmed by comparing projected adoption rates with actual adoption rates given technology fees.

The factors that will influence grower demand are: CRW infested acres, comparative yields and costs of competing technologies for CRW insect control, U.S. and global market acceptance and approval, and other regulatory constraints (e.g., refuge requirements).

About 30% of the corn acreage (twenty-four million acres) was treated with 12 million pounds of insecticides to control pests over the last several years. For the year 2000, almost 8 million pounds of CRW insecticide costing $172 million were applied to 14 million acres ($12.29 per acre). The EPA estimates and projections use the submitted comparative performance studies and yield enhancements discussed in the previous section which indicate an increased yield of 1.5 to 4.5% for use of MON 863 over chemical pesticides when infestation levels are high.

7.1.1. Methodology and parameter estimates

The Agency predicted mature market adoption rates based on a demand simulation model and

pricing behaviors based on revenue maximization (marginal cost = 0). The demand curve measures adoption at alternative technology fees for MON 863 corn. A discussion of the simulation model is found in Section II.E. of EPA's 2001 Bt Plant-Incorporated October 15, 2001 Biopesticides Registration Action Document (EPA, 2001). Briefly, individual circumstances and pest pressure affect the return from MON 863. The distribution of MON 863 grower value (willingness to pay) is assumed to be uniformly distributed from 0 to a maximum value. Unique costs (other than technology fees) associated with MON 863 are also assumed to be uniformly distributed from 0 to a maximum cost. Two parameter estimates are required to run the simulation model, maximum value and maximum cost. The model calculates adoption rate, gross benefits and net benefits for any given technology fee. Maximum grower value is derived from estimates of 1) improvements to yield, 2) reductions in chemical costs, and 3) the perceived value of a less toxic product. The costs unique to MON 863, such as marketability discounts/risks and refuge requirements, are more difficult to estimate directly so an indirect approach is used. It is the maximum cost consistent with the percent of growers who would not select MON 863 at technology fee of zero.

The maximum value reflects the acreage with the highest values due to greatest pest pressures and cost of rootworm control. An estimate of $15.75 is used for the model and is based on a 4.5% yield improvement on $350/acre gross income for corn, including government payments, which is characteristic of expectations for 2001. The cost savings from insecticides includes the out of pocket costs of $12.50 per acre plus a maximum of $2.50 per acre due to perceived value as a general use product with less toxic effects to the local environment. The perceived value of a less toxic product is not an out of pocket cost and is probably of lessor importance to growers. The high market share for Chlorpyrifos (25 % of the CRW market) may be due in part to the fact that it is only major alternative registered for general use (i.e., no restricted uses).

Value due to product performance (Yield)	$15.75
Value from lower chemical costs	$12.50
Value from easier and safer to use	$ 2.50
Max Value of Bt corn rootworm	$30.75

It is unlikely based on past experience with biotechnology products that European Union approval will occur in less than three years after launch (Vaughn et al., 2002). This international regulatory constraint imposes an additional cost on adoption of MON 863 corn. Just as was done for Roundup Ready® corn, until full European (or global) regulatory approval occurs, Monsanto plans to continue its channeling program with growers, dealers, and grain handlers to help ensure that MON 863 corn is directed into appropriate global markets (markets with regulatory approval). The simulation model can be used to assess the impact of access to global markets. The negative costs associated with limited marketability is reflected by the percent of growers who would not use MON 863 even if there was no technology fee. That is, adoption would not be 100% even if MON 863 is given away. Based on the results of a Survey on grower attitudes toward GMO's (genetically-modified organisms) conducted by the American Corn Growers Association (2001), sixteen percent of respondents stated they would not be willing to grow more non-GMO corn varieties. The sixteen percent of growers is consistent with a maximum costs of

286

$10 per acre as compared to a maximum cost of $5 per acre the 9% (91% adoption) at $0/acre technology fee.. A 16% removal from the target market would reduce adoption rates from 43% to 35% which would translate to lower grower benefits and less use reduction.

The demand curve and derived marginal revenue curve provide a basis for predicting a technology fee. The estimate of $15 per acre technology fee is based on revenue maximization behaviors which is equivalent to profit maximization if marginal cost is zero. The actual technology fee would vary from this estimate based on licensed seed companies perceptions of the demand curves for specific hybrids and marginal costs associated with marketing and sales.

EPA's economic assessment and eleven (11) year projection of aggregate grower benefits is based on current chemical prices of alternatives. It does not anticipate or include any price changes from competing technologies as MON 863 corn is introduced. It does not anticipate or include the effects of new active ingredients registered for corn rootworm.. The economic assessment is limited to grower benefits and does not estimate the reduced cost passed through to final consumers (though in the long run, economic theory suggests that the 2% improvement in returns on gross revenue would be passed through to consumers). No assessments are made of impacts to foreign trade or agricultural practices. MON 863 corn may lower the costs of rootworm control and therefore have some effect on acres grown to continuous corn which would increase pest pressures and reduce the environmental benefits of MON 863 (UCS 2002).

7.1.2. Corn Rootworm (CRW) infested acres

Scouting for the range and level of infestation is done by measuring the density of adult beetles and larvae. Gray (2000) noted that CRW infested acres are increasing due to the geographical expansion of the WCRW soybean variant. Based on both the likely geographic spread of the WCRW soybean variant and the NCRW extended diapause variant, it is likely the total infested acres will move from approximately 28 million acres to closer to 39 million acres in 13 years. This assessment projects the range of infestation to increase uniformly by 2.6% per year and the density to remain such that only one half of the acreage infested is above the economic threshold. To the extent that market forces reduce the economic threshold (increased corn prices, increased yields or reduced cost of CRW control), acreage adoption and conventional insecticide use would be higher than currently forecast and the environmental and grower benefits of MON 863 would also be higher than projected in this review.

EPA has considered the rate of increase of CRW infested aces as input into its simulation model that was used to predict technology fee, adoption rates, and grower benefits of MON 863 corn.

7.1.3. MON 863 corn hybrid supply

Adoption of MON 863 corn (acres) is dependent on supply as well as demand. The supply is constrained by corn seed hybrid availability; both for a single hybrid as well as the total number of hybrids available. The commercial hybrid development process requires sequential development that will take several years after commercial launch.

387

In the first two to three years after commercial launch, adoption of MON 863 corn is predicted to be relatively slow because there will only be a limited number of MON 863 corn seed hybrids available, grower acceptance, price, and global market approval of the technology. Monsanto has projected that MON 863 corn adoption will be similar to Roundup Ready® corn in that it will not be available in Pioneer or Syngenta brands nor have European Union approval at the time of launch (Vaughn et al., 2002). Roundup Ready® corn had approximately 1% (790,000 A), 2.5% (2 million A), and 5% (4 million A) acreage penetration in percent of total corn acres in year 1, 2, and 3 from commercial release, respectively. These data were used by EPA in its economic analysis.

7.1.4. Estimating the demand curve for MON 863

The demand curve for MON 863 for year 2013 is shown in Table 7. It is based on a simulation of adoption at alternative technology fees for MON 863 as described in section 7.1. Marginal revenue is computed using a technology fee of $15 based on revenue maximization. It is based on pricing behavior where the marginal costs of increasing seed production are negligible (assumes that all seed hybrids are in place). It is the last point where marginal revenue is positive (see bolded line in Table 7 below). The total revenue with a $15 technology fee is $252 million for 16.9 million acres of MON 863 corn planted in the year 2013. Actual technology fees are certain to vary from this estimate. At a $15 technology fee, MON 863 adoption is predicted to be 43% of infested acreage. If only 50% of infested acreage is treated, then only 7% of infested acreage will still be treated by conventional chemical controls (not including any refuge acres required as part of an Insect Resistance Management plan).

Table 7. Simulated demand curve for the year 2013.

Tech Fee Schedule ($)	Percent Adoption (Model Calculation)	Acres Adopted (X 10^6)	Total Revenue (X 10^6)	Marginal Revenue (X 10^6)	Marginal Revenue per Acre ($)
27	5%	1.95	52.65		
24	14%	5.46	131.04	78.39	22.33
21	23%	8.97	188.37	57.33	16.33
18	34%	13.26	238.68	50.31	11.73
15	**43%**	**16.77**	**251.55**	**12.87**	**3.67**
12	53%	20.67	248.04	(3.51)	(0.90)
9	63%	24.57	221.13	(26.91)	(6.90)
6	72%	28.08	168.48	(52.65)	(15.00)
3	83%	32.37	97.11	(71.37)	(16.64)
0	91%	35.49	0.00	(97.11)	(31.12)

7.1.5. Projecting grower benefits

Grower benefits are calculated as the sum of the difference between what the grower is willing to pay and the actual technology fee. The EPA simulation model computes the average gross

288

benefits for adopters and Bt-related costs due to MON 863 corn adoption. The estimated net benefits per acre are $6.56 are based on a $15 technology fee (see Table 7 above). The estimated gross benefits (primarily, yield and insecticide cost reduction) per acre for adopters are $23.94 per acre and estimated Bt-related costs for adopters are $2.38 per acre.

The annual change in infested acres, adoption, conventional chemical use and associated grower benefits are projected for each year from 2003 to 2013 (Table 8 below). Projected acres infested and conventional chemical treatments are based on growth rates for infested acreage with a fixed treatment percentage of 50% of the total projected CRW infested acres.

Table 8. Projected acreage infested, MON 863 adoption and conventional treatments 2003 to 2013

Year	Acres Infested ($X 10^6$)	Acres treated ($X 10^6$)	MON 863 acres ($X 10^6$)	Conventional treatments ($X 10^6$)
2000	28.0	14.0	0.0	14.0
2002	29.5	14.7	0.0	14.7
2003	30.2	15.1	1.0	14.1
2004	31.0	15.5	2.5	13.0
2005	31.8	15.9	4.0	11.9
2006	32.6	16.3	6.0	10.3
2007	33.5	16.7	7.2	9.5
2008	34.3	17.2	8.6	8.5
2009	35.2	17.6	10.4	7.2
2010	36.1	18.1	11.9	6.1
2011	37.1	18.5	13.7	4.8
2012	38.0	19.0	15.8	3.2
2013	39.0	19.5	16.8	2.7
Annual growth rate	**2.58%**	**2.58%**		**-14.36%**

Annual grower benefits (see Table 9) are based on a constant $6.56 per acre, and the growth in total annual benefits are due to the availability of hybrid seed containing Cry3Bb over a greater areas of CRW infestations. Actual grower benefits may be higher in the early years if supply is first available in those areas with highest CRW pest pressure.

The cumulative sum of the grower benefits for the first three years (2003 to 2005) is $49.2 million and for eleven years (2003 to 2013) is $642.7 million (Table 9). The discounted aggregate benefits for year 2003 to 2013 are $385.31 million assuming a discount rate of 7%. The 7% discount rate represents the Office of Management and Budget rate (see Circular No. A-94, Transmittal Memo No. 64, October 29, 1992), a relatively risk-free rate of return similar to that assumed for a long-term Treasury bond. The discount rate is defined as the interest rate used in calculating the present value expected yearly for benefits and costs.

289

Table 9: Annual Grower Benefits of MON 863

Year	Target Acres (X 10^6)	Adoption Acres (X 10^6)	Grower Benefits ($ X 10^6)
2003	2.3	1.0	6.6
2004	5.8	2.5	16.4
2005	9.3	4.0	26.2
2006	14.0	6.0	39.4
2007	16.7	7.2	47.2
2008	20.1	8.6	56.7
2009	24.1	10.4	68.0
2010	27.7	11.9	78.2
2011	31.9	13.7	90.0
2012	36.7	15.8	103.5
2013	39.0	16.8	110.5
Cumulative		**98.0**	**$ 642.7**

7.1.6. Projecting chemical use with MON 863 (MRID#s 456530-01 and 450297-01)

The insecticides used to control corn rootworm in conventionally grown (non-Bt) corn consist mainly of organophosphates (9), carbamates (3), synthetic pyrethroids (6), and phenyl pyrazole (1) classes of chemistry (see Table 1 above). Table 10 shows that there have been significant shifts in the use of insecticides. Synthetic pyrethroids have increased at the expense of organophosphates and carbamates. The clear shift is away from OP insecticides and toward synthetic pyrethroids, especially the effective, relatively new product tefluthrin, now the market leader (Benbrook, 2002). Fipronil was introduced in 1998 and accounts for the other category. Table 11 projects future acre treatments by chemical class using past trends and the projected conventional treatments shown in Table 8.

Table 10: Historical market shares for Corn Rootworm

Percent of total acre treatments

Chemical class	1995	2001
Carbamate	4.5%	2.1%
Synthetic Pyrethroid	21.9%	41.0%
Organophosphate	73.4%	45.8%
Other	0.1%	11.0%
total.	100.0%	100.0%

Table 11: Projected treatments for Corn Rootworm

Millions of acre treatments for Corn Rootworm

Chemical class	2002	2003	2004	2005	2006	2007
Carbamate	0.3	0.3	0.2	0.2	0.2	0.1
Synthetic Pyrethroid	6.2	6.2	5.9	5.6	5.0	4.8

290

Chemical class	2002	2003	2004	2005	2006	2007
Organophosphate	6.5	6.0	5.3	4.6	3.8	3.3
Other	1.7	1.7	1.6	1.5	1.4	1.3
MON 863	0.0	1.0	2.5	4.0	6.0	7.0
Total CRW-treated	14.7	15.1	15.5	15.9	16.3	16.5

Table 12: Projected use reduction associated with MON 863
Millions of acre treatments for Corn Rootworm

Chemical class	2002	2003	2004	2005	2006	2007
Carbamate	0.0	0.0	0.0	0.1	0.1	0.1
Pyrethroid	0.0	0.4	1.1	1.9	2.9	3.5
Organophosphate	0.0	0.4	1.0	1.5	2.2	2.5
Other	0.0	0.1	0.3	0.5	0.8	0.9
total reduction	0.0	1.0	2.5	4.0	6.0	7.0

These pesticides have varying application rates and many are toxic to humans and non-target wildlife and have restricted use or specific, mandatory mitigation measures to minimize exposure. The average active ingredient rate applied (lb/A) has been steadily decreasing reflecting the shift from OP's and carbamates to synthetic pyrethroids and fipronil. Rates have gone from an average of 0.7 lb/A in 1995 to 0.4 lb/A in 2001. The application rates shown in Table 1 for terbufos, chlorpyrifos, carbofuran, tebupirimfos, and phorate are closer to 1 lb/A. Synthetic pyrethroids and fipronil, for example, are newer chemistries that are used at 0.1 (or less) lb/A. The use reductions shown in Table 12 indicate that as MON 863 CRW-protected corn adoption increases in the next five years, acre treatments will be reduced for all currently registered CRW insecticides. The greatest use reductions are seen in both the organophosphate and synthetic pyrethroid classes. In 2005, approximately 1.5 million acre treatments of organophosphate insecticides, 1.9 million acre treatments of synthetic pyrethroid insecticides, 0.1 million acre treatments of carbamate insecticides, and 0.5 million acre treatments of other chemical insecticides including members of the phenyl pyrazole class (e.g., fipronil) will be reduced based on 2003 figures. In 2007, the extent of insecticide use reduction will be even greater, approximately 2.5 million acre treatments of organophosphate insecticides, 3.5 million acre treatments of synthetic pyrethroid insecticides, 0.1 million acre treatments of carbamate insecticides, and 0.9 million acre treatments of other chemical insecticides are expected to be reduced.

7.2. Comparing estimates of grower benefits from other studies

Reported estimates may differ with respect to the entities included as well target year. Some include the grower and chemical producer and reflect the total societal value of MON 863. EPA's assessment is limited to grower benefits. Estimates made by Alston et al. (2002) are based on an ex ante assessment assuming that MON 863 was available in the year 2000. It is necessary to adjust estimates to the extent possible to create valid comparisons.

The one-year total benefits (in the year 2000) with 100 percent adoption of MON 863 corn in year 2000 of $460 million (Alston et al. 2002). This benefit includes $171 million to Monsanto and other seed companies and $231 million to farmers from yield gains, and $58 million to farmers from reduced risk and time savings, and other benefits associated with the reduced use of insecticides. The EPA assessment for year 2013 is based on a higher level of infested acreage. Adjusting Alton et al's analysis of the ex ante total benefits for the year 2000 of $460 million to the year 2013 by an additional 39% gives $640 million in total benefits.

The EPA estimate of total benefits can be calculated assuming a zero technology fee. This is essentially the total area under the demand curve. Total benefits would be $507 million in 2013. This compares with Alston et al's adjusted estimate of $640 million total benefits.

In a separate analysis, Gray (2000) states that if farmers invested $400 million in this technology (technology fees are assumed to $15 per acre), these resources would prevent an economic loss of approximately $600 million, for a net gain of $200 million to farmers. Adjusting this estimate to coincide with infestation levels in 2013 (a 39% increase) provides and total benefits of $834 million.

EPA's estimate of total benefits in 2013 of $507 million in 2013 is 20% lower than Alston et al.'s projections and 40% lower than Gray's projections.

8. Suggested measures to monitor environmental and grower benefits

The amount of use reduction attributed to MON 863 cannot be directly observed. For example, if the economic threshold for CRW treatment is reduced by the increased competition created by MON 863, then total infested acres treated at all would increase. The effect of MON 863 on chemical use reduction would be less then 1 acre per adopted acre of MON 863. A survey of growers who adopted MON 863 would be helpful to directly estimate use reduction.

Translating the environmental benefits of use reduction is a topic that is given increased attention for the purpose of strategic planning and measuring results. Measures most likely affected by the reduction in cotton insecticide use are reported incidents to workers, accidental spills, and mortality to non target wildlife.

EPA Environmental Fate and Effects Division in the Office of Pesticide Programs has identified the most toxic active ingredients to birds, based on risk assessments of based on all available information. The list contains 10 insecticides currently being used on agricultural crops: aldicarb, methyl parathion, dicrotophos, carbofuran, phorate, oxamyl, diazinon, disulfoton, methamidophos, and ethoprophos. Methyl parathion, carbofuran, phorate, diazinon, and ethoprophos are active incredients registered as formulated products for the control of corn rootworm (i.e., alternatives to MON 863). The use of methyl parathion, carbofuran, and phorate for corn rootworm control accounts for 1.2 million acres which is 6.6% of the total use of the ten insecticides on all agricultural crops. Thus, MON 863 alone can have a significant impact on reducing the use of insecticides posing the highest risk to birds.

National findings from the National Water Quality Assessment Program (NAWQA) found that primarily herbicides were found in agricultural streams and shallow ground water. " Insecticides typically were detected in urban areas, sometimes at high concentrations. Urban areas, covering less than 5 percent of land in the continental United States, traditionally have not been recognized as important contributors to pesticide contamination, especially when compared to agricultural land, which covers more than 50 percent of the United States." Chlorpyrifos has the largest amounts applied to corn rootworm of any other conventional insecticide. However, it's common occurrence in the NAWQA data is thought to be related to use on home lawns instead of field corn. (It's use on lawns was discontinued with stop sale date of 12-31-02.) The environmental benefits of MON 863 are expected to be minor as measured by detections in water of chemical alternatives since these occurrences are infrequent.

Funds from the OPP budget for outcome development under GPRA were used to investigate the suitability of the North American Bird Breeding Survey (BBS) to measure the effects of changes in insecticide use. Changes in bird populations were considered both as an environmental indicator as well as an environmental outcome. Indicators only denote change while an outcome measure has a right and wrong directional change. Cotton was selected as a pilot study since Bt cotton, introduced in 1996, has helped reduce insecticide use by up to 80%.

The North American Bird Breeding Survey produces an index of relative abundance rather than a complete count of breeding bird populations. The data analyses assume that fluctuations in these indices of abundance are representative of the population as a whole (Sauer *et al.* 2001). Since 1966, the information has been collected on a national basis and is of high quality. Routes are randomly selected which reduces bias and permits statistical inference. The availability of roads in most areas is so extensive that studies have shown the sample to be representative of habitats of the entire region (personal communication with Laura Jackson, RTP, ORD - December 2002) Other requirements such as consistent methodology and observer expertise, visiting the same stops each year, and conducting surveys under suitable weather conditions have been applied to produce comparable data over time. Hames *et al.* (2002) show that BBS data can be successfully related to short-term trends if the necessary variables are included in the assessment

Forty two (42) counties in eleven (11) states were selected to examine if bird population trends were affected by the amount of Bt cotton. Six years of avian populations prior to 1996 were compared with the changes in avian trends for five years after Bt cotton was introduced While the study focused on changes in six year trend lines, another statistic that appears most useful is the percent of species with increasing (or decreasing trends). In most cases, the percent of species that had statistically significant trend changes was what would be expected by chance along. That is, 5% of species were significant at the 0.05 test level. However, certain situations appeared very different. For example, the Hot Continental ecoregion had 15% of the species found in cotton fields with a significant positive trend change. The Prairie and Tropical Steppe ecoregion had significant positive trend changes for 16% of avian species likely to be found in cotton fields. Significant differences in positive trends appeared for 2 of the 5 ecoregions studied. Ten (10%) percent of insectivores had significant positive trends. Bt cotton may increase available food supply as well as reduce avian mortality.

The study also showed the need to obtain more detailed geographic information since there were no significant differences between high Bt cotton and low Bt cotton counties. Funds for a follow up study were received through an internal EPA competition for measures development (OPEI/OCFO competition). The study will gather additional data on detailed land use characterization and integrate avian demographics from the Institute for Bird Populations Monitoring Avian Productivity and Survivorship program (MAPS). The study completion date is May 30, 2003.

Differing bird behaviors in regard to feeding and nesting requirements are sensitive in different ways to environmental change. Grouping species of birds reduces variability and smooths trends and offers numerous opportunities to create avian environmental indicators. For all species, the percent of trends that are increasing is 55% for the 1966 to 2002 period. However, only 21% of the 14 grassland breeding species have had increasing trends, according to USGS analyses. This assessment is confirmed by the recently released Heinz Center report on the State of the Nations Ecosystems. It quotes a study by W. Mark Roberts, an independent researcher, who found that the pattern of species populations growth trends has only recently changed very dramatically. For most of the past 35 years, populations of invasive and native, non invasive bird species were increasing in about the same proportion. However, from 1996 to 2000, about 80% of invasive species increased in population while only 30% of native species increased. Invasive species are believed to be indicative of agricultural land conversion, habitat fragmentation and the spread of exotic vegetation. In addition to BBS, other collected data exist that may be useful to study the effect of Bt corn on any number of aggregate indices, such as percent of invasive species, native species of management concern, or any other grouping of public interest.

This EPA review assesses the potential to measure predicted impacts of insecticide use reductions. To measure the actual environmental benefits of MON 863 corn, one could use changes in reported incidence of adverse affects to non target wildlife (incident data reports), National Water Quality Assessment Program (NAWQA), environmental monitoring results, and changes in trends in avian populations species sensitive to insecticide use.

References

Alston, J. M., J. Hyde, and M. C. Marra. 2002. An Ex Ante Analysis of the Benefits from the Adoption of Monsanto's Corn Rootworm Resistant Varietal Technology – YieldGard® Rootworm. May 18, 2002. Unpublished study, submitted by Monsanto Company (Ref. No. MSL-17993). MRID# 456923-01.

American Corn Growers Association "2001 Corn Producers Survey GMO's & Markets, July 9, 2001. http://www.acga.org/programs/2001CPS/.

Benbrook, C. 2002. Public comments submitted to OPP Docket OPP-30509.

Gray, M.E. 2000. Prescriptive Use of Transgenic Hybrids for Corn Rootworms: An Ominous Cloud on the Horizon? Proceeding of the January 5-6, 2000 Illinois Crop Protection Technology Conference. University of Illinois at Urbana-Champaign, College of Agricultural, Consumer, and Environmental Sciences, University of Illinois Extension. Pp. 97-103.

Hames, R.S., K.V. Rosenberg, J.D. Lowe, S.E. Barker, and A.A. Dhondt. 2002. Adverse effects of acid rain on the distribution of the wood thrush *Hylocichla mustelina* in North America. Proceedings of the National Academy of Science 99(17): 11235-11240.

Krysan, J. L., D. E. Foster, T. F. Branson, K. R. Ostlie, and W. S. Cranshaw. 1986. Two years before the hatch: rootworms adapt to crop rotation. Bull. Entomol. Soc. Amer. 32: 250-253.

Levine, E. and H. Oloumi-Sadeghi. 1991. Management of Diabroticite Rootworms in Corn. Annu. Rev. Entomol. 36: 229-255.

Levine, E., H. Oloumi-Sadeghi, and C. R. Ellis. 1992a. Thermal Requirements, Hatching Patterns, and Prolonged Diapause in Western Corn Rootworm (Coleoptera: Chrysomelidae) Eggs. J. Econ. Entomol. 85: 2425-2432.

Levine, E., H. Oloumi-Sadeghi, and J. R. Fisher. 1992b. Discovery of Multiyear Diapause in Illinois and South Dakota Nothern Corn Rootworm (Coleoptera: Chrysomelidae) Eggs and Incidence of the Prolonged Diapause Trait in Illinois. J. Econ. Entomol. 85: 262-267.

Meinke, L. J., B. D. Siegfried, R. J. Wright, and L. D. Chandler. 1998. Adult Susceptibility of Nebraska Western Corn Rootworm (Coleoptera: Chrysomelidae) Populations to Selected Insecticides. J. Econ. Entomol. 91: 594-600.

Miller, D. P. 2000. Public Interest Document Supporting the Registration and Exemption from the Requirement of a Tolerance for the Plant-Incorporated Protectant, *Bacillus thuringiensis* Cry3Bb Protein, and the Genetic Material Necessary for its production in Corn (Vectors ZMIR12L, ZMIR13L, and ZMIR14L). January 13, 2000. Unpublished study, submitted by Monsanto Company (Ref. No. 99-781E). MRID# 450297-01.

Mitchell, P.D. 2002. Yield Benefits of Corn Event MON 863. March 28, 2002. Unpublished study, submitted by Monsanto Company (Ref. No. MSL-17782). MRID# 456430-02.

Sauer, J. R., J. E. Hines, and J. Fallon. 2001. *The North American Breeding Bird Survey, Results and Analysis 1966 - 2000. Version 2001.2,* USGS Patuxent Wildlife Research Center, Laurel, MD

Scharf, M.E., L.J. Meinke, B.D. Siegfried, R.J. Wright, and L.D. Chandler. 1999. Carbaryl Susceptibility, Diagnostic Concentration Determination, and Synergism for U.S. Populations of Western Corn Rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 92: 33-39.

Union of Concerned Scientists. 2002. Public comments submitted to OPP docket-30509B.

U. S. Environmental Protection Agency (U.S. EPA), 2001. Biopesticides Registration Action Document: *Bacillus thuringiensis* Plant-Incorporated Protectants (10/16/01), located at http://www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm.

Vaughn, T., D. Ward, J. Pershing, G. Head, and J. McFerson. 2001. An Interim Insect Resistance Management Plan for Corn Event MON 863: A Transgenic Corn Rootworm Control Product. Unpublished study, submitted by Monsanto Company (Ref. No. MSL-17556). MRID# 455770-01.

Ward, D.P. 2002. Public Interest Assessment Supporting Registration of *Bacillus thuringiensis* Cry3Bb1 Protein and the Genetic Material (Vector ZMIR13L) Necessary for its Production in Corn Event MON 863. April 9, 2002. Unpublished study, submitted by Monsanto Company (MSL-17766). MRID# 456530-01.

Zhu, K. Y., G. E. Wilde, R. A. Higgins, P. E. Sloderbeck, L. L. Buschman, R.A. Shufran, R. J. Whitworth, S. R. Starkey, and F. He. 2001. Evidence of Evolving Carbaryl Resistance in Western Corn Rootworm (Coleoptera: Chrysomelidae) in Areawide-Managed Cornfields in North Central Kansas. J. Econ. Entomol. 94: 929-934.

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

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM MAR 29 2002

SUBJECT: Assessment of the product characterization of the Cry3Bb1 insect control protein as expressed in maize, a *Bacillus thuringiensis* -based plant-incorporated protectant for control of the corn rootworm.

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., Senior Scientist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511C

ACTION REQUESTED

To review the product characterization considerations associated with the approval of a Section 3 registration for the insect control protein Cry3Bb1 from *Bacillus thuringiensis* as expressed in maize.

BACKGROUND

Monsanto corporation has submitted a registration for the Cry3Bb1 δ-endotoxin in maize as a means of controlling the corn rootworm complex, *Diabrotica* spp. These products are based on the insecticidal protein of *B. thuringiensis* strains EG11098 and EG11231, a modified Cry3Bb1 protein, which represents the active ingredient of this plant-incorporated protectant. Maize plants are also intended to express neomycin phosphotransferase for tolerance to the antibiotic kanamycin during *in vitro* culture. Expression of the Cry3Bb1 protein provides protection against certain coleopteran insect pests.

297

Recycled/Recyclable • Printed with Vegetable Oil Based Inks on 100% Recycled Paper (40% Postconsumer)

RECOMMENDATIONS:

1.) **454240-09 - Safety Assessment of Cry3Bb variants in Corn Rootworm Protected Corn** - A statistical analysis of means is needed to determine whether the differences noted are indeed significant statistically speaking (*e.g.*, $\alpha = 0.05$) with respect to CPB LC_{50} values.

DATA REVIEW RECORD

Active Ingredient:	*Bacillus thuringiensis* Cry3Bb1 protein as expressed in maize
Product Name:	Event MON 863, Cry3Bb1 protein as expressed in maize
ID No.:	000524-LEI
Submission No.:	S600950
Chemical No.:	006484 *Bacillus thuringiensis* Cry3Bb1 protein and genetic material for its production.
DP Barcode:	D276610
Sponsor:	Monsanto Corporation, 700 Chesterfield Parkway, St. Louis, MO 63198
MRID/Study Titles:	454240-09 - Safety Assessment of Cry3Bb variants in Corn Rootworm Protected Corn 454240-10 - Additional Characterization of the Cry3Bb1 Protein Produced in Corn Event MON 863 454240-11 - Primary Structural Protein Characterization of Corn Event MON 863 Cry3Bb1.11098 Protein Using –terminal Sequencing and MALDI Time of Flight Mass Spectrometric Techniques.

REVIEW SUMMARIES

MRID# - 454240-09- Safety Assessment of Cry3Bb variants in Corn Rootworm Protected Corn - Plants transformed for corn rootworm control (Event MON 863) contained a total of seven amino acid changes within the Cry3Bb1.11098 δ-endotoxin when compared to the sequence as found in wild type *B. thuringiensis. B.t.* strains EG11231 and EG11098 contain variants of the Cry3Bb1 protein which differ from the wild type δ-endotoxin by 4 and 5 amino acids, respectively. Two further alterations in amino acid sequence were made for Cry3Bb1.11098 during cloning and insertion into the maize genome. Structural data indicate that these alleles of this protein maintained a very similar structure to the native form. The initial transformation event used to evaluate the rootworm protected maize was MON 853, which encodes Cry3Bb1 variant 11231. Protein produced by fermentation of *B. thuringiensis* cells expressing variant 11231 was used in toxicology studies for environmental and mammalian concerns. Functional and physicochemical equivalence between variant 11231 produced in *B.t.* and that produced in MON 853 were demonstrated. Maize was also transformed with variant 11098 resulting in transformation event MON 863. These two variants, 11098 and 11231, were

shown to be physicochemically and functionally equivalent. The registrant stated that an examination of toxicity toward catfish, bobwhite quail, *Daphnia magna*, Collembola (*Folsomia candida*), adult and larval honeybees, a ladybird beetle, a green lacewing, a parasitic wasp, and earthworms resulted in a NOEC (No Observable Effect Concentration) being established which exceeded the concentration of Cry3Bb1 toxins expected in the maximum environmental exposure. NOECs surpassed maximum predicted environmental concentrations by 3 to 141 fold, hence, the risk to non-target organisms from the culture of MON 863 is indicated to be minimal. However, this aspect is the subject of another review and outside the purview of this report. Given the lack of known mechanisms of mammalian toxicity from *B.t.* δ-endotoxins, their widespread use in agriculture, the rapid digestibility of Cry3B proteins, their lack of homology to known toxins and allergens, and the safety of the microbial biopesticide Raven®, which expresses Cry3B proteins, the Cry3Bb1 protein is expected to have a reasonable certainty of causing no harm in its aggregate exposure.

454240-10 - Additional Characterization of the Cry3Bb1 Protein Produced in Corn Event MON 863 - Two genetic variants designated as *cry3Bb1.11098* and *cry3Bb1.11231* produce the δ-endotoxin proteins Cry3Bb1.11098 and Cry3Bb1.11231, respectively. Cry3Bb1.11098 differs from the wild type *B.t.* protein by 5 amino acids, while the Cry3Bb1.11231 protein differs by 4 amino acids. The *cry3Bb1.11098* gene was used to develop maize line MON 863 and variant *cry3Bb1.11231* was used in the development of MON 853 for control of the corn rootworm complex. Further manipulations during cloning and insertion into the maize genome brings the total amino acid differences for these two transformants to seven and five for the 11098 (MON 863) and 11231 (MON 853) Cry3Bb1 proteins, respectively. Cry3Bb1 protein was purified from event MON 863 grain by immunoaffinity chromatography and then analyzed by N-terminal sequencing and MALDI-TOF. Trypsin fragments subjected to MALDI-TOF / MS provided for identification or verification of 38 % of the total protein by mass matching when coupled with sequencing of 29 N-terminal amino acids. Data from MALDI-TOF / MS and N--terminal sequencing indicate that the deduced amino acid sequences of Cry3Bb1.11098, as present in MON 863 and in *B.t.* strain EG11098, are accurate. A comparison of functionality and physicochemical characteristics strongly suggests that the two protein variants are nearly equivalent. Proteins from the fermentation of *B.t.* strains EG11098 and EG11231 were used for mammalian and ecotoxicology studies as well as in assays relying on immunorecognition of proteins. These proteins are considered as biologically suitable for these studies based upon structural data indicating only minor changes in the shape of the δ-endotoxin proteins.

454240-11 - Primary Structural Protein Characterization of Corn Event MON 863 Cry3Bb1.11098 Protein Using N--terminal Sequencing and MALDI Time of Flight Mass Spectrometric Techniques - Transformation event MON 863 (maize) produces the 74 kDa Cry3Bb1.11098 protein for control of the corn rootworm complex. Modifications to this protein for expression *in planta* bring the differences between the wild type and MON 863 expressed variant to seven amino acids. Grain from event MON 863 was used as a source of

Cry3Bb1.11098 protein for MALDI-TOF / MS and N-terminal sequence analyses. Of the 653 amino acids present in the 74 kDa form of the Cry3bb1 protein, 225 were identifiable as to position based upon mass matching. Three fragments from the N–terminal region of the protein were also among those matched, representing 43 amino acids. One fragment included the N–terminus indicating the loss of the terminal methionine and the acetylation of the alanine added at position two. This potentially explains the difficulty in sequencing the N-terminus of the 66 kDa form of the protein eluted from PVDF blots. Protein samples obtained from elution off of PVDF membranes of both the 74 kDa and 66 kDa proteins were subjected to Edman degradation chemistry, but the larger peptide revealed no sequence data, presumably due to blockage of the terminal amino acid residue. When the bacterially produced version of this protein was subjected to N–terminal sequencing procedures, N–terminal sequence data was obtained successfully. The presumed reason for this rests with the post-translational modifications that are typical of eukaryotes (*e.g.*, plants) which are lacking in prokaryotes (*e.g.*, bacteria). Such modification could explain the blockage noted during the attempt to sequence the N-terminus of the com-derived Cry3Bb1.11098 protein.

DATA EVALUATION REPORT

Reviewed by: Chris A. Wozniak, Ph.D., Biologist, OPP / BPPD

Secondary Reviewer: John L. Kough, Ph.D., Biologist, OPP / BPPD

MRID No.:	454240-09
Active Ingredients:	*Bacillus thuringiensis* Cry3Bb protein as expressed in maize
Product Name:	Event MON 863, Cry3Bb as expressed in maize
Confidentiality:	No claim of confidentiality is made under FIFRA 10(d)(1)(A), (B) or (C).
ID No.:	000524-LEI
Submission No.:	S600950
Chemical No.:	006484 *Bacillus thuringiensis* Cry3Bb protein and genetic material for its production.
DP Barcode:	D276610
Sponsor:	Monsanto Corporation, 700 Chesterfield Parkway, St. Louis, MO 63198.
Authors:	J.D. Astwood, R.E. Hileman, M.J. McKee, T.J. Rydell, J.W. Seale, and L. English
Testing Facility:	Monsanto Corporation, Product Safety Center, 800 North Lindbergh Blvd., St. Louis, MO 63197; Monsanto Company, Plant Protection, 700 Chesterfield Parkway North, St.. Louis, MO 63198
Study Titles:	Safety Assessment of Cry3Bb variants in Corn Rootworm Protected Corn
Study Date:	April 19, 2001
Study No.:	17225
Conclusion:	Plants transformed for corn rootworm control (Event MON 863) contained a total of seven amino acid changes within the Cry3Bb1.11098 δ-endotoxin when compared to the sequence as found in wild type *B. thuringiensis*. *B.t.* strains EG11231 and EG11098 contain variants of the Cry3Bb1 protein which differ from the wild type δ-endotoxin by 4 and 5 amino acids, respectively. Two further alterations in amino acid sequence were made for Cry3Bb1.11098 during cloning and insertion into the maize genome. Structural data indicate that alleles of this protein maintained a very similar structure to the native form. According to the registrant, an examination of toxicity toward catfish, bobwhite quail, *Daphnia magna*, Collembola (*Folsomia candida*), adult and larval honeybees, a ladybird beetle, a green lacewing, a parasitic wasp, and earthworms resulted in a NOEC (No Observable Effect Concentration) being established which exceeded the concentration of Cry3Bb1 toxins expected in the maximum environmental exposure. NOECs surpassed maximum predicted environmental concentrations by 3 to 141 fold, hence, the risk to non-target organisms from the culture of MON 863 is indicated to be minimal. Given the lack of known mechanisms of mammalian toxicity from *B.t.* δ-endotoxins, their widespread use in agriculture, the rapid digestibility of

Cry3B proteins, their lack of homology to known toxins and allergens, and the safety of the microbial biopesticide Raven®, which expresses Cry3B proteins, the Cry3Bb1 protein is expected to have a reasonable certainty of causing no harm in its aggregate exposure

Classification: Acceptable.

Good Laboratory Practices:
This submission does not represent a study and, therefore, was not conducted in compliance with the Certification of Good Laboratory Practices as outlined in 40 CFR 160.

Purpose: To describe the alterations in the MON 853 and MON 863 proteins, and to summarize the previously performed toxicity tests as they relate to health and environmental safety.

METHODS:
Introduction - In order to develop maize plants resistant to the corn rootworm complex, a series of Cry3Bb1 variants were evaluated *in planta* for their ability to protect the plants from feeding damage. Cry3Bb1 protein is the insecticidal crystal protein derived from *Bacillus thuringiensis* (*B.t.*) with activity against certain coleopteran insects. *B.t.* strains EG11098 and EG11231 provided the genetic material necessary for the production of these proteins, in modified form, into maize. All these Cry3Bb1 proteins had an additional alanine at amino acid position (codon) two in the ORF due to an introduced *Nco* I restriction site for cloning purposes. The Cry3Bb1.11098 protein produced in *B. thuringiensis* is different from the wild type form of this δ-endotoxin at 5 positions. Two additional amino acid changes were added when the gene was cloned for use in the transformation of maize. Hence, there are a total of seven amino acid differences between the protein (*B.t.* 11098) expressed in MON 863 plants transformed for corn rootworm complex (CRC) control and the wild type δ-endotoxin in the native bacterium and 5 amino acid differences between the *B.t.* 11231 protein and the δ-endotoxin of the native bacterium. The *B.t.* expressed proteins are 652 amino acids in length, while the corn expressed variants of Cry3Bb1 are 653 amino acids long.

Variants of the Cry3Bb1 protein were examined for their potential for enhanced activity toward the Southern Corn Rootworm (SCR), part of the CRC. The alteration of several amino acids within these variants can impact the activity of the δ-endotoxin relative to insect species specificity and the toxicity per amount of toxin (*i.e.*, LD_{50}). The toxins do remain, however, within the Cry3Bb1 designation since the levels of sequence homology are still high and the basic spectrum of insect activity is common to all members of the Cry3B group.

The initial transformation event used to evaluate the CRC protected maize was MON 853, which encodes Cry3Bb1 variant 11231. Protein produced by fermentation of *B. thuringiensis* cells

expressing variant 11231 was used in toxicology studies for environmental and mammalian concerns. Functional and physicochemical equivalence between variant 11231 produced in *B.t.* and that produced in MON 853 were demonstrated. Maize was also transformed with DNA encoding the variant 11098 resulting in transformation event MON 863. These two variants, 11098 and 11231, were shown to be physicochemically and functionally equivalent. Based on the expected similar potency to CRC, the conclusions from the ecological risk assessment (not reviewed herein) for MON 863 were based upon Cry3Bb1 variant 11231 protein produced in *B.t.* Acute oral toxicity and *in vitro* digestibility studies were completed using protein produced in *B.t.* variant 11098. Both Cry3Bb1.11231 and 11098 were found to digest in two minutes or less in a simulated gastric environment.

Table I.

Cry3Bb1 allele	Amino Acid Substitutions relative to wild type Cry3Bb1 proteins[a]						
B.t. strain EG11098 Cry3Bb1.11098 protein		D165G	H231R	S311L	N313T	E317K	
B.t. strain EG11098 (Q348R) Cry3Bb1.11098 (Q348R) protein		D165G	H231R	S311L	N313T	E317K	Q348R
B.t. strain EG11231 Cry3Bb1.11231 protein			H231R	S311L	N313T	E317K	
Corn Event MON 863 Cry3Bb1.11098 protein[b]	A2	D166G	H232R	S312L	N314T	E318K	Q349R
Corn Event MON 853 Cry3Bb1.11231 protein	A2		H232R	S312L	N314T	E318K	

a - The amino acid positions are shown relative to the wild type sequence (GenBank Accession No. M89794). The first letter of the IUPAC-IUB single letter code for amino acids corresponds to the wild type amino acid, the number indicates the position of the amino acid residue, and the second letter corresponds to the changed amino acid.
b - Amino acid positions are shifted by one amino acid when compared to the *B.t.* expressed proteins as an alanine residue was added at position 2 during a cloning procedure.

Following DNA sequence analysis and assessment of the expressed Cry3Bb1 protein in maize, it was noted that an amino acid substitution occurred at position 349 (Q to R, glutamine replaced by arginine). This is in reference to variant 11098 when comparing production in the bacterial (*B.t.*) system versus the plant. When a comparison of the structural data between the two was performed, the two variants (*i.e.*, 11098 and 11098/Q348R) were considered as superimposable, suggesting a strong similarity in three dimensional structure and presumably the biological activity. When tested against the SCR, similar specific activities were found for the two protein sources. It was concluded that the use of protein variant 11098 produced by fermentation of *B.t.*

was adequate for the food and feed safety evaluations as conducted for MON 863 in maize.

All of the Cry3Bb1 alleles known (36 examined) can vary in biological activity against the Southern Corn Rootworm by 2 to 8 fold. The alleles described in this submission all vary by less than 5 % in amino acid sequence, which by nomenclatural definition makes them variants of one protein (as opposed to members of a different class). In order to obtain this magnitude of change in biological activity, it is not necessary to dramatically alter the tertiary structure of the protein molecule. While the 11231 variant of Cry3Bb1 was demonstrated to have 8 fold higher activity toward SCR than the wild type protein, the comparison of their two structures suggests that H231R, S311L, N313T, and E317K (mutations) did not alter the basic shape of the protein significantly. The D165G mutation also induced minor alterations in the shape of the protein or at least provided for greater degrees of freedom at position 165 by substituting glycine, the smallest of the amino acids, for a larger, charged (aspartic acid) residue. Q348R is within a hydrophobic pocket of the protein and did not produce an obvious structural change in the δ-endotoxin structure as viewed by superimposition of the two structural representations of the molecule. While Q348R introduces a highly charged residue (R) into the hydrophobic pocket, the tertiary structure of the region was not compromised and the net hydrophobicity of the pocket was maintained. Small changes in protein structure were noted for all mutations to the wild type allele, however, all resulted in apparently minor alteration to the protein backbone and three dimensional structure. Some of the noted differences may have been partially attributable to the temperature of the sample during the X-ray diffraction process.

Based upon the lack of structural alterations in the Cry3Bb1 variant Q349R when compared to the proteins produced in the *B.t.* expression system for the 11098 variant, the basic properties of the δ-endotoxin should be very similar if not equivalent. The Cry3Bb1 variants were shown to digest rapidly in a simulated gastric environment and have no homology to known toxins or allergens. In addition, no acute oral toxicity was demonstrated using these variants as the test substance.

The absence of gut receptors in mammals for *B.t.* δ-endotoxins, the lack of any effects seen in the acute oral toxicity study, the rapid digestibility of the proteins in simulated gastric fluids, the absence of any homology to known allergens or toxins, and the safe use of this protein in microbial products all suggest that mammalian safety issues are not a concern for Cry3Bb1 proteins expressed in maize.

Since the Cry3Bb1 variants were considered as toxicologically equivalent with respect to non-target effects by the registrant, the 11231 variant produced in *B.t.* fermentation was used to assess non-target effects, even though the protein variant produced in MON 863 was the 11098 protein. The minor alterations in amino acid sequence would not generally be expected to change the toxicity profile of the variant proteins from that seen in the coleopteran insects already demonstrated to be susceptible. Exposure levels for non-target organisms were based upon the expression levels of Cry3Bb1 protein found in leaves, pollen, grain and other tissues of MON

863. Studies examining toxicity to non-target organisms included protein from both microbially fermented and plant produced (11098 / Q349R) proteins.

Studies conducted included examination of toxicity toward catfish, bobwhite quail, *Daphnia magna*, Collembola (*Folsomia candida*), adult and larval honeybees, a ladybird beetle, a green lacewing, a parasitic wasp, and earthworms. For all of these, a NOEC (No Observable Effect Concentration) was established which exceeded the concentration of Cry3Bb1 toxins expected in the maximum environmental exposure, according to the registrant. NOECs surpassed maximum predicted environmental concentrations by 3 to 141 fold, and it was concluded by the authors that the risk to non-target organisms from the culture of MON 863 is indicated to be minimal. Ecotoxicology studies and non-target issues are the subject of a separate review within the Agency and are not covered herein.

Further evidence that environmental exposure to Cry3Bb1.11098 toxin will be minimized from the culture of MON 863 comes from the rapid degradation of the toxin in soil. Results from the aerobic soil degradation study yielded estimates of DT_{50} (time to 50 % degradation) of 2.37 and 2.76 days for the ELISA-based and insect bioassay determined estimates, respectively. DT_{90} estimates were 7.87 and 9.16 days for ELISA and insect bioassay results. By themselves, these estimates of degradation do not determine the potential for exposure to non-target soil organisms, but they do indicate that the protein is degrading at a rapid rate. However, this aspect of the risk assessment is the subject of another review and outside the purview of this report.

B.t. δ-endotoxins are noted for their degree of host specificity. Typically a class of these insecticidal proteins will show activity against an order of insects, at most two, and vary greatly within the order as to the individual activity noted on a species. The Cry3Bb1 protein has high activity against species within the Chrysomelidae, a family in the order Coleoptera. Comparisons of LC_{50} values for various alleles of Cry3Bb1 proteins as determined with Colorado Potato Beetle, a coleopteran insect, are shown in Table II. While there are differences between the *B.t.* produced proteins Cry3Bb1.11231 and 11098 and their homologs produced *in planta*, numerically the differences appear to be small. What is needed, however, is a statistical analysis of means to determine whether the differences noted are indeed significant statistically speaking (*e.g.*, $\alpha = 0.05$). The comparison of the mean LC_{50} for MON 863 produced protein versus that from *B.t.* Cry3Bb1.11098 suggests that although mutations in δ-endotoxins may alter the host specificity (within the Coleoptera) and the specific activity of the protein molecule may increase, the values noted for the CPB suggest the sequence changes discussed previously in this review have not altered the activity of this protein in the domains responsible for its insecticidal activity to any great degree.

Given the lack of known mechanisms of mammalian toxicity from *B.t.* δ-endotoxins, their widespread use in agriculture, the rapid digestibility of Cry3B proteins, their lack of homology to known toxins and allergens, and the safety of the microbial biopesticide Raven®, which expresses Cry3B proteins, it is reasonable to expect MON 863 maize to be similar to traditional

maize with respect to food, feed and environmental safety. The assessment of MON 863 corn in this review for use in food or feed commodities is covered by the exemption from a requirement for a food tolerance as indicated in 40 CFR §180.1214.

Table II.
LC_{50} values (µg/mL) for Cry3Bb1 protein variants against Colorado Potato Beetles (CPB)

Study	*B.t.* Cry3Bb1.11231	Corn Event MON 853 Cry3Bb1.11231	*B.t.* Cry3Bb1.11098	Corn Event MON 863 Cry3Bb1.11098
98-01-39-18[a]	2.87	2.51	2.75	1.62
	—[b]	—[b]	1.96	1.19
	—	—	3.25	3.96
Mean (SD)	—	—	**2.56 (0.65)**	**2.26 (1.49)**

a - Two additional bioassays were conducted with Colorado Potato Beetles (*Leptinotarsa decemlineata*) and resulted in slightly higher LC_{50} values for the *B.t.* produced protein relative to the data presented here. These data were viewed as exploratory and were not included here.
b - Not determined.

Table III.
LC_{50} values (µg/mL) for *B.t.* strain EG11231 Cry3bb1.11231 protein against CPB

Study	*B.t.* Cry3Bb1.11231
CA-98-169	1.55
WL-98-298	2.24 1.31
SB-98-296	1.29 2.07 1.90 2.03
WL-99-013	1.73
99-0139-17	2.28 2.40

306

98-01-62-02	5.33 3.07 3.46
Mean (SD)	**2.36 (1.09)**

DATA EVALUATION REPORT

Reviewed by: Chris A. Wozniak, Ph.D., Biologist, OPP / BPPD CAW

Secondary Reviewer: John L. Kough, Ph.D., Biologist, OPP / BPPD JLK

MRID No.:	454240-10
Active Ingredients:	*Bacillus thuringiensis* Cry3Bb protein as expressed in maize
Product Name:	Event MON 863, Cry3Bb as expressed in maize
Confidentiality:	A claim of confidentiality is made under FIFRA 10(d)(1)(A), (B) or (C) and confidential materials have been moved to a confidential appendix.
ID No.:	000524-LEI
Submission No.:	S600950
Chemical No.:	006484 *Bacillus thuringiensis* Cry3Bb protein and genetic material for its production.
DP Barcode:	D276610
Sponsor:	Monsanto Corporation, 700 Chesterfield Parkway, St. Louis, MO 63198.
Authors:	R.E. Hileman and J.D. Astwood
Testing Facility:	Monsanto Corporation, Product Safety Center, 800 North Lindbergh Blvd., St. Louis, MO 63197
Study Titles:	Additional Characterization of the Cry3Bb1 Protein Produced in Corn Event MON 863
Study Date:	April 13, 2001
Study No.:	17137
Conclusion:	Two genetic variants designated as *cry3Bb1.11098* and *cry3Bb1.11231* produce the δ-endotoxin proteins Cry3Bb1.11098 and Cry3Bb1.11231, respectively. Cry3Bb1.11098 differs from the wild type Cry3Bb1 *B.t.* protein by 5 amino acids, while the Cry3Bb1.11231 protein differs by 4 amino acids. The *cry3Bb1.11098* gene was used to develop maize line MON 863 and variant *cry3Bb1.11231* was used in the development of MON 853 for control of the corn rootworm complex. Further manipulations during cloning and insertion into the maize genome brings the total amino acid differences for these two transformants to seven and five for the 11098 (MON 863) and 11231 (MON 853) Cry3Bb1 proteins, respectively. Trypsin fragments subjected to MALDI-TOF / MS provided for identification or verification of 38 % of the total protein by mass matching when coupled with sequencing of 29 N-terminal amino acids. Data from MALDI-TOF / MS and N–terminal sequencing indicate that the deduced amino acid sequences of Cry3Bb1.11098, as present in MON 863 and in *B.t.* strain EG11098, are accurate. A comparison of functionality and physicochemical characteristics strongly suggests that the two protein variants are nearly equivalent. These proteins produced by bacteria or corn plants are considered as biologically suitable for toxicology studies based upon structural data indicating only minor changes in the shape of the δ-endotoxin alleles.

Classification: Acceptable.

Good Laboratory Practices:
This study was not conducted in compliance with the Certification of Good Laboratory Practices as outlined in 40 CFR 160.

Purpose: To provide information regarding the DNA and amino acid sequence of the Cry3Bb1 protein and its variants.

METHODS:
Background - The focus of this report is the Cry3Bb1 protein and its variants used as a means of corn rootworm complex (*Diabrotica* spp.) control as developed for use in maize. The wild type gene (*cry3Bb1*; GenBank Accession No. M89794) was initially modified for us in a *B. thuringiensis* (*B.t.*) expression system. Two of the variants developed during this modification step had enhanced activity toward corn rootworm when expressed in *B.t.* The two variants are designated as *cry3Bb1.11098* and *cry3Bb1.11231* and they produce the proteins Cry3Bb1.11098 and Cry3Bb1.11231, respectively. Cry3Bb1.11098 differs from the wild type protein by 5 amino acids, while the Cry3Bb1.11231 protein differs by 4 amino acids. The *cry3Bb1.11098* gene was used to develop maize line MON 863 and variant *cry3Bb1.11231* was used in the development of MON 853 for control of the corn rootworm complex. See the table below for a summary of the amino acid sequence alterations.

Table I.

Cry3Bb1 allele proteins[a]	Amino Acid Substitutions relative to wild type Cry3Bb1						
B.t. strain EG11098 Cry3Bb1.11098 protein		D165G	H231R	S311L	N313T	E317K	
B.t. strain EG11098 (Q348R) Cry3Bb1.11098 (Q348R) protein		D165G	H231R	S311L	N313T	E317K	Q348R
B.t. strain EG11231 Cry3Bb1.11231 protein			H231R	S311L	N313T	E317K	
Corn Event MON 863 Cry3Bb1.11098 protein[b]	A2	D166G	H232R	S312L	N314T	E318K	Q349R
Corn Event MON 853 Cry3Bb1.11231 protein	A2		H232R	S312L	N314T	E318K	

a - The amino acid positions are shown relative to the wild type sequence (gen Bank Accession

No. M89794). The first letter of the IUPAC-IUB single letter code for amino acids corresponds to the wild type amino acid, the number indicates the position of the amino acid residue, and the second letter corresponds to the changed amino acid.
b - Amino acid positions are shifted by one amino acid when compared to the *B.t.* expressed proteins as an alanine residue was added at position 2 during a cloning step.

During a cloning step prior to introduction of the *cry3Bb1.11098* and *cry3Bb1.11231* genes into maize, three nucleotides were added in each gene at the 5' end of the coding sequence to introduce an *Nco*I restriction site for purposes of DNA manipulation. Hence, GCC was added to *cry3Bb1.11098* and GCA to *cry3Bb1.11231* at nucleotide positions 4, 5, and 6. This resulted in an alanine residue being added to each protein at position 2 in the ORF. Other genetic elements were included on the plant transformation vector, including a neomycin phosphotransferase gene (*nptII*) allowing selection of transformed cells *in vitro* on media containing kanamycin.

For transformation events utilizing the *cry3Bb1.11098* gene (*e.g.*, MON 863), further DNA sequencing indicated that an additional amino acid change occurred via a substitution at position 349 (Q349R). The net result is that a total of seven amino acids are different when one compares the sequence of the wild type Cry3Bb1 protein with that expressed in MON 863. The Cry3Bb1.11098 protein expressed in *B.t.* EG11098 differs from that in maize MON 863 by an additional two amino acids (the alanine addition at the 5' coding end and the substitution at position 349). Those transformation events using the Cry3Bb1.11231 variant do not have this substitution at position 349, although they do contain the alanine addition at the 5' end.

Molecular characterization of the *cry3Bb1.* Insert in corn event MON 863 - Since events MON 852, MON 858, MON 859, MON 862 and 96.040 were all produced using the *cry3Bb1.11098* gene in the same plasmid vector, all of these lines are considered to contain the same amino acid alterations as described above for MON 863. Events using the *cry3Bb1.11231* gene in maize resulted in a deduced amino acid sequence which contains the additional alanine residue at the 5' end, but not the alteration at position 349 or the D166G substitution. These events include MON 853, MON 854, MON 855 and MON 860. The Cry3Bb1.11231 protein as produced in maize differs from the *B.t.* produced Cry3Bb1.11231 protein by 1 amino acid and by 5 amino acids from the wild type protein.

Characterization of the Cry3Bb1 protein produced in corn event MON 863 - Cry3Bb1 protein was purified from event MON 863 grain by immunoaffinity chromatography and then analyzed by N-terminal sequencing and MALDI-TOF (matrix assisted laser desorption ionization- time of flight) mass spectrometry (MS). Trypsin fragments subjected to MALDI-TOF / MS provided for identification or verification of 38 % of the total protein by mass matching when coupled with sequencing of 29 N-terminal amino acids. Data from the MS analysis were able to confirm the presence of the alanine residue at position 2 and the Q349R substitution.

BPPD Comment: Data from MALDI-TOF / MS and N–terminal sequencing indicate that the

deduced amino acid sequences of Cry3Bb1.11098, as present in MON 863 and in *B.t.* strain EG11098, are accurate. A comparison of functionality and physicochemical characteristics strongly suggests that the two protein variants are nearly equivalent. The Q349R substitution noted in the MON 863 transformation event was not intended and is absent from the events using *cry3Bb1.11231*. Proteins from the fermentation of *B.t.* strains EG11098 and EG11231 were used for mammalian and ecotoxicology studies as well as in assays relying on immunorecognition of proteins. These proteins are considered as biologically suitable for these studies based upon structural data indicating only minor changes in the shape of the δ-endotoxin proteins.

DATA EVALUATION REPORT

Reviewed by: Chris A. Wozniak, Ph.D., Biologist, OPP / BPPD CAW

Secondary Reviewer: John L. Kough, Ph.D., Biologist, OPP / BPPD JLK

MRID No.:	454240-11
Active Ingredients:	*Bacillus thuringiensis* Cry3Bb protein as expressed in maize
Product Name:	Event MON 863, Cry3Bb as expressed in maize
Confidentiality:	No claim of confidentiality is made under FIFRA 10(d)(1)(A), (B) or (C).
ID No.:	000524-LEI
Submission No.:	S600950
Chemical No.:	006484 *Bacillus thuringiensis* Cry3Bb protein and genetic material for its production.
DP Barcode:	D276610
Sponsor:	Monsanto Corporation, 700 Chesterfield Parkway, St. Louis, MO 63198.
Authors:	R.S. Thoma, G. Holleschak, R.E. Hileman, J.D. Astwood
Testing Facility:	Monsanto Corporation, 700 Chesterfield Parkway, St. Louis, MO 63198
Study Titles:	Primary Structural Protein Characterization of Corn Event MON 863 Cry3Bb1.11098 Protein Using N–terminal Sequencing and MALDI Time of Flight Mass Spectrometric Techniques.
Study Date:	April 13, 2001
Study No.:	17154
Conclusion:	Transformation event MON 863 (maize) produces the 74 kDa Cry3Bb1.11098 protein for control of the corn rootworm complex. Modifications to this protein for expression *in planta* bring the differences between the wild type and MON 863 expressed variant to seven amino acids. Grain from event MON 863 was used as a source of Cry3Bb1.11098 protein for MALDI-TOF / MS and N-terminal sequence analyses. Of the 653 amino acids present in the 74 kDa form of the Cry3Bb1 protein, 225 were identifiable as to position based upon mass matching. Three fragments from the N–terminal region of the protein were also among those matched, representing 43 amino acids. One fragment included the N–terminus indicating the loss of the terminal methionine and the acetylation of the alanine added at position two. This potentially explains the difficulty in sequencing the N-terminus of the 66 kDa form of the protein eluted from PVDF blots.
Classification:	Acceptable.

Good Laboratory Practices:

This study was not conducted in compliance with the Certification of Good Laboratory Practices as outlined in 40 CFR 160.

Purpose: To provide peptide mass and immunoaffinity data derived from the Cry3Bb1.11098 protein, as found in MON 863 maize, and compare it to the deduced amino acid sequence indicated by the DNA sequence, thereby confirming the identity of this protein.

METHODS:

Introduction - Transformation event MON 863 (maize) produces the 74 kDa Cry3Bb1.11098 protein. Initial attempts to obtain N–terminal sequence data from this insect control protein resulted in no useful data using the standard Edman degradation chemistry. This variant of the Cry3Bb1 protein from *Bacillus thuringiensis* was modified to enhance the bioactivity toward the corn rootworm, a coleopteran pest. The modified protein contained five amino acid differences from the wild type protein when expressed in recombinant *B.t.* strain EG11098. During cloning and vector construction prior to introduction to the plant, an additional alanine residue was added at the 5' end of the coding sequence to provide for a restriction enzyme site useful in cloniug procedures. An additional amino acid alteration in this protein brings the total differences between the wild type and MON 863 expressed variant to seven amino acids.

Trypsin digestion of the Cry3Bb1.11098 protein - Grain from event MON 863 was used as a source of Cry3Bb1.11098 protein. Immunocapture from an antibody-based chromatographic matrix provided protein for electrophoresis on a 4 to 20 % polyacrylamide gradient gel . Proteins were stained with Coomassie Brilliant Blue R and gel pieces containing the Cry3Bb1.11098 protein (based on M_r and gel migration) were cut out and then destained with 40 % (v/v) methanol and 10 % (v/v) acetic acid. Incubation in 100 mM ammonium carbonate solution followed for 30 minutes, after which time the proteins were reduced with 10 mM dithiothreitol in the same buffer at 37 °C for 2 hours. Protein was then alkylated using iodacetic acid (200 mM) in the dark at ambient temperatures for 20 minutes. Samples were reincubated in ammonium bicarbonate for 30 minutes and then incubated in acetonitrile (50 %) for 30 minutes at room temperature. Following a repeat of the previous washing steps, the gel pieces were dried in a Speed-Vac and gel pieces rehydrated in 25 mM ammonium carbonate containing trypsin (33 μg/mL). Digestion proceeded for 16 h at 37 °C, at which time the gel pieces were placed into 70 % acetonitrile containing 0.1 % (v/v) trifluoroacetic acid (TFA) for 1 hour at room temperature. This extraction procedure was repeated twice and the supernatants combined. Samples were dried in a Speed-Vac and then reconstituted in 0.1 % (v/v) TFA.

N–Terminal sequence analysis - Protein samples were recovered from PVDF membranes and subjected to automated Edman degradation using a Procise™ 494 Protein Sequencing System from Perkin-Elmer. Chromatographic analysis was calibrated using a PTH standard supplied by the manufacturer (Perkin-Elmer) prior to each sequence analysis.

MALDI-TOF and Mass Spectrometry analysis - Trypsin digested stock of porcine somatotropin was used as a standard to calibrate the Perseptive™ Voyager DE - RP MALDI-TOF instrument (Perkin-Elmer). Each peptide sample was co-crystallized with α-cyano-4-hydroxy cinnamic acid (α-cyano) and then analyzed in reflector mode at a laser power

attenuation step setting value of 1620, analyzing from 400 to 3000 mass units. Linear mode was also used to detect larger fragments (2000 to 5000 mass units) with power attenuation setting values of 1620 and 1800. MacBioSpec™ software was used to compare the mass data obtained to the sequence deduced from the DNA sequence.

RESULTS:

N–Terminal sequencing - Protein samples obtained from elution off of PVDF membranes of both the 74 kDa and 66 kDa proteins were subjected to Edman degradation chemistry, but the larger peptide revealed no sequence data, presumably due to blockage of the terminal amino acid residue. When the bacterially produced version of this protein was subjected to N–terminal sequencing procedures, N–terminal sequence data was obtained successfully. The presumed reason for this rests with the post-translational modifications that are typical of eukaryotes (*e.g.*, plants) which are lacking in prokaryotes (*e.g.*, bacteria). Such modification could explain the blockage noted during the attempt to sequence the corn-derived Cry3Bb1.11098 protein.

It was possible to obtain N–terminal sequence from the smaller 66 kDa protein band. The band was treated as two separate samples for sequencing purposes since the band appeared on the gel as two separate entities of closely matching M_r (*i.e.*, a doublet). The upper band of this pair contained peptides with N–termini starting at positions 47 and 50, predominantly. The lower band contained peptides with a terminus at position 61, but also containing some fragments with termini at positions 47 and 50 as well. The loss of this N–terminal sequence up to 61 amino acid residues approximates the apparent loss of 8 kDa based upon mobility fo the protein during gel electrophoresis.

MALDI-TOF / MS Data - Both bands (66 kDa and 74 kDa) were also subjected to MALDI-TOF / MS analysis for verification that the Cry3Bb1.11098 protein produced in maize event MON 863 was equivalent to that deduced from the known DNA sequence. Using the reflector mode setting at 400 to 3000 mass units during MS analysis yielded 34 protein digestion products (fragments) from the 74 kDa band and an additional three fragments were detected in linear mode. Using the sensitivity of this technique (*i.e.*, the ability to detect mass differences of 1 Da), 24 of the 37 peptides were discerned as matching the predicted fragments based upon selective digestion by trypsin (*i.e.*, cleavage after lysine and arginine). Those peptides which were not matched to predicted fragments may represent autocatalytic products of the trypsin itself, contaminants of the trypsin preparation as purchased, or non-specific cleavage of the Cry3Bb1.11098 protein at sites other than after lys or arg.

Of the 653 amino acids present in the 74 kDa form of the Cry3bb1 protein, 225 were identifiable as to position based upon mass matching. Three fragments from the N–terminal region of the protein were also among those matched, representing 43 amino acids. One fragment included the N–terminus indicating the loss of the terminal methionine and the acetylation of the alanine added at position two. Since the remaining fragments identified as to position were scattered across the length of the protein, the trypsin treatment is thought to have significantly digested the

Cry3Bb1.11098 protein.

Large peptides do not elute efficiently from gel pieces, so they may not be accurately represented in the analysis by MALDI-TOF / MS. In addition, smaller peptides (*e.g.*, below 6 amino acids) are not accurately detected since the effect of the matrix (α-cyano) cannot be discerned from that of the small fragments.

Data derived from the 66 kDa protein were very similar to those from the 74 kDa band, except for the absence of three peptides representing the N–terminal region. Since these fragments were from the region between positions 2 and 44, they would not be represented in the shorter 66 kDa protein which does not contain the residues at positions 1 to 47 (or 1 to 61 depending on the band sequenced).

The percent coverage of the Cry3Bb1.11098 protein from maize following MALDI-TOF / MS analysis was approximately 34 %. The additional N–terminal sequencing brings this total to approximately 38.5 %. The precise matching of sequence fragments by MALDI-TOF and sampling of peptides from varied regions / domains of the insecticidal protein indicate that the peptide found in maize event MON 863 is the same protein as found in the *B.t.* strain EG11098; exceptions to this include post-translational modifications which may be present in the plant produced protein as opposed to the bacterial production system and the known additions of alanine at position 2 and substitution of arginine at position 349.

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

MAR 29 2002

MEMORANDUM

SUBJECT: EPA Review of the Application for an Exemption from the Requirement of a Tolerance for Cry3Bb1 Protein Expressed in all Food Commodities, Submitted by Monsanto Company [Submission: S600950; DP Barcode: D276610; ID# 000524-LEI; MRID#'s:453484-03, 454240-01 through 454240-11 & 455382-01 through 455382-03; 455382-09; 455770-02].

TO: Michael Mendelsohn (PM-90)
Regulatory Action Leader
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511C)

FROM: Chris Wozniak, Ph.D., Plant Pathologist
John L. Kough, Senior Scientist
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511C)

ACTION REQUESTED: To review product data submitted for several Cry3Bb1 protein variants to determine if it supports inclusion of these variants under an existing exemption from the requirement of a tolerance for Cry3Bb1 protein expressed in food and feed commodities of field, sweet and popcorn.

DATA REVIEW RECORD

Active Ingredient:	Cry3Bb1 Protein (*Bacillus thuringiensis*) and the DNA which encodes the Cry3Bb1 protein
Product Name:	Food commodities containing Cry3Bb protein
Company Name:	Monsanto Company, St. Louis, MO
ID No:	000524-LEI
Chemical Number:	006484
Submission Number:	S600950
DP Barcode:	D276610
MRID No:	453484-03 Product Characterization (agronomic performance)-

316

	885.1100
454240-01	Product Characterization (protein levels) - 885.1100
454240-02	Protein Levels (DNA characterization) - 885.1100
454240-03	Product Characterization (sequencing & immunoreactivity) - 885.1100
454240-04	Product Characterization (protein equivalence) - 885.1100
454240-05	Product Characterization (protein equivalence) - 885.1100
454240-06	*In vitro* Digestibility - 885.1100
454240-07	Product Characterization (heat stability)-885.1100
454240-08	Product Characterization (allergen sequence database comparison) - 885.1100
454240-09	Safety Assessment of Cry3Bb1 Variants in Corn Rootworm Protected Corn
454240-10	Product Characterization (protein equivalence)-885.1100
454240-11	Product Characterization (amino acid sequencing)-885.1100
455382-01	Product Characterization (protein equivalence)-885.1100
455382-02	Acute Oral Toxicity - 885.3050
455382-03	*In vitro* Digestibility - 885.1100
455382-09	Product Characterization (heat stability)-885.1100
455770-02	*In vitro* Digestibility - 885.1100

BACKGROUND:

Cry3Bb1 protein is a δ-endotoxin from *Bacillus thuringiensis* spp. *kumamotoensis* and has activity against Coleopteran species, especially those in the Chrysomelidae family. Wild-type Cry3Bb1 protein (GenBank Accession No. M89794) was genetically modified to enhance its activity against the corn rootworm complex (*Diabrotica* spp.). Two Cry3Bb1 variants were engineered for expression in *B.t.* strains EG11098 and EG11231. These two variant proteins (Cry3Bb1.11098 and Cry3Bb1.11231) contain 5 and 4 amino acid changes, respectively, compared to the Cry3Bb1 wild-type endotoxin. Corn has been genetically modified to express the Cry3Bb1.11231 variant (MON 853) or the Cry3Bb1.11098 variant protein (MON863). Both these corn events also express neomycin phosphotransferase (*nptII* gene - for protein selectable marker). As part of the EUP for corn expressing Cry3Bb1 protein, data was submitted and reviewed to support an exemption from the requirement for a food tolerance. That data was reviewed (memoranda from M. Watson to M. Mendelsohn, January 7, 2000 & April 25, 2001) and a tolerance exemption (40CFR§180.1214) was published May 11, 2001. After this tolerance publication, the company discovered an additional amino acid change in the sequence of the introduced DNA which coded for an arginine residue at position 349 in the Cry3Bb1 protein instead of glutamine. The company generated another complete package of characterization and toxicology data for this variant identified in the submissions as Cry3Bb1.11098(Q349R).

DISCUSSION:

Protein variants with the same activity are a well described biological phenomenon examined both for studies of genetic relatedness and protein evolution (Klug, W.S. & Cummings, M.R., (1997) Concepts of Genetics, p. 695). However, the relationship between these protein

variations and changes in bioactivity to a target pest or toxicity of the protein to non-target organisms is not easily established without extensive data. The relationship is certainly not so well understood as to be predicted without confirmation by some evidence. Some changes can be reasonably expected to be neutral with respect to both bioactivity and toxicity. However, minor modifications with resulting enhanced bioactivity are the basis for directed protein engineering and are probably the reason for the choice of these variant Cry3Bb1 proteins over the wild-type for use against the corn rootworm complex. The extensive data, both in this package and that already reviewed (memoranda from M. Watson to M. Mendelsohn, January 7, 2000 & April 25, 2001), provide additional support to the notion that minor modifications for enhanced efficacy can be made to a protein without necessarily altering the resulting mammalian toxicity.

RECOMMENDATIONS:

The data provided in this submission supports the contention that the Cry3Bb1.11098, Cry3Bb1.11098(Q349R) and Cry3Bb1.11231 proteins are variants of the Cry3Bb1 protein. Since these variants do not differ significantly from the Cry3Bb1 protein in terms of biochemical or toxicological characteristics, the Cry3Bb1.11098, Cry3Bb1.11098(Q349R) and Cry3Bb1.11231 protein variants are all covered by the exemption from the requirement for a food tolerance (40CFR§180.1214).

SUMMARY OF SUBMITTED DATA:

453484-03 **Product Characterization (agronomic performance)-885.1100** -Results of the study show that there are some differences in the properties of the transgenic plants versus the control lines used in the tests. Some of the variation identified included a variety of differences in corn ear height, plant height, weight, grain moisture and yield, but in each case, the difference was small. However, based upon the data provided, it appears that none of these differences would have a significant agronomic impact on the crops and are likely similar to typical differences seen in different plant lines and/or those differences caused by differing ecological effects. The data included in this submission appear to support the agronomic equivalency of corn event MON 863 hybrids.

454240-01 **Product Characterization (protein levels) - 885.1100** -The protein titer data provided shows the ranges of Cry3Bb1 protein in various parts of the plant, as well as geographical variation. Overall, based upon the ranges provided, there appears to be significant variation between the samples analyzed on different days post-planting and at different sites. CLASSIFICATION: Acceptable.

454240-02 **Protein Levels (DNA characterization) - 885.1100** -The data presented in this submission describe the DNA insert for event MON 863. The data provided support the finding that event MON 863 contains 1 intact copy of the insert which encodes for both Cry3Bb1 and NPTII proteins. CLASSIFICATION: Acceptable.

454240-03 **Product Characterization (sequencing & immunoreactivity) - 885.1100-** The N-terminal sequence analysis and the immunoreactivity to Cry3Bb1 polyclonal antisera confirm the relationship of Cry3Bb1.11098 and Cry3Bb1.11231 to wild-type Cry3Bb1. Further confirmatory data include protein molecular weight analysis and bioactivity. There are some amino acid changes (four or five) in the two test proteins compared to wild-type. However, these changes do not appear to significantly affect the bioactivity nor the immunoreactivity of the variant proteins. Based upon the data submitted, the two proteins produced by fermentation - Cry3Bb1.11098 and Cry3Bb1.11231 - have been confirmed as Cry3Bb1 protein variants. CLASSIFICATION: Acceptable.

454240-04 **Product Characterization (protein equivalence) - 885.1100** -This report compares the physical (MW, N-terminal sequencing) and functional (bioassay) characteristics of Cry3Bb1.11098 and Cry3Bb1.11231 proteins produced in *E. coli* and CRW protected corn. The data provided show that the proteins have equivalent molecular weight, immunological reactivities, N-terminal sequences and comparable LC_{50} values. This data supports the determination of the equivalence of the bacteria- and plant-produced proteins, and the use of the bacterially-produced proteins to support registration of the CRW corn product. CLASSIFICATION: Acceptable.

454240-05 **Product Characterization (protein equivalence) - 885.1100** -Based upon the data provided, it appears that both the Cry3Bb1.11098 and NPTII proteins produced in event MON 863 have equivalent molecular weights and antigenic properties with these same proteins produced in *B.t.* and *E. coli* respectively. CLASSIFICATION: Acceptable.

454240-06 ***In vitro* Digestibility - 885.1100** -The tests performed in this study show that the Cry3Bb1 proteins are not stable to digestion in simulated gastric fluid. Incubation of Cry3Bb1.11098 and Cry3Bb1.11231 in SGF results in the loss of detectable protein by the 30 and 15 second observation points, respectively, as detected by SDS-PAGE. Insect bioassay data indicated that the protein loss insecticidal activity within 2 minutes of incubation in SGF. Incubation in the SIF resulted in a ~59 kDa digestion product that retained its insecticidal activity after at least 30 minutes incubation. CLASSIFICATION: Acceptable.

4542240-07 **Product Characterization (heat stability)-885-1100** -Heating the corn flour samples at 204°C for 30 minutes destroys both the immunoreactivity and insect bioactivity of the Cry3Bb1.11098 found in MON 863 corn. The Cry3Bb1 immunoreactivity was not detectable in both an immunoblot and ELISA format for MON 863. For MON 853, Cry3Bb1 was not recognizable in an immunoblot and reduced more than 1000-fold in an ELISA format. Since the rabbit anti-Cry3Bb1 antibody employed was polyclonal IgG, it is also suggestive that epitopes were destroyed and not just rendered

unrecognized by alteration of the three dimensional configuration.

454240-08 **Product Characterization (allergen sequence database comparison) - 885.1100** -Several amino acid database comparison tools were employed to compare the amino acid sequence of Cry3Bb to known protein allergens and gliadins. The UPDATE2 database was compiled to allow for comparison of Cry3Bb1.11098 and Cry3Bb1.11231 to these proteins. The level of similarity identified does not indicate significant similarity to any of the proteins or gliadins contained in the database. In addition, no contiguous stretch of 8 identical amino acids was identified in either the FASTA or IDENTITYSEARCH algorithms suggesting a lack of immunological similarity. Based upon this data, it does not appear that Cry3Bb1 shares significant structural, biological or immunological similarity with known protein allergens or gliadins. CLASSIFICATION: Acceptable.

454240-09 **Safety Assessment of Cry3Bb1 Variants in Corn Rootworm Protected Corn** -Plants transformed for corn rootworm control (Event MON 863) contained a total of seven amino acid changes within the Cry3Bb1.11098 δ-endotoxin when compared to the sequence as found in wild type *B. thuringiensis*. *B.t.* strains EG11231 and EG11098 contain variants of the Cry3Bb1 protein which differ from the wild type δ-endotoxin by 4 and 5 amino acids, respectively. Two further alterations in amino acid sequence were made for Cry3Bb1.11098 during cloning and insertion into the maize genome. Structural data indicate that these alleles of this protein maintained a very similar structure to the native form. The initial transformation event used to evaluate the rootworm protected maize was MON 853, which encodes Cry3Bb1 variant 11231. Protein produced by fermentation of *B. thuringiensis* cells expressing variant 11231 was used in toxicology studies for environmental and mammalian concerns. Functional and physicochemical equivalence between variant 11231 produced in *B.t.* and that produced in MON 853 were demonstrated. Maize was also transformed with variant 11098 resulting in transformation event MON 863. These two variants, 11098 and 11231, were shown to be physicochemically and functionally equivalent. The registrant stated that an examination of toxicity toward catfish, bobwhite quail, *Daphnia magna*, Collembola (*Folsomia candida*), adult and larval honeybees, a ladybird beetle, a green lacewing, a parasitic wasp, and earthworms resulted in a NOEC (No Observable Effect Concentration) being established which exceeded the concentration of Cry3Bb1 toxins expected in the maximum environmental exposure. NOECs surpassed maximum predicted environmental concentrations by 3 to 141 fold, hence, the risk to non-target organisms from the culture of MON 863 is indicated to be minimal. However, this aspect is the subject of another review and outside the purview of this report. Given the lack of known mechanisms of mammalian toxicity from *B.t.* δ-endotoxins, their widespread use in agriculture, the rapid digestibility of Cry3B proteins, their lack of homology to known toxins and allergens, and the

safety of the microbial biopesticide Raven®, which expresses Cry3B proteins, the Cry3Bb1 protein is expected to have a reasonable certainty of causing no harm in its aggregate exposure.

454240-10 **Product Characterization (protein equivalence)-885.1100** -Two genetic variants designated as *cry3Bb1.11098* and *cry3Bb1.11231* produce the δ-endotoxin proteins Cry3Bb1.11098 and Cry3Bb1.11231, respectively. Cry3Bb1.11098 differs from the wild type *B.t.* protein by 5 amino acids, while the Cry3Bb1.11231 protein differs by 4 amino acids. The *cry3Bb1.11098* gene was used to develop maize line MON 863 and variant *cry3Bb1.11231* was used in the development of MON 853 for control of the corn rootworm complex. Further manipulations during cloning and insertion into the maize genome brings the total amino acid differences for these two transformants to seven and five for the 11098 (MON 863) and 11231 (MON 853) Cry3Bb1 proteins, respectively. Cry3Bb1 protein was purified from event MON 863 grain by immunoaffinity chromatography and then analyzed by N-terminal sequencing and MALDI-TOF. Trypsin fragments subjected to MALDI-TOF / MS provided for identification or verification of 38 % of the total protein by mass matching when coupled with sequencing of 29 N-terminal amino acids. Data from MALDI-TOF / MS and N–terminal sequencing indicate that the deduced amino acid sequences of Cry3Bb1.11098, as present in MON 863 and in *B.t.* strain EG11098, are accurate. A comparison of functionality and physicochemical characteristics strongly suggests that the two protein variants are nearly equivalent. Proteins from the fermentation of *B.t.* strains EG11098 and EG11231 were used for mammalian and ecotoxicology studies as well as in assays relying on immunorecognition of proteins. These proteins are considered as biologically suitable for these studies based upon structural data indicating only minor changes in the shape of the δ-endotoxin proteins.

454240-11 **Product Characterization (amino acid sequencing)-885.1100** - Transformation event MON 863 (maize) produces the 74 kDa Cry3Bb1.11098 protein for control of the corn rootworm complex. Modifications to this protein for expression *in planta* bring the differences between the wild type and MON 863 expressed variant to seven amino acids. Grain from event MON 863 was used as a source of Cry3Bb1.11098 protein for MALDI-TOF / MS and N-terminal sequence analyses. Of the 653 amino acids present in the 74 kDa form of the Cry3bb1 protein, 225 were identifiable as to position based upon mass matching. Three fragments from the N–terminal region of the protein were also among those matched, representing 43 amino acids. One fragment included the N–terminus indicating the loss of the terminal methionine and the acetylation of the alanine added at position two. This potentially explains the difficulty in sequencing the N-terminus of the 66 kDa form of the protein eluted from PVDF blots. Protein samples obtained from elution off of PVDF membranes of both the 74 kDa and 66 kDa proteins were subjected to Edman degradation chemistry, but the larger peptide revealed no

sequence data, presumably due to blockage of the terminal amino acid residue. When the bacterially produced version of this protein was subjected to N–terminal sequencing procedures, N–terminal sequence data was obtained successfully. The presumed reason for this rests with the post-translational modifications that are typical of eukaryotes (*e.g.*, plants) which are lacking in prokaryotes (*e.g.*, bacteria). Such modification could explain the blockage noted during the attempt to sequence the N-terminus of the corn-derived Cry3Bb1.11098 protein.

455382-01 **Product Characterization (protein equivalence)**-885.1100 -MALDI-TOF analysis of the microbial and corn Cry3B1.11098(Q349R) proteins yielded an agreement of from 42 to 50 amino acid fragments predicted from the theoretical sequence. The N-terminus of the microbial form lacked the terminal methionine which is commonly cleaved in expressed proteins. The corn form was apparently not only lacking the terminal methionine but the N-terminal alanine residue was acetylated as indicated by a 42 Dalton greater weight. The N-terminal amino acid sequence analyses were flawed in that unequivocal determinations were not possible due to the presence of multiple residues in most cycles. However, by comparison to the expected sequences, several different start sites for N-terminal sequencing could be detected. In the *E. coli* Cry3Bb1, the sequence started at both position 2 and 32. In the corn Cry3Bb1, three different starts were detected at position 19, 25 and 36. The immunoblot analysis gave similar positive band patterns that indicated the Cry3Bb1 protein produced in both corn and *E. coli* had essentially the same electrophoretic mobility and immunoreactivity. The positive bands themselves were sometimes rather broad (74-66kDa) but no series of distinct bands could be discerned from the photographs provided. The molecular weight and purity analyses for the corn and microbial extracts indicate that the microbially produced samples were nearly twofold higher purity in Cry3Bb1 proteins compared to the corn extracts. The purity for Cry3Bb1 was 92.6% and 53.9% for microbial and corn extracts, respectively. Total protein concentrations for the two extracts were determined as 0.58 mg/ml and 0.46 mg/ml for microbial and corn extracts, respectively, by colormetric assays. The glycosylation analysis for the Cry3Bb1 extracts gave no positive carbohydrate staining regions for either the microbial or corn samples in the expected regions for Cry3Bb1 protein. The results of the bioassays for the two Cry3Bb1extracts against Colorado potato beetle larvae (table 5 attached) indicate that there was a dose/response in all tests and the LC_{50} values were similar and had overlapping 95% confidence intervals.

455382-02 **Acute Oral Toxicity - 885.3050** -There did not appear to be significant adverse affects to animals resulting from exposure to Cry3Bb1.11098 at dose amounts of 300, 900 & 2700 mg/kg body weight. Observations included some minor clinical affects and a relatively insignificant lack of weight gain in two animals, however, these do not appear to be related to exposure to the test

substance, because these occurred in the various test groups. Based upon the data contained in this submission, the LD_{50} for Cry3Bb1.11098 is greater than 3200 mg/kg body weight in mice. CLASSIFICATION: Acceptable. Toxicity Category III.

455382-03 ***In vitro* Digestibility - 885.1100** -The tests performed in this study show that the Cry3Bb1 proteins are degraded in simulated gastric fluid. Incubation of corn-produced and *E. coli*-produced Cry3Bb1 protein in SGF results in the loss of detectable protein by the 15 second observation point, as detected by SDS-PAGE. CLASSIFICATION: Acceptable.

455382-09 **Product Characterization (heat stability) 885.1100** -The immunoblot shows that extraction of the MON863 corn grain spiked with NPTII yielded an immunoreactive band that comigrated with the *E. coli* produced NPTII. The blot also showed that, regardless of the extraction buffer used, the heat treatment effectively removed any immunoreactive bands from the samples. The results suggest that, even if detectable levels of NPTII were present in MON863 corn grain, the heat treatment would remove them. Unfortunately, the use of a mouse monoclonal antibody limits the ability of this data to be extrapolated. A heat treatment significantly above the 95.8°C used for sample preparation for SDS-PAGE destroyed the epitope(s) recognized by the anti-NPTII antibody used.

455770-02 ***In vitro* Digestibility - 885.1100** -Simulated intestinal fluid activity was verified to be present and at a level deemed acceptable by SOP GEN-PRO-058-01. The gels provided indicate that the Cry3Bb1.11098 (Q349R) protein is present as a single band at 74 kDa which rapidly degraded to two bands of 68 and 57kDa at the first assay time point of 1 minute. The subsequent samples (from 5 minute to 24 hours) all gave a single 57 kDa band which did not appear to decrease in intensity. This lack of degradation by intestinal fluids is similar to the majority of Cry proteins which are resistant to the action of trypsin.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist

Secondary Reviewer: John L. Kough, Ph.D., Biologist

STUDY TYPE:	Protein Characterization (885.1100)
MRID NO:	454240-03
TEST MATERIAL:	Cry3Bb Protein
PROJECT NO:	98-01-39-05; MSL-17219
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Amended Report for MSL-15531: Characterization of *B.t.* Protein 11098 and *B.t.* Protein 11231 Produced by Fermentation
AUTHOR(S):	Ronald E. Hileman, Ph.D., Paul D. Pyla, B.S., Thomas C. Lee, Ph.D., and James D. Astwood, Ph.D.
STUDY COMPLETED:	April 10, 2001 (amendment1)
CONCLUSION:	The N-terminal sequence analysis and the immunoreactivity to Cry3Bb1 polyclonal antisera confirm the relationship of Cry3Bb1.11098 and Cry3Bb1.11231 to wild-type Cry3Bb1. Further confirmatory data include protein molecular weight analysis and bioactivity. There are some amino acid changes (four or five) in the two test proteins compared to wild-type. However, these changes do not appear to significantly affect the bioactivity nor the immunoreactivity of the variant proteins. Based upon the data submitted, the two proteins produced by fermentation - Cry3Bb1.11098 and Cry3Bb1.11231 - have been confirmed as Cry3Bb1 protein variants.
CLASSIFICATION:	ACCEPTABLE
GOOD LABORATORY PRACTICE:	This study was conducted in compliance with Good Laboratory Standards with the following exception: egg receipt, sterilization, and incubation to the first instar larvae stage for the insect bioassay portion of the analytical procedure was not conducted in accordance with the GLPs.

I. STUDY DESIGN

Test Material: Test Samples: Cry3Bb1.11098 (lot# 6312803) and Cry3Bb1.11231 (lot# 6312812) produced in genetically modified *B. thuringiensis* strains EG11098 and EG11231, respectively. The proteins were determined to be

92.3% and 96% pure, respectively. Total protein concentrations of the stock solutions were 1.66 mg/ml for Cry3Bb1.11098 and 2.20 mg/ml for Cry3Bb1.11231. The materials were stored at -80 °C.

Methods:

1. Protein Purification: proteins were purified as described in Appendix 2.
2. Moisture Analysis: the amount of water present in the protein powder was determined using a Mettler DL18 titrator with an attached Mettler AE100 analytical balance (SOP PC-92-SOP-161-1 - not attached).
3. Protein Concentration: total protein (% weight) was determined as the mean of the separate solutions of the proteins. The protein concentration was determined using the Bio-Rad colorimetric dye-binding protein assay to provide a weight percent protein value. Amino acid analysis was also used to estimate protein concentration.
4. Salt Content: proteins were separately lyophilized after dialysis against ~5 mM sodium bicarbonate (pH 10.6).
5. Amino Acid Composition: vapor phase hydrolysis of the proteins in 6 N HCl (1% phenol v/v) was performed according to SOP 400.249 (not attached). Each solution was examined in quadruplicate and the mean protein concentration determined.
6. SDS/PAGE/Imaging/Densitometry: Laemmli sample buffer was added to the protein solutions (0.5 mg/ml protein concentration) and the proteins were applied to a 4→20% gradient polyacrylamide gel (SOP-PB-EQP-005-01 - not attached). The protein gels were stained with Colloidal Brilliant Blue G and imaging and densitometry performed using a Bio-Rad GS-700 (Quantity One Software, v 4.0). Molecular weight was determined based upon protein standards and protein concentration determined using densitometric analysis.
7. N-terminal Amino Acid Sequence Analysis: proteins were added to polyacrylamide gels as described above and electroblotted to Immobilon-P^{SQ} PVDF membranes visualized with Ponceau S and cut from the membranes. The proteins were eluted from the membrane and sequenced using automated cycles of Edman degradation (Applied Biosystems Model 494 Procise Protein Sequencing System (SOP 400.324 - not attached).
8. Immunoblot Analysis: protein samples were diluted in Laemmli buffer and separated on 4→20% acrylamide gels. The proteins were transferred to Immobilon membranes and exposed to polyclonal antibody. The proteins were visualized using the ECL detection system (Amersham) and exposure to Amersham Hyperfilm.
9. Storage Stabilility: protein stability was determined at 4, -20 and -80° C over 80 days. 50 µl aliquots of each protein were stored at the respective temperatures and removed at 0, 11, 19, 28, 39, 69 & 80 days and analyzed by gel electrophoresis as described in #6 above.
10. Solubility: protein solubility was determined by dissolving approximately 60 mg of each protein in 1 ml of water. The proteins were divided into 3 aliquots and allowed to solubilize for 1 hr at room temperature (mixing every 15 mins). The solutions were centrifuged and the supernatant was removed to a new vial and assayed using

329

the Bio-Rad dye-binding protein assay (SOP GEN-PRO-015-00 - not provided). The mean concentration and average deviation of the three aliquots were calculated.

11. Insect Bioassay: protein bioassay was determined using first instar western corn rootworm larvae (*Diabrotica virgifera virgifera*). The larvae (16 per dose level) were fed a diet contain various amounts of the proteins - for Cry3Bb1.11098 dose levels were: 738, 369, 185, 92, 46, 23, & 12 μg/ml - for Cry3Bb1.11231 dose levels were: 768, 384, 192, 96, 48, 24 & 12 μg/ml). Individual larvae were scored for survival and weight after 7 days and the EC_{50} determined:

$$\text{weight(mg)} = \frac{w_0}{1 + (dose/EC_{50})^b}$$

where: w_0 and b correspond to the fitted larval weight at 0 dose and a fitting parameter respectively.

II. RESULTS

1. Protein identity: the N-terminal sequence of each protein is shown in Table 2. The N-terminal sequences for each protein, as expected, show that they were derived from wild-type Cry3Bb1. Both a major and minor sequence is provided for each protein which according to the registrant, is probably due to multiple "protease-sensitive" sites in the proteins.

N-terminal sequencing was conducted on the major ~67kDa polypeptide of both Cry3Bb1.11098 and Cry3Bb1.11231, the results of which indicated that the proteins both were derived from the wild-type Cry3Bb1 protein (Figure 1-attached). When the sequences of these two proteins are compared to wild-type, the Cry3Bb1.11098 and Cry3Bb1.11231 sequences show 5 and 4 amino acid changes respectively.

Amino Acid Changes from Wild-type

Cry3Bb1.11098	Cry3Bb1.11231
D165G	-
H231R	H231R
S311L	S311L
N313T	N313T
E317K	E317K

Table 2 shows the results of sequence analysis of the ~59 kDa polypeptide of the two modified proteins. In addition to the ability to distinguish the proteins using the ~67 kDa protein N-terminal sequence, the D165G change in Cry3Bb1.11098 and the lack of a sequence change in Cry3Bb1.11231 (i.e., identical to wild-type at position 165), also provides a tool to distinguish the two proteins.

Figure 3 shows the results of the Western analysis. Anti-Cry3Bb1 wild-type protein polyclonal antibodies recognize both the ~67kDa and ~59 kDa proteins. No other proteins were obviously recognized by the antisera.

2. Protein Concentration: total protein solutions were prepared from lyophilized samples of Cry3Bb1.11098 and Cry3Bb1.11231. Each protein was prepared at a concentration of 1.00 mg/ml based upon colorimetric assays using the Bio-Rad colorimetric dye-binding assay. Amino acid compositional analysis showed that there were only minor differences between the colorimetric-determined and actual concentration of the proteins. Using the amino acid compositional analysis, the protein concentrations were determined to be 1.03 mg/ml and 1.02 mg/ml for Cry3BB1.11098 and Cry3Bb1.11231 respectively. Based upon these results, the registrant indicated that the colorimetric assay was used because of its relative accuracy and ease of use.

3. Protein Purity: Figure 2 shows the results of a SDS-PAGE gels stained with Colloidal Brilliant Blue G. The relative optical density (OD) was quantitated using densitometry and the purity calculated as the average relative OD of three separate load levels (1, 2, & 3 μg) for the ~67 and ~59 kDa protein bands for both Cry3Bb1.11098 and Cry3Bb1.11231, as a proportion of the total OD for all visible protein bands within a lane. These data are summarized in Table 3. The report does note that the identity of two observed proteins - 60.4 and 35.5 kDa - was not determined due to low abundance and therefore, these were not included in the purity estimate.

4. Insect Bioassay: the bioactivity of the proteins was determined against western corn rootworm. A 50% reduction in larvae weight (i.e., EC_{50}) was determined for Cry3Bb1.11098 at 96± 40 μg/ml and at 88 ± 44 μg/ml for Cry3Bb1.11231. The standard error results here are fairly significant, however, the report indicated that this may be due to some difficulty in accurately weighing the small individual larvae, which appears to be logical.

5. Stability: Protein stability was measured using densitometric analysis of Colloidal Brilliant Blue G stained SDS-PAGE gels. The relative optical density for Cry3Bb1.11098 samples stored at 4 °C, decreased approximately 15% over the 80 day storage period. There was no apparent decrease for the Cry3Bb1.11231 under any of the storage conditions.

6. Protein Properties:

Property	Method	Cry3Bb1.11098	Cry3Bb.11231
Identity	N-terminal Seq.	Cry3Bb1.11098 - confirmed	Cry3Bb1.11231- confirmed, but not distinguishable from Wild-type
Purity	Densitometry	92.3%	96%
Bioactivity	WCRW bioassay	EC50 = 96 µg/ml	EC50 = 88 µg/ml
Immunoreactivity	Immunoblot Analysis	Confirmed	Confirmed
Composition (lyophilized powder)	Protein assay Moisture analysis Calculation	32.9% protein 18.4% water 56.1% salt	41.2% protein 16.2% water 48.5% salt
Solubility	Protein assay	15.0 mg/ml	14.1 mg/ml

III. DISCUSSION

The tests described in this report were reviewed to confirm the identities of the two Cry3Bb1 test proteins. The N-terminal sequence analysis and the immunoreactivity to Cry3Bb1 polyclonal antisera confirm the relationship of Cry3Bb1.11098 and Cry3Bb1.11231 to wild-type Cry3Bb1. Further confirmatory data include protein molecular weight analysis and bioactivity. There are some amino acid changes (four or five) in the two test proteins compared to wild-type. However, these changes do not appear to significantly affect the immunoreactivity of the protein. The bioactivity based on an assay tracking western corn rootworm larval weight reduction between these two Cry3Bb1 protein variants was similar. Other safety data test submitted to support this product allow for assessment of any potential adverse affects from these proteins because of the amino acid sequence changes. Based upon the data submitted, the two proteins produced by fermentation - Cry3Bb1.11098 and Cry3Bb1.11231 - have been confirmed as Cry3Bb1 proteins.

CLASSIFICATION: ACCEPTABLE

Reg. # 524-528
Volume 2 of 4

Page ___ is not included in this copy.

Pages 331 through 336 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist MK for

Secondary Reviewer: John L. Kough, Ph.D., Biologist JLK

STUDY TYPE:	Product Characterization - Protein Equivalence (885.1100)
MRID NO:	454240-04
TEST MATERIAL:	Cry3Bb1 Protein variants
PROJECT NO:	Study 98-01-39-18; MSL-17222
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Amendment Report for MSL-15835: Assessment of the equivalence of *B.t.* Protein 11098, *B.t.* protein 11231 and NPTII Protein Expressed in Corn Events MON 853 and MON 860 to Microbial Sources
AUTHOR(S):	Gyula Holleschak, B.S., Thomas C. Lee, Ph.D., Paul D. Pyla, B.S., Ronald E. Hileman, Ph.D. and James D. Astwood, Ph.D.
STUDY COMPLETED:	April 10, 2001, Amendment 1
CONCLUSION:	This report compares the physical (MW, N-terminal sequencing) and functional (bioassay) characteristics of Cry3Bb1.11098 and Cry3Bb1.11231 proteins produced in *E. coli* and CRW protected corn. The data provided show that the proteins have equivalent molecular weight, immunological reactivities, N-terminal sequences and comparable LC_{50} values. This data supports the determination of the equivalence of the bacteria- and plant-produced proteins, and the use of the bacterially-produced proteins to support registration of the CRW corn product.
CLASSIFICATION:	ACCEPTABLE
GOOD LABORATORY PRACTICE:	This study was conducted in accordance with Good Laboratory Practice guidelines with the following exception: egg receipt, sterilization and incubation to the first instar larvae stage for the insect bioassay portion of this analytical procedure was not conducted in accordance with the GLPs. No impact on the study was expected.

I. STUDY DESIGN

Test Materials and Methods:

A. *Equivalence Assessment of Bacterial-Produced and Plant-Produced Cry3Bb1.11098 and Cry3Bb1.11231 Proteins*:

Materials:

- Test material - Cry3Bb1.11098 from corn event 96.040 (Lot# 6321003) and Cry3Bb1.11231 from corn event MON 853 (Lot# 6321004). Both proteins were produced in corn rootworm (CRW) protected corn. Both proteins were purified using immunoaffinity chromatography from extracts of root tissues of greenhouse-grown plants. Protein purity was ~ 93% and 80% for Cry3Bb1.11098 (~ 1.5 mg) and Cry3Bb1.11231 (~ 0.2 mg) respectively. It was necessary to purify additional Cry3BB1.11098 protein (Lot# 6321045) from event MON 859. This protein was purified to 73% purity and stored as a 0.32 mg/ml solution.
- Reference Materials - Cry3Bb1.11098 (Lot# 6312803) and Cry3Bb1.11231 (Lot# 6312812) proteins purified from *Bacillus thuringiensis*. These proteins which were previously characterized (Hileman et al., 1999) were purified and lyophilized. The protein powder was found to be 30.7% and 39.6% pure for Cry3Bb1.1098 and Cry3Bb1.11231 protein respectively. Total protein stock solutions were estimated to be 1.66 mg/ml for 11098 and 2.20 mg/ml for 11231, with purities of 92.3% and 96% for Cry3Bb1.11098 and Cry3Bb1.11231 respectively.

Methods:

1. N-terminal Amino Acid Analysis: bacterially-produced proteins were prepared for N-terminal sequencing according to Hileman et al. (1999). Plant-produced proteins were diluted with 2x Laemmli buffer and separated on NOVEX 4→20% gradient polyacrylamide gels (SOP PB-EQP-005-01 - not submitted) and electroblotted to Immobilon-P^{SQ} PVDF membranes. Proteins were visualized by Ponceau S staining, cut from the membrane and sequenced by an automated Edman degradation on an Applied Biosystems model 494 Procise Protein Sequencing System (SOP 400.324).
2. SDS-PAGE: aliquots of purified protein was diluted or concentrated as necessary and added to an equal amount of 2x Laemmli buffer, heated to 100 °C (~ 3 min) and loaded onto the NOVEX 4→20% polyacrylamide gels (SOP PB-EQP-005-01). Proteins were visualized with Colloidal Coomassie Brilliant Blue dye and molecular weight estimated by comparison to molecular weight standards.
3. Immunoblot Analysis: performed according to SOP GEN-PRO-002-03. SDS-PAGE was performed as described above. Plant and microbial proteins were loaded at approximately 10, 20 and 50 ng/well each to allow for visual comparison of immunological response versus concentration for both proteins. Proteins were transferred to 0.45 µm nitrocellulose membrane in buffer (25 mM Tris, 192 mM glycine and 20% methanol). Immunoblots were blocked with 2% BSA in TBST and incubated with a ~ 1:5000 dilution of rabbit anti-Cry3Bb1 IgG polyclonal antibody (Lot#783)

raised against *B.t.* produced Cry3Bb1 wild-type protein. Rabbit antibody bound to the blot was detected using ~ 1:7500 dilution of goat anti-rabbit IgG polyclonal antibody conjugated to horseradish peroxidase. Immunoreactive bands were visualized with the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech).

4. Staining for Carbohydrate: purified proteins were diluted or concentrated as necessary and added to an equal amount of 2x Laemmli buffer, heated to 100 °C (~ 3 min) and loaded onto the NOVEX 4→20% polyacrylamide gels (SOP PB-EQP-005-01). Samples of horseradish peroxidase and ovalbumin were analyzed on the same gel to serve as positive (glycosylation) controls. These two proteins were loaded at 50 and 250 ng/well. Cry3Bb1 proteins were loaded at 750 ng/well. Proteins were transferred to PVDF membrane (SOP GEN-PRO-002-03) and carbohydrates covalently associated with the blotted proteins were detected by GlycoTrack™ kit (Oxford GlycoScience, Inc.). In short, any bound carbohydrates are first oxidized by periodate treatment, then react with hydrazine linked to biotin. Any bound biotin is then detected by streptavidin linked to alkaline phophatase conjugate and typical colorimetric reactions.

5. Insect Bioassay: Cry3Bb1 proteins were diluted to a 5 fold excess in 2 ml deionized water. The 2 ml samples were subsequently diluted with 8 ml of a liquid test diet to provide final concentrations between 0.0 and 10.0 µg/ml. Colorado potato beetle (*Leptinotarsa decemlineata*, CPB) larvae were grown on test diets containing the plant or microbial Cry3Bb1 proteins and incubated at approximately 28 °C for one week. CPB larvae were scored for mortality (SOP BR-ME-0044-02 - not submitted). Five separate bioassays were conducted with 16 larvae per dose. The insect mortality results from the five assays were analyzed together in the multi-intercept logistic regression model (Agresti, 1996):

1) $$\ln \frac{\theta_{ijk}}{1-\theta_{ijk}} = \alpha_{ijk} + \beta \ln X$$

where θ_{ijk} is the probability of death for an insect in assay i on a diet of concentration X of protein variant j from source k. The logistic slope parameter, β, is constant, but the intercept can vary from assay, variant, and source. The logistic model can also be expressed in terms of mortality probability as:

341

2) $$\theta_{ijk} = \frac{\exp(\alpha_{ijk} = \beta \ln X)}{1 + \exp(\alpha_{ijk} = \beta \ln X)}$$

Here, the LC_{50}, is computed from the slope and intercept as:

3) $$LC_{50} = X_{ijk} = \exp\left(-\frac{\alpha_{ijk}}{\beta}\right)$$

The generalized linear model procedure, GENMOD, in SAS was used to fit the model to the data. The slope and intercepts were used to compute the LC50 for each variant-source combination in all 5 assays. The likelihood ratio test was implemented in GENMOD by testing the hypothesis

4) $$\alpha_{ijk>Bt} - \alpha_{ij,corn} = 0$$

B. *Bridging Equivalence of Cry3Bb1.11231 Protein Produced by Corn Event MON 860 to Cry3Bb1.11231 Protein from Corn Event MON 853 and Bacillus thuringiensis:*

Materials:

- Test Material - test material was prepared from grain of MON 860 obtained from plants grown in the field (Production Plan# 98-01-39-01- not submitted).
- Control Materials - grain extracts from non-transgenic lines MON 846 and MON 847 were used to evaluate the specificity of the antibodies used for immunoblot analysis. This grain was obtained from plants grown under the plan identified above.
- Reference Materials for Event Bridging - Cry3Bb1.11231 from *Bacillus thuringiensis* (Lot# 6312812) and CRW protected corn event MON 853 (Lot# 6321004).

Methods:

1. Extraction of Cry3Bb1.11231 from Corn Event MON 860: approximately 1 g of frozen corn grain from event MON 860 was extracted in 10 ml of 2x Laemmli buffer using an Omni 2000 hand held homogenizer (1 min, high speed @ 4 °C). The sample was centrifuged (10 min @ 14,000 x g) and the supernatant was removed and heated at 100 °C for ~ 3 min. The sample compared to corn produced Cry3Bb1.11231 (corn event MON 853 - Lot# 6321004) and bacterial-produced Cry3Bb1.11231 (Lot# 6312812) using SDS-PAGE (NOVEX 4→20% polyacrylamide gels) according to SOP PB-EQP-005-01 (not submitted). The proteins were transferred to

342

0.45μm nitrocellulose membrane (25 mM Tris, 192 mM glycine and 20% methanol buffer). The membrane was blocked with 2% BSA and TBST, incubated with ~ 1:5000 dilution of rabbit anti-Cry3Bb1 polyclonal antibody (Lot# 783) raised against *B.t.* produced Cry3Bb1 protein. A ~1:7500 dilution of goat-anti-rabbit polyclonal antibody conjugated to horseradish peroxidase and the ECL system were used for detection.

Bridging Equivalence of NPTII Protein Produced in CRW Protected corn to NPTII Protein Produced by E. coli:

- Test Materials - extracts from root tissue of corn events MON 853 and MON 860. These were obtained from plants grown in the field (Production Plan# 98-01-39-01- not submitted).
- Control Materials - extracts from root tissue from non-transgenic lines MON 846 and MON 847. These extracts were used to confirm specificity of antibodies. These extracts were obtained from plants grown in the field (Production Plan# 98-01-39-01- not submitted).
- Reference Materials - *E. coli*-produced NPTII protein standard (Lot# 4821020, batch reference 6340505).

Methods:

1. Extraction of NPTII Protein from Plant Tissues: root samples of MON 846, MON 847, MON 853 and MON 860 were separately homogenized in extraction buffer [25 mM sodium phosphate, pH 7.04, 1.0 mM EDTA, 2.5 mM DTT, 0.5 mM PMSF, 1 mM benzamidine-HCl, 0.05% N-lauroyl sarcosine and 0.05% triton X-100] - tissue to buffer ratio of 1 mg tissue/5 μl buffer. Samples were homogenized with the Omni hand-held homogenizer at medium speed for ~ 30 seconds, clarified by centrifugation for ~ 20 min (14,500 x g) and the supernatants were concentrated in a Speed-Vac for ~ 3 hours. All samples were concentrated about 5x and a 100 μl aliquot of each was mixed with 100 μl of 2x Laemmli buffer. The samples were heated (~ 5 min, 100 °C) and stored at -80 °C until used.
2. SDS-PAGE: two identical NOVEX 4→20% polyacrylamide gels were used so that two immunoblots could be produced - one each for polyclonal and monoclonal antibody detection. Color molecular weight markers and biotinylated molecular weight markers were loaded on each gel.
3. Immunoblot Analysis: proteins separated by SDS-PAGE were transferred to PVDF membranes. The membranes were blocked with 5% non-fat dry milk (NFDM) in 1x TBST for 1.5 hours at room temperature. One blot was incubated overnight at 4 °C with a combination of two anti-NPTII protein monoclonal antibodies (mAb7b and mAb9, each at ~ 1:300 dilution) in 1 x TBST with 1%

NFDM. The second membrane was incubated in a ~1:5000 dilution of anti-NPTII protein polyclonal antibody (Lot# 6340503). Mouse or rabbit IgG bound to the protein was probed with ~1:10000 dilution of anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase in 1x TBST with 1% NFDM for 1 hour. Each of the secondary antibody solution also contained ~1:10000 dilution of Neutravidin as a probe for the biotinylated MW markers. Immunoreactive bands were visualized with the ECL system.

D. *Bridging Equivalence of Cry3 proteins found in commercial microbial B.t. products to the bacterial forms of Cry3Bb1 used in the above studies:*

-Test materials: Raven™(lot DRV-5088-109) and Foil™ (lot DFO-6355-109) microbial insecticides based on *Bacillus thuringiensis* and known to express endotoxins in the Cry3 family (Cry3Bb1 and Cry3A, respectively) were used to establish the immunological and molecular similarity between these wild type proteins and those expressed in MON 863 corn.
-Reference materials: Cry3Bb1.11098 (lot 6312803) and Cry3Bb1.11231 (lot 6312812) proteins isolated from bacteria served as the reference standards.

Methods

1. Extraction methods- Raven™ and Foil™ were suspended in an extraction buffer (50mM carbonate/bicarbonate, 10mM DTT, 1mM benzamidine-HCl, 1mM EDTA, 0.5mM PMSF, pH 10.0). The amounts were 74.1 mg Raven Technical powder to 40ml buffer and 50.3 mg Foil technical powder to 40 ml buffer. The suspensions were vortexed and held on a rocker table overnight at 4°C. These extracts were mixed with 2x Laemmli buffer, heated to 100°C for five minutes then loaded onto the gels for electrophoresis.
2. Electrophoresis- A 4→20% NOVEX SDS-PAGE mini-gel system was loaded with the samples and run according to SOP PB-EQP-005-01. The separation was done at a constant 160V for approximately 1.5 hours until the dye front reached the bottom of the gel.
3. Colloidal Blue Staining- The protein bands were visualized by staining with colloidal Coomassie Blue.
4. Immunoblot Analysis- The SDS-PAGE gels were electroblotted onto a PVDF membrane which was subsequently blocked for non-specific binding with either 2%(w/v) BSA or 5% (w/v) NFDM in PBST. The procedures for the analysis were to be found in SOP GEN-PRO-002-03. The membranes were probed with three different primary

antibodies: a rabbit polyclonal anti Cry3Bb1 (lot 783, bleed 12/6/96); a rabbit polyclonal anti-Cry3Aa (lot DD78, bleed 6/5/92) and a mouse monoclonal anti-Cry3Bb1 (lot 214.10C-3-4, cell line 4247.LC9.2.2.2). The specific binding of these primary antibodies was detected by the binding of either goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma A9169, lot 77H4807) or an undescribed anti-mouse IgG conjugate (Sigma A8924, lot 46H8848). The reaction was visualized by Amersham Pharmacia's enhanced electrochemiluminescence.

II. RESULTS

A. *Equivalence Assessment of Bacterial-Produced and Plant-Produced Cry3Bb1.11098 and Cry3Bb1.11231 Proteins*:

1. Protein Identity: the identities for Cry3Bb1.11098 and Cry3Bb1.11231 were confirmed by using N-terminal sequencing data to compare to the bacterial-produced proteins (Table 2). This sequencing was performed on the two major visible bands (~ 66 and 56 kDa) and produced identical sequences for both the *B.t.* and plant-produced proteins. Note that the AA residues 1-57 were not evident and the identity of many of the assigned AA residues for the 55kDa moiety were claimed as tentative due to equivocal peak presence.

2. SDS-PAGE Analysis: figures 1 & 2 show SDS-PAGE results for the Cry3Bb1 proteins. Figures 1 compares plant and *B.t.* produced Cry3Bb1.11098 and Figure 2 compares plant and *B.t.* produced Cry3Bb1.11231. The quality of the copies of the gels do not allow for adequate comparison of the Cry3Bb1.11098 samples. Arrows are shown at the positions where the proteins were apparently visualized by the registrant, but several of these protein bands are not visible. For Cry3bb1.11231, the upper band (~66 kDa) is visible for each sample, but not for the ~ 56 kDa protein. Assuming that the arrows shown appropriately indicate the position of the proteins, then the data show that the proteins migrate similarly on the SDS-PAGE gel system. It should be noted that in figure 2 the lower loading amounts appear to give more intense staining results suggesting that perhaps the legend or the gel is actually in reverse order. Better copies of each gel should be submitted to allow the Agency to make a definitive determination of this issue. Table 3 shows the approximate molecular weights of each of the proteins based upon data obtained using a Bio-Rad densitometer.

3. Comparison of Immunoreactivity: figures 4 and 5 show the results of immunoblot analysis of each protein. These results provide

support for the determinations made by the registrant in #2 above. The immunoblot analysis shows the similar migration pattern for each visible protein. A few of the proteins are not visible on the copies provided, but this appears to reflect the amount of protein loaded in each case. For each missing protein, the lane with a higher amount of protein loaded shows the appropriate protein band. Based upon these data, the *B.t.* and plant produced protein in each case appear to have identical migration. It should be noted that for the Cry3Bb1.11098 that there appears to be a preponderance of the higher weight species produced in bacteria versus the lower weight species in the corn event 96.040.

4. Glycosylation Test: Figure 6 shows the results of the GycoTrack blot showing an apparent lack of glycosylation. Control samples (horseradish peroxidase and ovalbumin) had bands visible after staining, but both the *B.t.* and plant-produced Cry3Bb1 proteins did not, indicating a lack of glycosylation. Lanes 5 and 7 of the figure show a positive staining smear as would be expected for a plant extract. However, there are no definitive carbohydrate-positive bands in the region where the Cry3Bb1 proteins would migrate.

5. Bioactivity:
The functional equivalence of the bacterially-produced and plant-produced proteins were analyzed using Colorado Potato Beetle (CPB). Cry3Bb1.11231 was used in one assay while Cry3Bb1.11098 was used in all five assays (assays 1, 2 & 5 were for equivalence between the bacterial and plant sources - assays 3 & 4 were exploratory investigations on Cry3Bb1.11098). The calculated LC_{50} values for each test are shown in Table 4. In assay 1, the bacterial- and plant-produced Cry3Bb1.11231 proteins were not significantly different (p=0.06089). However, the Cry3Bb1.11098 from corn had a significantly different LC_{50} than the bacterially-produced protein (p=0.0340). These two proteins were re-tested in assay2 with a larger number of concentrations, and the significant difference between the proteins's bioactivity was confirmed. (P=0.0147). In assay 5, a different lot of plant-produced Cry3bb1.11098 was compared with the same bacterial-produced protein used previously and there was no significant difference, indicating some variation in lot to lot, assay to assay, or both. Some of these differences were attributed to the presence of protein aggregates.

6. Equivalence of Cry3Bb1.11231 Protein Produced by Corn Event MON 860 to Cry3Bb1.11231 Protein from Corn Event MON 853 and *Bacillus thuringiensis*:
The relative MW of Cry3Bb1 from MON 860 was compared to that from MON 853 using immunoblot analysis (Figure 7). The extract from MON 860 produced bands of the expected MW (~ 66 kDa and ~ 55 kDa) which co-migrated with those from MON 853. Lane 7 of Figure 7 shows extract of event MON 854 which was dropped by amendment after the analysis.

7. Bridging Equivalence of NPTII Protein Produced in CRW Protected corn to NPTII Protein Produced by *E. coli*:
Figure 8 shows the immunoblot which compares the relative molecular weight of plant- and bacteria-produced NPTII proteins. Based upon the results shown in Figure 8, the NPTII protein produced in *E. coli*, MON 853 and MON 860, show similar electrophoretic mobility indicating equivalent molecular weight.

8. Bridging Equivalence of Cry3 proteins found in commercial microbial B.t. products to the bacterial forms of Cry3Bb1 used in the above studies:
Figures A and B demonstrate that Cry3Bb1 protein variants 11098 and 11231 had similar molecular weights and immunoreactivity to the δ-endotoxins found in the commercial *Bacillus thuringeinsis* products Raven and Foil. The immunoreactivity was distinguishable between Cry3B and Cry3A proteins when probed with immunoreagents developed by immunization with Cry3Aa. There was some cross-reactivity between Cry3A and Cry3B proteins with the rabbit polyclonal anti-Cry3Bb1 antiserum.

III. DISCUSSION

The purpose of this study is to show the equivalence of bacteria- and plant-produced proteins. Because of the limited amount of protein present in CRW protected plants, production of the Cry3Bb1 protein in a surrogate bacterial host allows for a significant increase in the amount of protein available for characterization and safety testing.

This report compares the physical (MW, N-terminal sequencing) and functional (bioassay) characteristics of Cry3Bb1.11098 and Cry3Bb1.11231 proteins produced in *E. coli* and CRW protected corn. The data provided show that the proteins have equivalent molecular weight, immunological reactivities, N-terminal sequences and comparable LC_{50} values. This data supports the determination of the equivalence of the bacteria- and plant-produced proteins, and the use of the bacterially-produced proteins to support registration of the CRW corn product.
CLASSIFICATION: ACCEPTABLE

Reg. # 524-528
Volume 2 of 4

Page ___ is not included in this copy.

Pages 346 through 371 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist MTW for

Secondary Reviewer: Chris A. Wozniak, Ph.D., Biologist CAW

STUDY TYPE:	Product Characterization (885.1100)
MRID NO:	453484-03
TEST MATERIAL:	Corn Event MON 863
PROJECT NO:	99-894E
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Agronomic Equivalency of Corn Event MON 863 Hybrids as Determined in Year 2000 Field Trials
AUTHOR(S):	Clinton D. Pilcher, John K. McFerson, and Dennis P. Ward
STUDY COMPLETED:	January 30, 2001
CONCLUSION:	Results of the study show that there are some differences in the properties of the transgenic plants versus the control lines used in the tests. Some of the variation identified included a variety of differences in corn ear height, plant height, weight, grain moisture and yield, but in each case, the difference was small. However, based upon the data provided, it appears that none of these differences would have a significant agronomic impact on the crops and are likely similar to typical differences seen in different plant lines and/or those differences caused by differing ecological effects. The data included in this submission appear to support the agronomic equivalency of corn event MON 863 hybrids.
CLASSIFICATION:	ACCEPTABLE
GOOD LABORATORY PRACTICE:	This study was not conducted in accordance with Good Laboratory Practice guidelines

I. STUDY DESIGN

Test Material: Corn Event MON 863

Methods: Six corn hybrids - H-1, H-2, H-3, H-4, H-5 and H-6 - were used for agronomic equivalency trials under EUP# 524-EUP-93. The H-1 through H-3 lines are hybrids derived from positive and negative isolines and were compared under typical field conditions at locations in Illinois, Iowa, and Nebraska using a randomized split-plot design (each plot was two rows of

between 88 and 115 ft^2). Each plot was replicated four times at each location. The H-4 through H-6 hybrids were derived from converted inbreds and these lines were evaluated to their parental controls at locations in Illinois and Iowa using a balanced lattice square design and two replications at each site (each plot had two rows of 115 ft^2). The positive and negative isolines of H-1 were also evaluated using the design of H-4 through H-6.

Herbicide and fertilizer applications were performed as for typical farming practices for each location. The soil was treated with an insecticide to control corn rootworm larvae. Also, the plots were sprayed with foliar insecticides and fungicides. A description of the evaluations are found in Table 4 (attached). Statistical analyses were based on location means for each hybrid pair and a standard Student's t-test was used to assess the significance of any differences observed - statistical significance was predetermined to be at the 5 % level ($p \leq 0.05$).

II. RESULTS

Summary results of the year 2000 trials are provided in Tables 5 through 11 (attached). According to the report, there were nine statistically significant differences observed in the 64 comparisons made. The report further indicates that 5% (3 of the 64) of the differences are expected based upon chance alone.

- Table 5 describes the agronomic equivalency trials conducted at each location with MON 863, hybrid H-1. The test line percent difference from the control line and p-values are included for the randomized split-plot design. The differences ranged from a high of 12.9% in seedling vigor to a low of 0.5% for both GDU (growth degree units) to pollen shed and GDU to silking.

- Table 6 describes the agronomic trials conducted with MON 863 hybrid H-2. The test line percent difference from the control line and p-values are included for the randomized split-plot design. The percent difference between the test and control lines ranged from a high of 9.5% for seedling vigor to a low of 0% difference in GDU to pollen shed.

- Table 7 describes the agronomic trials conducted with MON 863, hybrid H-3. The test line percent difference from the control line and p-values are included for the randomized split-plot design. The percent difference between the test and control lines ranged from a high of 14.3% for seedling vigor to a low of 0.4% for GDU to pollen shed.

- Table 8 describes the agronomic trials conducted with MON 863, hybrid H-4. The test line percent difference from the control line and p-values are included for the balanced lattice square design where MON 863 was compared to its parental line.

The percent difference between the test and control lines ranged from a high of 24.1% for seedling vigor to a low of 0% for GDU to silk.

- Table 9 describes the agronomic trials conducted with MON 863, hybrid H-5. The test line percent difference from the control line and p-values are included for the balanced lattice square design where MON 863 was compared to its parental line. The percent difference between the test and control lines ranged from a high of 9.7% for seedling vigor to a low of 0.08% for GDU to silk.

- Table 10 describes the agronomic trials conducted with MON 863, hybrid H-6. The test line percent difference from the control line and p-values are included for the balanced lattice square design where MON 863 was compared to its parental line. The percent difference between the test and control lines ranged from a high of 3.5% for % grain moisture to a low of 0% for seedling vigor.

- Table 11 describes the agronomic trials conducted with MON 863, hybrid H-1. The test line percent difference from the control line and p-values are included for the balanced lattice square design where MON 863 was compared to its negative isogenic control. The percent difference between the test and control lines ranged from a high of 5.0% for yield to a low of 0% for seedling vigor.

III. DISCUSSION

Results of the study show that there are some differences in the properties of the transgenic plants versus the control lines used in the tests. However, according to the registrant, most of these differences are not agronomically important. The data presented appear to support this position. Seedling vigor was significantly different ($p \leq 0.05$) for the H-3 / MON 863 comparison and at $p \leq 0.10$ for the H-5 comparison. These results may be explained by an associated linkage drag that was absent from the other converted lines. There were a variety of differences in ear height, plant height, weight, grain moisture and yield, but each of these differences were numerically small. These differences are typical of biological systems under field comparisons and would not be expected to have a major agronomic impact on the crops. The data included in this submission, as presented, support the agronomic equivalency of corn event MON 863 hybrids.

CLASSIFICATION: ACCEPTABLE

Reg. # 524-528
Volume 2 of 4

Page ___ is not included in this copy.

Pages 375 through 377 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist

Secondary Reviewer: John L. Kough, Ph.D., Biologist

STUDY TYPE:	Product Characterization (DNA Molecular Identity) 885.1100
MRID NO:	454240-02
TEST MATERIAL:	Corn Event MON 863
PROJECT NO:	Study 99-01-39-27; MSL-17152
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Amended Report for MSL-16505: Molecular Analysis of Corn Event MON 863
AUTHOR(S):	Tracey A. Cavato, Ellen C. Rigden, Donald W. Mittanck, Ronald P. Lirette
STUDY COMPLETED:	April 10, 2001 (amendment 1)
CONCLUSION:	The data presented in this submission describe the DNA insert for event MON 863. The data provided support the finding that event MON 863 contains 1 intact copy of the insert which encodes for both Cry3Bb1 and NPTII proteins.
CLASSIFICATION:	ACCEPTABLE
GOOD LABORATORY PRACTICE:	This study complies with Good Laboratory Practice guidelines with the following exception: sequence information used in the study was generated by the Monsanto Sequencing Center, which does not generate its data in compliance with GLP regulations. However, this should not affect the data presented.

I. STUDY DESIGN

Test Material: DNA sequence of corn rootworm event MON 863 - includes both *cry3Bb1* and *nptII* genes.

Control: DNA sequence of non-transgenic corn line MON 846 (A1xA634).

References: DNA sequence information from plasmid PV-ZMIR13 (parent of DNA fragment used in MON 863 transformation). Molecular size markers (Gibco BRL and Boehringer Mannheim).

Methods:

1. DNA Isolation: DNA extracted from leaf tissue was used to obtain the 5' and 3' insert-to-plant junction sequences for MON 863. DNA extracted from grain was also used where grain samples (harvested from individual plots from four different field sites) were blended separately into a fine powder using a commercial blender. Approximately 6 g of the processed grain was transferred to a pre-chilled centrifuge tube (30-35 ml) and ~ 16 ml of CTAB buffer was added (CTAB - 1.5% CTAB, 75 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 1.05 M NaCl and 0.75% PVP). The samples were mixed, incubated at 65 °C for 30 minutes and cooled to room temp. An equal volume of chloroform:isoamyl alcohol (24:1) was added and the suspension was mixed and separated by centrifugation (13, 8000 x g). The aqueous layer was removed and 1/10 volume of 10% CTAB buffer (10% CTAB, 0.7 M NaCl) and an equal volume of chloroform:isoamyl alcohol was added. The samples were mixed, separated by centrifugation and the aqueous phase was removed and mixed with an equal volume of CTAB precipitation buffer (1% CTAB, 50 mM Tris pH 8.0 and 10 mM EDTA pH 8.0). After 1 hour at room temperature, the samples were centrifuged (8,800 x g) to pellet the DNA. The supernatant was removed and the pellet was re-dissolved in approximately 2 ml high salt TE (10 mM Tris pH 8.0, 10 mM EDTA pH 8.0 and 1 M NaCl) at 37 °C for two hours. The samples were subjected to centrifugation at 20, 000 x g to pellet impurities and the supernatant was removed to a clean 13 ml tube with 150 µl 3M NaOAc and 2 volumes chilled 100% ethanol. Precipitated DNA was removed using a sealed/hooked glass Pasteur pipet and washed with 1 ml 75% ethanol. The DNA was pelleted by centrifugation at 14, 000 x g, vacuum dried and dissolved in TE (pH 8.0).
2. DNA Quantitation and Restriction Enzyme Digestion: DNA quantitation was performed using a Hoefer DnYA Quant 200 Fluorometer using Boehringer Mannheim molecular size marker IX or plasmid pBR322 as a calibration standard (for genomic or plasmid DNA quantitation respectively). Approximately 10 µg digested control DNA was loaded into one lane and 20 µg test DNA was split into two lanes of an agarose gel. Restriction enzyme digests were performed as 500 µl solutions using 100 units of restriction enzyme, overnight at 37 °C. After digestion, samples were ethanol-precipitated, washed (75% ethanol) and dried. Finally, the DNA was dissolved in TE, pH 8.0.
3. DNA Probe Preparation: plasmid DNA was isolated from *E. coli* cultures. Most probe templates were prepared by PCR using plasmid PV-ZMIR12 as template - PV-ZMIR12 is identical to PV-ZMIR13 except that it contains the small subunit chloroplast transit

360

peptide (SSU CTP) immediately adjacent to the 5' end of the *cry3Bb1* coding region. Linearized whole PV-ZMIR13 plasmid and PV-ZMIR13L were also used for probe templates. Approximately 25 ng of each probe template was labeled with ^{32}P-dCTP (6000 Ci/mmol) using random priming. The NOS 3' polyadenylation sequence was labeled using PCR. All labeled probes were purified using a Sephadex G-50 column.

4. Southern Blot Analysis: DNA samples were digested and separated on 0.6% agarose gels. A "long" and "short" run was performed for most gels to provide greater separation and retention of larger and smaller molecular weight fragments, respectively. Gels were photographed and DNAs were transferred to Hybond-N nylon membrane using a Turboblotter (Schleicher & Schuell). DNAs were cross-linked using a UV Stratalinker (Stragene). Blots were pre-hybridized for 2 to 72 hours (500 mM Na2HPO4-7H2O, 7% SDS , 1 mg/ml tRNA) followed by hybridization for 8 to 24 hours at 65 °C. Following hybridization, blots were washed (0.1% SDS, 0.1X SSC) 2 X (15 min each) at room temperature and 2X (20 min each) at 65 °C. Blots were exposed to Kodak Biomax MS film.
5. Verification of 5' and 3' Genomic Flanking Sequences: 5' and 3' insert-to-plant genomic DNA junctions were verified using PCR and DNA sequencing (primers described in Figure 14). PCR reactions were conducted with 100 ng genomic DNA as template in a 50 µl reaction. PCR products were separated on 1% agarose gels and sequenced by the Monsanto Genomic Sequencing Center.

II. RESULTS

Figures 1 & 2 and Table 1 describe Plasmid PV-ZMIR13 and the DNA fragment used to generate event MON 863. The two *Mlu*I sites on either side of the DNA fragment were used to remove the DNA fragment from the plasmid for use in the transformation event. Included in the DNA fragment were the *cry3Bb1* and *nptII* genes, as well as other genes necessary for *in planta* expression (Table 1).

Analysis of MON 863:

1. Insert Number: the insert number was determined using *Nde*I which digests out side of the insert DNA and results in a fragment which contains both the insert DNA and some adjacent plant genomic DNA. The number of copies were then visualized by Southern hybridization (Figure 3). The presence of only one DNA band indicates that there was only one insertion of the DNA and therefore, only one copy of the gene.
2. Copy Number: DNA samples were digested with *Eco*RV and the Southem blot was probed with PV-ZMIR13. Figure 4 shows the presence of two distinct bands for MON 863 (~9.6 and ~ 3.7 kb). As *Eco*RV cuts only once within the DNA insert,

381

the expected two DNA bands are produced by this digestion indicating the presence of only one copy of the insert.

3. *cry3Bb1* Cassette Intactness: Figures 5-7 show the results of Southern analysis to determine the intactness of the insert DNA. Only one DNA band was produced for each of the regions probed for: 4-AS1 promoter-wt CAB leader-ract1 intron (Figure 5); *cry3Bb1* Coding Region (Figure 6); and tahsp17 3' polyadenylation sequence (Figure 7). The lack of any unexpected DNA bands indicates none of these regions contained any additional sequence.

Analysis of nptII intactness:

Figures 8-10 show the Southern results for the identification of the intactness of the *nptII* cassette. As with MON 863, there were not any unexpected DNA bands for any of the regions of the cassette which indicates an intact *nptII*-containing cassette.

Border Sequence Confirmation: PCR and DNA sequencing were used to confirm the expected border sequences using genomic DNA. The junction sequences at the 5' and 3' ends of the MON 863 insert were verified. PCR results (Figure 13) show the correct size PCR products based upon the primer sets used. Sequence analysis of this PCR products (Figure 14) were also used to verify the presence of the proper DNA sequence.

III. DISCUSSION

Based upon the data provided, it appears that event MON 863 contains 1 copy of the intended DNA cassette. Further analysis of the intactness of the DNA insert and respective genes revealed that they were stably inserted.

CLASSIFICATION: ACCEPTABLE

Reg. # 524-528
Volume 2 of 4

Page ___ is not included in this copy.

Pages 363 through 364 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist

Secondary Reviewer: John L. Kough, Ph.D., Biologist

STUDY TYPE:	Product Characterization - Protein Equivalence (885.1100)
MRID NO:	454240-05
TEST MATERIAL:	Cry3Bb1 Protein (Event MON 863)
PROJECT NO:	99-01-39-31, MSL-17220
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Amended Report to MSL-16596: Assessment of the Physicochemical Equivalence of Cry3Bb1.11098 and NPTII Proteins in Corn Event MON 863 to Microbial Sources
AUTHOR(S):	Gyula Holleschak, B.S., Ronald E. Hileman, Ph.D. and James D. Astwood, Ph.D.
STUDY COMPLETED:	April 10, 2001 (amendment 1)
CONCLUSION:	Based upon the data provided, it appears that both the Cry3Bb1.11098 and NPTII proteins produced in event MON 863 have equivalent molecular weights and antigenic properties with these same proteins produced in *B.t.* and *E. coli* respectively.
CLASSIFICATION:	ACCEPTABLE
GOOD LABORATORY PRACTICE:	This study was conducted in accordance with Good Laboratory Practice guidelines .

I. STUDY DESIGN

Test Materials and Methods:

A. *Equivalence Assessment of Bacterial-Produced and Plant-Produced Cry3Bb1.11098 and Cry3Bb1.11231 Proteins*:

Materials:

- Test Material - Root extract from event MON 863 which contained both cry3Bb1.1098 and NPTII proteins. Roots were obtained from plants grown in the field under a production plan 99-01-08. Gels also included plant material from MON 862..
- Control Materials - Root extracts from non-transgenic corn lines MON 846 and MON 847 were used for antibody evaluation. Roots were also obtained from plants grown in the field.

- Reference Materials - Cry3Bb1.11098 protein (Lot# 6312803) produced in *B.t.* strain EG1098 and Cry3Bb1.11098 protein (lot# 6321003) expressed in event 96.040; NPTII protein expressed in *E. coli* (Lot# 6340505).

Methods:

A. Bridging Equivalence of Cry3Bb1.11098 Protein Produced in IPC Events MON 862 and MON 863 to Cry3Bb1.11098 Protein from IPC Event 96.040 and *B.t.*

1. Extraction of Cry3Bb1.11098 Protein from Root Tissue of IPC Event MON 862 and MON 863: Root samples of IPC events MON 862 and MON 863, as well as control lines MON 846 and 847 were homogenized separately in 15 ml tubes with extraction buffer (50 mM sodium borate, pH 9.8, 1 mM EDTA, 2.5 mM DTT, 0.5 mM PMSF, 1 mM benzamidine) at a 1:5 ratio (1 g tissue:5 ml buffer). An Omni 2000 homogenizer was used at high speed for 1 minute. The samples were clarified by centrifugation at 14,500 x g for 20 min and stored as 100µl aliquots at -80 °C.
2. SDS-PAGE: Aliquots of the supernatant with protein were added to an equal amount of 2x Laemmli buffer, heated to 100 °C (~ 3 min) and loaded onto the NOVEX 4→20% polyacrylamide gels (SOP PB-EQP-005-01). Proteins were transferred to 0.45 µm nitrocellulose membrane and membranes were blocked with 5% NFDM (non-fat dry milk) in PBST. The blot was then incubated for 45 minutes at 25 °C with anti-Cry3Bb1 wild type rabbit IgG polyclonal antibody (Lot# 783) at a 1:5000 dilution. The membrane was probed using a 1:7500 dilution of goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Sigma # 1-9169, Lot# 77H4807). Immunoreactive bands were visualized with the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech).

B. Bridging Equivalence of NPTII Protein Produced in IPC Events MON 862 and MON 863 to NPTII Protein Produced by *E. coli*:

1. Extraction of NPTII Protein from Plant Tissues: Root samples of MON 846, MON 847, MON 862 and MON 863 were separately homogenized in extraction buffer [25 mM sodium phosphate, pH 7.00, 2.5 mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 0.05% N-lauryl sarcosine and 0.05% triton X-100] - tissue to buffer ratio of 1 g root tissue/5 ml buffer. Samples were homogenized with the Omni hand-held homogenizer at high speed for ~ 1 min, clarified by centrifugation for ~ 20 min (14,500 x g) and stored as 100 µl aliquots at -80 °C.
2. SDS-PAGE: NOVEX 4→20% polyacrylamide gels were used. Proteins separated by SDS-PAGE were transferred to nitrocellulose

401

membranes. The membranes were blocked with 5% non-fat dry milk in 1x TBST for 45 minutes at room temperature. The membrane was incubated overnight at 4 °C with an anti-NPTII protein mouse monoclonal antibody (Lot# 6340503) at 1:300 dilution in PBST containing 1% NFDM. Mouse IgG bound to the protein was probed with ~1:5000 dilution of goat anti-mouse IgG secondary antibody conjugated to horseradish peroxidase in 1x TBST with 1% NFDM for 45 min. Immunoreactive bands were visualized with the ECL system.

II. RESULTS

A. Equivalence Assessment of Bacterial-Produced and Plant-Produced Cry3Bb1.11098 Protein:

Figure 1 shows the Western blot results comparing bacterial-produced and plant-produced Cry3Bb1 protein. Lanes 2-4 represent protein from *B.t.* (Lanes 3 & 4 spiked with non-transformed MON 846 & 847 respectively) and lanes 8 & 9 represent MON 862 & 863 respectively. Each of these protein bands appear co-migrate and therefore to be of the same molecular weight (~ 66 kDa). The 59 kDa band is also present (although the quality of the gel figure provided does not show the same band intensity in all lanes as expected). There are no protein bands visible in the negative control lanes (5 & 6) which both indicate the specificity of the antibodies and the lack of the Cry3Bb1 protein in these lines. The report also indicates that a larger (74 kDa) band is also present in lanes 8 & 9 (MON 862 & 863). This is apparently the full-length form of the Cry3Bb1.1098 protein which is apparently proteolytically process to the 66 kDa fragment which is usually observed. (This reviewer notes that the 74kDa band was not visible in the gel reproductions provided.)

B. Equivalence of NPTII Protein Produced in MON 862 & 863 to NPTII Protein Produced in *E. coli*:

Figure 2 shows the results of the comparison of the bacterial-produced and plant-produced NPTII protein. Comparison of lanes 2-4 (*E. coli*-produced) to lanes 7 & 8 show that the proteins co-migrated at the same rate and appear to share the same molecular weight. A lack of a signal in the control lanes (lanes 5 & 6) indicate that the antisera used in this study is specific for NPTII protein.

III. DISCUSSION

The results of this study indicated that the plant-produced Cry3Bb1 and NPTII proteins comigrated in SDS-PAGE gels with their respective bacterial-produced proteins. The plant-produced and bacterial-produced proteins appear to share the equivalent molecular weights (based on gel comigration) and have similar immunoreactivity with antibodies specific for each protein. These same antibodies to Cry3Bb1 and NPTII did not produce a signal in the negative controls. Based

402

upon this data, it appears that the molecular weights and antigenic properties of the plant-produced and bacterial-produced proteins are equivalent.

CLASSIFICATION: ACCEPTABLE

Reg. # 524-528

Volume 2 of 4

Page ___ is not included in this copy.

Pages 404 through 405 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist

Secondary Reviewer: John L. Kough, Ph.D., Biologist

STUDY TYPE:	*In vitro* Digestibility
MRID NO:	454240-06
TEST MATERIAL:	Cry3Bb1.11098 and Cry3Bb1.11231 Proteins
PROJECT NO:	Study Number: 98-01-39-02
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Amended Report for MSL-15704: Assessment of the *in vitro* digestibility of *B.t.* Protein 11098 and *B.t.* Protein 11231 Utilizing Mammalian Digestive Fate Models
AUTHOR(S):	John N. Leach, Ronald E. Hileman, Ph.D., John W. Martin, B.A., Richard S. Thoma, M.S. and James D. Astwood, Ph.D.
STUDY COMPLETED:	April 10, 2001 (amendment 1)
CONCLUSION:	The tests performed in this study show that the Cry3Bb1 proteins are not stable to digestion in simulated gastric fluid. Incubation of Cry3Bb1.11098 and Cry3Bb1.11231 in SGF results in the loss of detectable protein by the 30 and 15 second observation points, respectively, as detected by SDS-PAGE. Insect bioassay data indicated that the protein loss insecticidal activity within 2 minutes of incubation in SGF. Incubation in the SIF resulted in a ~59 kDa digestion product that retained its insecticidal activity after at least 30 minutes incubation.
CLASSIFICATION:	ACCEPTABLE. Neither Cry3Bb1.11098 nor Cry3Bb1.11231 appear to be stable to digestion in simulated gastric fluid for more than 30 seconds. However, there appears to be a discrepancy between the results discussion and figures 2 & 4. The Cry3Bb1.11098 protein appeared to be significantly digested within 15 seconds and completely digested in less than 30 seconds based upon the figures provided. This differs somewhat with the discussion provided by the registrant which indicates that the protein was digested to undetectable levels between 30 and 60 seconds. These do not represent drastically different digestion times, but the discrepancy should be addressed.
GOOD LABORATORY PRACTICE:	This study was conducted in accordance with Good Laboratory Practice guidelines with four exceptions as described in the

406

submission. These exceptions should not have a significant impact on the outcome of the study.

I. STUDY DESIGN

Test Material:

1. The test substances were: Cry 3Bb.11098 protein (lot# 6312803) and Cry3Bb1.11231 protein (lot# 6312812) which were isolated from transformed *Bacillus thuringiensis* strains EG11098 and EG11231 respectively.
2. The reference substance for the simulated gastric fluid (SGF) assay was hemoglobin. The reference substance for the simulated intestinal fluid (SIF) assay was resorufin-labeled casein.
3. The control substances were prepared by using purified water (vehicle) instead of the Cry3Bb proteins.

The recovery of the test substances and effect of buffers/pH conditions alone were demonstrated by trials which served as reference treatments. These reference treatments were prepared in SGF without pepsin or SIF without pancreatin.

Methods:

Test Treatments:

1. Simulated gastric and intestinal fluids were prepared and confirmed to be functionally active on the day of use. SGF was measured in accordance with SOP No. GEN-PRO-057-01 (not submitted) which relates to the amount of pepsin activity to the rate of hemoglobin digestion. SIF activity was measured in accordance with SOP No. GEN-PRO-058-01 (not submitted) which relates to the amount of proteolytic activity to the rate of resorufin released from resorufin-labeled casein. Both assays determine the amount of digestion by comparison to the change in absorbance. Digestion is directly related to increased absorbance due to the amount of small fragments present in TCA protein precipitated solutions.
2. The rate of Cry3Bb digestion in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was examined in two sets of digestion trials (based upon volume), small scale and large scale. The large scale digestions were conducted for use in insect bioassays.
3. Timed incubations began with the addition of Cry3Bb protein to individual incubation tubes in a shaking 37° C waterbath. [zero incubation time trials were quenched prior to addition of the test substance].
4. SGF digestions were quenched by addition of sodium carbonate and SIF digestions were quenched by addition of 1X Laemmli buffer.

5. Small scale SGF trials were further diluted in 5X Laemmli protein sample loading buffer, then heated to 100 °C for 5 min. Small scale SIF trials were diluted with Laemmli sample buffer followed by heating to 100 °C for 5 min.
6. Large scale SGF trials were placed in dry ice-methanol baths until storage at -15 °C or colder. Large scale SIF trials were diluted in purified water followed by freezing in a dry-ice methanol bath, then stored at -15° C.

Test Treatments:

Treatment	Incubation Times
SGF	0, 15, 30 s and 1, 2, 4, 8; 15, 30, 60, and 120 min
SIF	0, 1, 5, 15, 30 min and 1, 2, 4, 8, 16, and 24 hr

Control and Reference Treatments:

1. Control treatments were prepared replacing the test substance with purified water vehicle in the SGF or SIF test system. Reference samples were placed in the SIF and SGF fluids without the pancreatin and pepsin respectively. Samples were collected at 0 and 120 min for SGF and 0 and 24 hr for SIF small scale trials. For large scale trials, the 0 min time point was used for both SGF and SIF.

SDS-PAGE Analysis:

1. *SDS-PAGE/Colloidal Brilliant Blue G Staining:*
   SDS-PAGE analysis was performed according to SOP No. BtC-PRO-026-01 (not submitted). Previous work identified the level of detection for Cry3Bb1.11098 and Cry3Bb1.11231 proteins using Brilliant Blue G staining to be 1 ng. The gels were loaded with 1 µg Cry3Bb1 total protein samples per lane according to the gel photos provided. Tricine gels and running buffers were used, which provide good resolution of peptides ≥ 2 kDa.. After separation of the proteins by electrophoresis, proteins were visualized by staining with Colloidal Brilliant Blue G concentrate. After electrophoresis, gels were fixed (methanol/acetic acid) for 30 minutes followed by overnight staining. Gels were then washed with 25% methanol solution, then photographed.

2. *SDS-PAGE/Immunoblot Analysis:*
   The limit of detection for this method was determined to be approximately 0.2 ng for the upper protein band (~67 kDa) and 1 ng

408

for the lower protein band (~59 kDa). The gels were loaded with 10 ng of Cry3Bb1 total protein per lane according to the gel photos provided. Proteins were separated by electrophoresis and transferred to 0.45μm PVDF membrane. The membrane was incubated in blocking solution (5% NFDM [nonfat dried milk] dissolved in TBST [TBST = Tris-buffered saline with Tween 20]). Protein specific monoclonal antibody was added at 1:5000 (antibody in 1% NFDM in TBST) and incubated (usually overnight at 4 °C). Membranes were washed in TBST and protein-bound antibody was detected using anti-rabbit IgG conjugated to horseradish peroxidase. The membrane was incubated with the secondary antibody (1:7500 dilution in 1% (w/v) NFDM dissolved in TBST) for at least 1 hour at room temperature, followed by TBST washing. Secondary antibody bound to the membrane was visualized by ECL (chemiluminescence) on X-Ray film.

Functional Assay:
The functional assay was an insect bioassay performed according to SOP No. BR-ME-0044-02 (not submitted) to evaluate the functional activity of the Cry3Bb1 proteins. An agar-based diet into which the digestion trials were added was used. Colorado potato beetle (CPB) larvae were placed onto wells containing the diet. Protein functional activity was determined by comparing the number of surviving insects and mean insect weight to a standard dose curve and other trials. Large-scale digestion trials were modified to maintain the functionality of the proteins. The Laemmli sample buffer and heating steps were eliminated.

N-terminal Amino Acid Sequence Analysis:
SDS-PAGE analysis of the Cry3Bb1 proteins identified minor small molecular weight bands which were analyzed by N-terminal sequencing to determine if they were Cry3Bb1 protein digestion products Searle SOP No. 400.324 (not submitted).

For preparation of a protein digestion product with a high enough amount of resulting peptide fragments protein for analysis, the experimental volumes, concentrations and pepsin to protein ratio were changed. A series of incubation trials were used to generate the same fragments as previously identified. SDS-PAGE gels were run followed by electro-transfer to 0.45 μm pore size PVDF membrane in CAPS transfer buffer (10 mM CAPS pH 11, 10% (v/v) methanol). 10.5μg of total protein was loaded into each of five lanes. Proteins were visualized by staining the membrane with Ponceau S.

II. RESULTS

Effectiveness of the Test Systems:

The proteolytic activity of SGF prepared for small scale digestions was 16,733 U/ml (where 1U = Δ0.001 $A_{280\ nm}$ min^{-1} at 37 °C) which, according to the registrant, was within the requirements of SOP No. GEN-PRO-057-01 (not submitted). The SGF prepared for the large scale digestions had an activity of 15,683 U/ml, which was not within the SOP requirements (SOP acceptability range for SGF not provided). However, this preparation was accepted by the registrant anyway as they believe that there was not any impact on the study based upon the following:

i) if the activity units are argued to be only valid to two significant figures, the low activity rounds up to the acceptable range.

ii) the same lot of pepsin was used to ensure comparable results between the small-scale and large-scale digestions.

iii) the variability within the activity assay is broad. One standard deviation is approximately 25% of the mean.

iv) the SGF which fell outside the accept criteria, was close to the activities of earlier preparations with the same lot of pepsin.

v) the acceptance criteria established in the SOP was based on only a few lots of pepsin and did not include data from the lot being used.

vi) the particular lot of pepsin used to prepared SGF had been observed to consistently produce low activities within the acceptable range

vii) validation experiments for the SGF model demonstrated that protein digestion rates are not sensitive to small variations in pepsin concentration.

The SIF proteolytic activity of SIF was 11,581 U/ml (1U=Δ0.001 A574nm min-1 at 37 °C) for the small scale digestions and 10,111 U/ml for large scale digestions. Each of these activities are within the SOP requirements according to the submission (data not provided).

Degradation of Cry3Bb1 Proteins in SGF/Insect Bioassay:

Figures 2 & 4 show the results of staining and immunoblot analysis for the small scale digestion of Cry3Bb1.11098 protein samples incubated in SGF. The Cry3Bb1.11098 protein was significantly digested within 15 seconds and apparently completely digested in less than 30 seconds based upon the figures provided. This differs somewhat with the discussion provided by the registrant which indicates that the protein was digested to undetectable levels between 30 and 60 seconds. These do not represent drastically

different digestion times, but the discrepancy should be addressed given that the submitted gels do not verify the claims.

Figures 3 & 5 show the results of staining and immunoblot analysis for the small scale digestion of Cry3Bb1.11231 protein samples incubated in SGF. The Cry3Bb1.11231 protein was apparently completely digested by the 15 second timepoint based upon the figures provided. This interpretation agrees with that provided by the registrant.

Further, the registrant indicates that there was a low molecular weight protein fragment (~ 3 kDa) present in SGF digestions as a faint band in the SDS-PAGE stained gels (Figures 2 & 3) up to the 4 min digestion. Increased sample load and gel analysis indicated that this band was a doublet. No sequence data could be obtained for these doublet bands for Cry3Bb1.11098 when examined by N-terminal sequencing. For the Cry3Bb1.11231 doublets, no sequence data was obtained for the upper doublet, but analysis of the lower doublet indicated that it originated from the Cry3Bb1.11231 protein beginning at aa 563:

^{563}LQRYRVRIKYAST(T)(N)^{577} - determined
^{563}LQRYRVRIRYASTTN^{577} - expected

Insect bioassay analysis indicated that the proteins subjected to large scale SGF digestions lost insecticidal activity within 2 minutes (Figure 1, graph A-D).

Degradation of Cry3Bb1 Proteins in SIF/Insect Bioassay:
Incubation of the Cry3Bb1 proteins in SIF appears to generate a stable ~59 kDa fragment of Cry3Bb1 which is present through the 24 hour timepoint (Figures 6 & 7). Insect bioassay of SIF-digested proteins indicated that insecticidal activity remained even after 30 minutes of SIF incubation (Figure 1). These results indicate that the ~59 kDa protein is biologically active (Figure 1, graph E-H).

Test Substance Stability:
The test substance reference samples (incubated in the absence of enzyme) were stable throughout the experiment as shown on Figures 2-7. These results indicate the enzymatic activity of the SGF or SIF alone is responsible for the breakdown of the Cry3Bb1 proteins. No effect of solution pH or chemical composition was evident.

Comparison of Large-Scale and Small-Scale Digestions:
Large and small-scale SGF digestions were compared by SDS-

411

PAGE/Colloidal Blue staining to ensure that the results obtained by each were comparable. Figures 8 & 9 show the results of this testing and indicate that based upon the figures provided, the small and large-scale digestions provided similar results for the Cry3Bb1.11231 protein. However, there appears to be a small difference in the digestion times for the Cry3Bb1.11098 protein in small and large-scale digestions. This protein had took slightly longer for complete digestion in the large-scale digestion. However, this difference does not appear to be significant enough to affect the insect bioassay results. Therefore, the results of the insect bioassay should be applicable to both sizes of digestions.

Figure 10 shows the results of SIF small and large-scale comparisons. There was some difference in the digestion times for the small and large-scale digestions. However, each produced the same ~59 kDa protein and therefore appear the insect bioassay data is applicable to both.

III. DISCUSSION

Protein stability in simulated digestive fluids, specifically SGF, is one characteristic for food allergenic proteins which has been described. Although most proteins which have been identified as food allergens are stable to digestion, not all proteins which are stable to digestion are known to be food allergens. The tests performed in this study show that the Cry3Bb1 proteins are degraded to small or undetectable fragments in simulated gastric fluid. Incubation of Cry3Bb1.11098 and Cry3Bb1.11231 in SGF results in the loss of detectable protein by the 30 and 15 second observation point, respectively, as detected by SDS-PAGE or immunoblot. Insect bioassay data indicated that the protein loss insecticidal activity within 2 minutes of incubation in SGF. Incubation in the SIF resulted in a ~59 kDa digestion product that retained its insecticidal activity after at least 30 minutes incubation. These results will be combined with amino acid similarity and heat stability data to determine the potential allergenicity of this protein.

CLASSIFICATION: ACCEPTABLE. Cry3Bb1.11098 and Cry3Bb1.11231 do not appear to be stable to digestion in simulated gastric fluid. However, there appears to be a discrepancy between the results discussion and figures 2 & 4. The Cry3Bb1.11098 protein appeared to be significantly digested within 15 seconds and completely digested in less than 30 seconds based upon the figures provided. This differs somewhat with the discussion provided by the registrant which indicates that the protein was digested to undetectable levels between 30 and 60 seconds. These do not represent drastically different digestion times, but the discrepancy should be addressed.

Reg. # 524-528

Volume 2 of 4

Page ___ is not included in this copy.

Pages 413 through 434 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD

Secondary Reviewer: Chris Wozniak, Ph.D., Plant Pathologist, BPPD

STUDY TYPE:	Product Characterization
MRID NO:	454240-07
CHEMICAL NO:	006484
TEST MATERIAL:	corn grain from MON863
STUDY NO:	99-01-39-32; MSL-17223
SPONSOR:	Monsanto Co., St. Louis, MO
TESTING FACILITY:	Monsanto Co., St. Louis, MO
TITLE OF REPORT:	Amended report for MSL-16597: Immunodetectability of Cry3Bb1.11098 and CryBb1.11231 Proteins in the Grain of Insect-Protected Corn Events MON 863 and Mon 853 After Heat Treatment
AUTHORS:	Gyula Holleschak, Ronald Hileman, James D. Astwood
STUDY COMPLETED:	April 10, 2001
CONCLUSION:	Heating the corn flour samples at 204°C for 30 minutes destroys both the immunoreactivity and insect bioactivity of the Cry3Bb1.11098 found in MON 863 corn. The Cry3Bb1 immunoreactivity was not detectable in both an immunoblot and ELISA format for MON 863. For MON 853, Cry3Bb1 was not recognizable in an immunoblot and reduced more than 1000-fold in an ELISA format. Since the rabbit anti-Cry3Bb1 antibody employed was polyclonal IgG, it is also suggestive that epitopes were destroyed and not just rendered unrecognized by alteration of the three dimensional configuration.
CLASSIFICATION:	ACCEPTABLE.

STUDY DESIGN

This study is designed to examine the integrity of the proteins expressed in corn after exposure to heat at levels similar to that expected during corn grain processing. The intergrity of the protein was defined as recognition by polyclonal antibodies in an ELISA or western blot format.

GLP STATEMENT This study meets the GLP requirements of 40 CFR Part 160.

Test material: Test material is grain from MON 863 and MON 853 corn expressing the Cry3Bb1.11098 and CryBb1.11231 variant protein endotoxins, respectively. These insect control protein genes are also expressed with the kanamycin resistance gene as a selection marker for cell culture.

Control material: Corn grain from line MON 846 and MON 847 represents the untransformed control background for the tests done here. The absence of the relevant introduced genes was

425

confirmed by PCR (MON 846) or by ELISA for the Cry3Bb1 protein (MON 847).

Reference material: The checks for reactivity of the antibodies in this system were bacterially produced proteins isolated from *B. thuringiensis* strains EG 11098 (Cry3Bb1.11098; lot 6312803) or EG 11231 (Cry3Bb1.11231; lot 6312812).

Test methods:
Heating- The corn grain samples were ground to a fine powder then subsamples were subjected to temperatures of 204°C for 30 minutes in a programmable electric furnace.

Extraction of the Cry proteins prior to analysis: The treated samples were separately homogenized with PBS-T and/or 2x Laemmli buffer[1] (1g flour /10ml buffer) using a Omni 2000 homogenizer at high speed for 20 seconds. The homogenate was clarified by centrifugation for about 10 minutes at 14,500 xg with 0.5ml of the supernatant being. Extracts in PBS-T were diluted with an equal volume of 2x Laemmli buffer; those in Laemmli buffer were diluted with a equal volume of milliQ water and stored at -80°C until analysis.

SDS-PAGE and immunoblots: Samples were heated to 100°C for 3 minutes before loading onto gels. The SDS-PAGE was in a 4-20% polyacrylamide NOVEX minigel. Electrophoresis was at constant 150 V for about 1.5 hours or until the dye front reached the bottom of the gel. The bansd were transferred to a NOVEX 0.45 m nitrocellulose membrane (150mA for 1.5hr). The membranes were blocked for non-specific bnding with 5% (w/v) nonfat dried milk in PBST for 45 min., then incubated with an anti-wild type Cry3Bb1 polyclonal rabbit IgG antibody (lot 783; diluted 1:5000 in 1% (w/v) nonfat dried milk in PBST) for 45 min. Goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:7500 in 1% (w/v) nonfat dried milk in PBST) detected any bound specific rabbit antibody and was visualized by enhanced chemiluminescence and exposed to high performance chemiluminescence film.

ELISA: Treated grain samples were submitted for analysis by ELISA according to SOP BR-ME-0059-04 (not included) so as to determine the extractable levels of Cry3Bb1 protein in the sample.

Insect bioassay: The treated corn flour samples were tested for bioactivity against Colorado potato beetle (CPB, *Leptinotarsa decemlibeata*) by a diet incorporation assay. One and two gram corn samples were diluted with 2ml of water and 8 ml of insect diet. Cry3Bb1 standards were also prepared at a final dose of 10 g/ml in insect diet. The prepared diets were infest with 16 larvae per assay point and scored according to SOP BR-ME-0044-02 (not included). CPB larva were fed on diet at 28°C for one week, then scored for mortality.

RESULTS AND DISCUSSION

---

[1]PBST= 1.54mM KH_2PO_4, 154mM NaCl, 5.11mM Na_2HPO_4, 0.05% (v/v) Tween 20; Laemmli2x= 500mN Tris, 4% (w/v)SDS, 20% 2-mercaptoethanol and 60% (v/v) glycerol)

Immunorecognition assays: The heat treatment of 204°C for 30 minutes baking removed any immunoreactive bands corresponding to the Cry3Bb1 proteins in corn flour on an immunoblot which included positively staining samples of both unbaked corn flour and bacterial Cry3Bb1 standards (figure 1 attached). The positive staining samples had either two or three bands. Two bands (approximately 66kD predominately and a 55kD band) were seen in the bacterial standards and three bands (a 74kD, a predominate 66kD band and a fainter band at approximately 50kD) were seen in the MON 863 samples. The 50kD band was also seen in the control untransformed MON 846 samples so was presumably a plant protein that cross reacts with the rabbit antibody. None of the baked corn flour samples had any immunoreactive positive bands. These results suggest that both the Cry3Bb1.11098 endotoxin and the cross reactive plant protein were destroyed by baking.
The immunoblots for the MON 853 corn samples gave similar results to those described above. However, no presumably cross reactive native corn protein band at approximately 50kD was seen in any MON 853 or control corn samples.

Insect bioassay: While no determination of the LD_{50} values was possible from the results of the insect bioassays on CPB larvae, it is apparent that baking at 204°C for 30 minutes has reduced the insecticidal activity to the background level found in the untransformed corn events (figure 3). This is a further indication that the Cry3Bb1 protein has been rendered inactive by the typical baking step found in corn processing.

Baking also destroyed any immunoreactive proteins in the MON 863 extracts examined by ELISA (stated limit of detection= 1ng/ml). However, the MON853 extracts did have ELISA detecable levels of Cry3Bb1 but apparently this was not quantifiable since the value given was >10ng/ml. The positive MON 863 and MON 853 unbaked corn flour extracts had Cry3Bb1 levels of >2.56 g/ml. Therefore, heating reduced the detectable Cry3Bb1 levels at least 1000x even in MON 853 corn flour. It should be noted that the corn protein giving the immunoreactive band seen in the control corn extracts by immunoblot was not detectable (<1ng/ml) in the ELISA format.

Reg. # 524-528

Volume 2 of 4

Page ___ is not included in this copy.

Pages 426 through 432 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist

Secondary Reviewer: John L. Kough, Ph.D., Biologist

STUDY TYPE:	Product Characterization (885.1100)
MRID NO:	454240-08
TEST MATERIAL:	Cry3Bb1 Protein
PROJECT NO:	Study # 01-01-39-23; MSL # 17140
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Bioinformatics Evaluation of the Cry3Bb1 Protein Produced in Corn Event MON 863 Utilizing Allergen, Toxin and Public Domain Protein Databases
AUTHOR(S):	Ronald E. Hileman, Ph.D., Elena a. Rice, Ph.D., Richard E. Goodman, Ph.D., and James D. Astwood, Ph.D.
STUDY COMPLETED:	April 17, 2001
CONCLUSION:	Several amino acid database comparison tools were employed to compare the amino acid sequence of Cry3Bb to known protein allergens and gliadins. The UPDATE2 database was compiled to allow for comparison of Cry3Bb1.11098 and Cry3Bb1.11231 to these proteins. The level of similarity identified does not indicate significant similarity to any of the proteins or gliadins contained in the database. In addition, no contiguous stretch of 8 identical amino acids was identified in either the FASTA or IDENTITYSEARCH algorithms suggesting a lack of immunological similarity. Based upon this data, it does not appear that Cry3Bb1 shares significant structural, biological or immunological similarity with known protein allergens or gliadins.
CLASSIFICATION:	ACCEPTABLE
GOOD LABORATORY PRACTICE:	As a bioinformatics computer-generated study, this submission is not be subject to GLP standards.

I. STUDY DESIGN

Test Material: Amino acid sequence of MON 863 Cry3Bb1 protein as expressed in corn (Cry3Bb1.11098). When the *cry3BB1.11098* gene was modified for expression in corn, the resulting gene is predicted to encode an additional

two amino acid changes relative to the B.t. Cry3Bb1.11098 protein sequence. Therefore, the Cry3Bb1.11098 produced in corn differs from its B.t.-produced relative by 2 amino acids and by 7 amino acids from wild type Cry3Bb1 protein.

Methods: The purpose of this study was to determine if there is any amino acid similarity between the Cry3Bb1 protein and the proteins known to be toxins, allergens, causes of celiac disease or other pharmacological effects. Structural similarities were determined using FASTA and immunologically relevant similarities were determined using a pair-wise comparison algorithm. A sequence containing 8 or more linearly contiguous amino acid identities are considered relevant and would suggest further examination of relatedness.

1. Sequence Database Preparation:

A preliminary sequence list was compiled using the STRINGEARCH function of GenBank with "allergen" as the keyword. This sequence list was compared to previously compiled allergen and gliadin databases (Hileman and Astwood, 1999a & Metcalfe et al.). Sequences present in the previous list but absent in the new list were added. Duplicate sequences were identified using the FASTA function and these sequences were removed. The database list was finalized by adding additional publically available allergen sequences (available at: **ftp://biobase.dk/resources/pub/who-iuis/allergen.list**) and by performing a search of current literature using Internet PubMed and Entrez systems. In cases where only the N-terminal sequence was available, the sequence was added when it contained 8 or more residues. The DATASET function was used to compile the allergen and gliadin database, called ALLERGEN3 and containing 659 protein sequences.

The toxin sequence database (TOXIN4) was constructed using public domain databases (GenBank and EMBL GenPept version 108, PIR and NRL3D version 56 and SwissProt version 36). The ALLPEPTIDES sequence database was used to represent all known publically available protein sequences.

2. Sequence Databse Searches:

DNA sequence obtained from corn event MON 863 was translated using the standard genetic code. Structural similarity of the deduced Cry3Bb1 protein sequence was compared to the the sequences in ALLPEPTIDES, TOXIN4 AND ALLERGEN3 using the FASTA function of GCG. This function provided the ability to assess the structural similarity. The specifica parameters used included an expectation threshold of 10, a word size (*k-tuple*) of 2, a gap creation penalty of 12 and a gap extension penalty of 2. The BLOSUM50 scoring matrix was used -

derived from a set of aligned, ungapped regions from protein families called BLOCKS database. Sequences from each block were clustered. Two sequences were put into the same cluster whenever the percent of identical residues exceeded the specified level. Therefore, the BLOSUM50 matrix would identify blocks of conserved residues which are at least 50% identical. The extent of similarity was evaluated by visual inspection of the alignment, the calculated percent identity and the *E* score.

In addition to the FASTA comparisons, an 8-mer search was performed as IDENTITYSEARCH was used to identify matches of 8 linearly contiguous amino acid identities. The program compares the query sequence to each protein sequence in the allergen database using a sliding window of 8 amino acids, with a 7 amino acid overlap relative to the preceding window.

II. RESULTS

Table 1 describes the differences between wild-type Cry3Bb1 and those proteins expressed in either corn events MON 853 or MON 864 or bacteria. The corn line under consideration for registration (corn Event 863-Cry3Bb1.11098) contains seven amino acid changes from the wild-type Cry3Bb1. This protein was subsequently used for the safety testing described in this submission.

Table 1. Cry3Bb1 Protein Variants:

Cry3Bb1 Allele	**Amino Acid substitutions relative to wild type Cry3Bb1 protein[a]**						
B.t. EG11098 Cry3Bb1.11098 Protein	-	D165G	H231R	S311L	N313T	E317K	-
B.t. EG11231 Cry3Bb1.11231 Protein	-	-	H231R	S311L	N313T	E317K	-
Corn Event MON863 Cry3Bb1.11098	A2	D166G	H232R	S312L	N314T	E318K	Q349R
Corn Event MON853 Cry3Bb1.11231	A2	-	H232R	S312L	N314T	E318K	-

[a] wild-type CryBb1 protein - Accession No. M89794.

Table 2 shows the resulting "best" similarities from protein sequence comparisons of Cry3Bb1.11098 to protein allergen/gliadin and toxin sequences available in public databases.

435

Table 2. Best Similarities

Database	Appendix	# Hits	Accesion #	Description	E Score	% ID	AA Overlap
ALLERGEN3	2	6	S83343	Cyn d1	3.8	23.6	55
TOXIN4	4	169	Q06117	Cry3Bb1	2.7×10^{-228}	99.1	651
ALLPEPTIDES	5	267	Q06117	Cry3Bb1	5.7×10^{-259}	99.1	651

****

Significant structural similarities to allergens/gliadins were evaluated using the FASTA sequence comparison tool and the proteins identified in this comparison are found in appendix 2. There was no significant sequence similarity identified in this comparison. The highest similarity shown was a 23.6% identity over a 55overlap (Bermuda grass pollen), with no significant regions of continuous identical amino acids (i.e., 8 contiguous amino acid identities). Other similarities in descending order are: midge globulin 35.1% in 37aa; latex allergen 30.9% in 55 aa; mite allergen 25.9% in 58aa; olive pollen 26% in 73aa and *Aspergillus fumigatus* allergen 32.3% in 65aa.

Appendix 3 shows the results of the Cry3Bb1 sequence with the ALLERGENS3 sequence database. As with the allergen/gliadin comparison, there were no significant protein similarities with known protein allergens identified.

Appendix 4 shows the FASTA sequence aliment tool was used to identify potential similarities between Cry3Bb1.11098 and known toxins. This sequence comparison identified similarities between Cry3Bb1.11098 abd Cry3Bb1, but not significant structural similarity to proteins other than *Bacillus thuringiensis* and *Clostridium* insecticidal toxins.

III. DISCUSSION

Several amino acid database comparison tools were employed to compare the amino acid sequences of Cry3Bb1.11098 and Cry3Bb1.11231 to known protein allergens and gliadins. The UPDATE2 database was compiled to allow for comparison of Cry3Bb1 to these proteins. The level of similarity does not indicate significant similarity to any of the proteins or gliadins contained in the database. In addition, no contiguous stretch of 8 identical amino acids was identified in either the FASTA or IDENTITYSEARCH algorithms suggesting a lack of immunological similarity. Based upon this data, it does not appear that Cry3Bb1.11098 nor Cry3Bb1.11231 share significant structural, biological or immunological similarity with known protein allergens or gliadins.

CLASSIFICATION: ACCEPTABLE

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD

Secondary Reviewer: Chris Wozniak, Ph.D., Plant Pathologist, BPPD

STUDY TYPE:	Product Characterization
MRID NO:	455382-01
CHEMICAL NO:	006484
TEST MATERIAL:	Cry3Bb1 Protein
STUDY NO:	01-01-39-30; MSL 17274
SPONSOR:	Monsanto Co., St. Louis, MO
TESTING FACILITY:	Monsanto Co., St. Louis, MO
TITLE OF REPORT:	Characterization and Equivalence of the Cry3Bb1 Protein Produced by *E. coli* Fermentation and Corn Event MON 863
AUTHOR:	Ronald E. Hileman, Gyula Holleschak, Larry A Turner, Richard S. Thoma, Christopher R. Brown, James D. Astwood
STUDY COMPLETED:	July 6, 2001
CONCLUSION:	MALDI-TOF analysis of the microbial and corn Cry3B1.11098(Q349R) proteins yielded an agreement of from 42 to 50 amino acid fragments predicted from the theoretical sequence. The N-terminus of the microbial form lacked the terminal methionine which is commonly cleaved in expressed proteins. The corn form was apparently not only lacking the terminal methionine but the N-terminal alanine residue was acetylated as indicated by a 42 Dalton greater weight. The N-terminal amino acid sequence analyses were flawed in that unequivocal determinations were not possible due to the presence of multiple residues in most cycles. However, by comparison to the expected sequences, several different start sites for N-terminal sequencing could be detected. In the *E. coli* Cry3Bb1, the sequence started at both position 2 and 32. In the corn Cry3Bb1, three different starts were detected at position 19, 25 and 36. The immunoblot analysis gave similar positive band patterns that indicated the Cry3Bb1 protein produced in both corn and *E. coli* had essentially the same electrophoretic mobility and immunoreactivity. The positive bands themselves were sometimes rather broad (74-66kDa) but no series of distinct bands could be discerned from the photographs provided. The molecular weight and purity analyses for the corn and microbial extracts indicate that the microbially produced samples were nearly twofold higher purity in Cry3Bb1 proteins compared to the corn extracts. The purity for Cry3Bb1 was 92.6% and 53.9% for microbial and corn extracts, respectively. Total protein concentrations for the two extracts were determined as 0.58 mg/ml and 0.46 mg/ml for

microbial and corn extracts, respectively, by colormetric assays. The glycosylation analysis for the Cry3Bb1 extracts gave no positive carbohydrate staining regions for either the microbial or corn samples in the expected regions for Cry3Bb1 protein. The results of the bioassays for the two Cry3Bb1extracts against Colorado potato beetle larvae (table 5 attached) indicate that there was a dose/response in all tests and the LC_{50} values were similar and had overlapping 95% confidence intervals.

CLASSIFICATION: ACCEPTABLE.

THIS REVIEW CONTAINS FIFRA CONFIDENTIAL INFORMATION

STUDY DESIGN The intent is to provide information on the similarity of the Cry3Bb1 protein isolated from either the MON 863 corn plant or bacterial fermentation to substitute one for the other in toxicology testing.

GLP STATEMENT The study was GLP compliant except for the lack of GLP equipment records (maintenence and calibration) and SOP for the MALDI-TOF mass spectrometer, densitometer and microplate scanning spectrophotometer.

Test material: Two forms of the Cry3Bb1.11098 (Q349R) protein were used as test material in this study: one isolated from MON863 corn grain (lot 6957088) and the other from *E. coli* fermentation (lot 6962478). The corn version was from grain derived from the corn hybrid RX 670 containing event MON 863 grown under the production plan 00-01-39-16 and 00-01-39-18. The bacterial Cry3Bb1 was made in *E. coli* containing the plasmid pET24d(+)/25097.

Test methods: Seven biochemical analytical methods were employed in this study: matrix assisted laser desorption and ionization-time of flight (MALDI-TOF) mass spectrometry, N-terminal amino acid sequencing, immunoblotting, insect bioassay, SDS-PAGE, glycosylation analysis and amino acid composition analysis. In addition, these procedures were performed on proteins chromatographically purified from each of the production methods; plant and microbe.

Purification of Plant Protein: The Cry3Bb1.11098 (Q349R) protein was isolated from corn kernels of the RX 760 hybrid with event MON863 by a two step procedure. The first step was extraction of the protein from kernels by grinding in liquid nitrogen and de-fatting by hexane extraction at 55°C. This solution was further purified by homogenization (two 30 second bursts at 13,000 rpm with a polytron PT-MR 3000 unit) followed by mixing in extraction buffer (1gm/10ml of 100mM sodium carbonate, 5mM EDTA, 1mM CHAPS with plant protease inhibitors (unspecified except for SIGMA cat number P-9599), pH 10). The insoluble plant proteins were removed by filtration through triple layer Miracloth and centrifugation (38,000 x g, 20 minutes at 4°C). This crude extract was then passed through an immuno-affinity column. The capture antibody (a polyclonal rabbit antibody serum MR877 developed by immunization

with a synthetic peptide of the N-terminal 74 amino acids) was covalently linked to a protein A agarose column packing. The plant extract was loaded in a recirculating manner overnight onto an approximately 5ml column. The bound Cry3Bb1 protein was eluted with 6ml of 100mM triethylamine and subsequently concentrated to about 2 ml with a Millipore 30kDa ultrafree centrifugal device. This plant Cry3Bb1 preparation was further purified by passage through a 4.6 x 100mm Source 15 PHE column equilibrated with 0.5M $(NH_4)_2SO_4$ then eluted with a linear $(NH_4)_2SO_4$ gradient to a final isocratic concentration of 0M $(NH_4)_2SO_4$. The fractions collected corresponding to Cry3Bb1 were concentrated to approximately 150 l using the Millipore centrifugal device then brought up to 10 ml with 100mM sodium carbonate, pH 10.0 with 1mM EDTA and again concentrated with the Millipore device to a final volume of approximately 150 l. Repeating this procedure and pooling the final fractions yielded the final preparation of plant Cry3Bb1 (lot 6957088).

Purification of Microbial Protein: The coding region of the plasmid pZMIR13L for *cry3Bb1.11098(Q349R)* was inserted into the expression vector pET24d(+) (Novagen) and used to transform STBL *Escherichia coli* competent cells (GIBCO Life Sciences). Incorporation of the appropriate DNA sequence was verified by generating nested PCR fragments staggered at approximately 300 bp intervals in both the sense and antisense direction and subsequently sequencing these purified DNA with an automated ABI 3700 capillary sequencer. Once the correct sequence for the *cry3Bb1.11098(Q349R)* gene was verified the expression plasmid (pET24d(+)/25097) was transformed into BL21(DE3) competent *E. coli* cells for large scale fermentation and recovery of the expressed Cry3Bb1 protein after induction with IPTG. The actual large scale fermentation was carried out at the Center for Bioprocessing Research, State College, PA and yeilded an approximately 110L batch (lot MON105). The cells were harvested by a Sharples centrifuge at 2L per min at 16,800 rpm to yeild about 6.04 kg of paste which was frozen and shipped on dry ice to Monsanto. The cell paste was thawed directly into buffer A (50mM sodium carbonate, 1mM EDTA, pH10) at a ratio of 1:1, homogenized and lyzed by two passages through a microfluidizer equipped with a pressure monitor. The cell debris was removed by centrifugation at 15,000 x g for 1 hour at 4°C and resulted in approximately 3L of supernatant. The protein fraction was enriched by ammonium sulfate fractionation at 20% saturation and centrifugation followed by 60% saturation and centrifugation. This cell pellet was suspended and dialyzed overnight in buffer A. The preparation was passed over a Millipore Vantage A2 Biochromatography column VA 130x500 packed with a bed volume of approximately 5.5L of Q-Sepharose Fast Flow. The column was loaded with approximately 2.5L of the ammonium sulfate cut until the effluent had an $absorbance_{280nm}$ reading of 0.1 then a linear gradient of 0 to 0.5M NaCl in buffer A was used to elute the protein. The fractions were monitored by SDS-PAGE to locate those most enriched in the 74kDa Cry3Bb1 protein (tubes #52-58, approximately 2.75 L). The protein in these fractions were then precipitated with ammonium sulfate, centirfuged at 8000rpm in a Beckman JLA 8.1000 rotor, resuspended in 10mM phosphate buffer, pH 7.0 and dialyzed overnight against the same buffer with a 14k MWCO cellulose membrane. This solution ws concentrated by covering the entire membrane with Aquacide I (carboxymethylcellulose, MW 70kDa). The final result was approximately 150ml of microbial Cry3Bb1.11098(Q349R) stored at 4°C in a polypropylene tub until needed.

439

The report refers to the lot number as 6962478 although this is not given in the description of the purification procedures.

MALDI-TOF: Mass spectral analyses employed a Perspective Voyager DE-Pro MALDI-TOF instrument with Data Explorer software. The instrument was calibrated using a tryptic digest of porcine somatotropin (lot 70003 PX). The samples of corn and microbial Cry3Bb1 (20 g total protein) were cut from SDS-PAGE gels at the 74kDa region, destained, reduced with dithiothreitol, alkylated with iodoacetic acid, dried, rehydrated in ammonium bicarbonate and subject to trypsin digest (33 l/ml) while still in the gel. The resulting peptides were extracted from the gel fragments three times with 50 l of 70% (v/v) acetonitrile with 0.1% trifluoroacetic acid (TFA), combined in a single tube and dried by a Speed Vac concentrator to 10 l. These samples were desalted with Millipore ZipTip$_{C18}$ pipette tips which had been pre-treated with a methanol wash and equilibrated with 0.1% TFA. Five l of each sample was washed with 5 l of a series of solutions with increasing concentrations of acetonitrile with 0.1% TFA (v/v). Then 0.3 l of the desalted solution was co-crystallized with 0.75 l of -cyano-4-hydroxycinnamic acid prior to analysis in the 500 to 5000 Dalton range in the reflector mode using 100 laser shots per spectrum. Protonated peptide masses were seen monoisotopically in the reflector mode. Computer software was used to generate theoretical trypsin digest fragments of the expected protein sequence along with calculations of the theoretical masses of these expected tryptic digest peptides. Peak acceptance was based on the presence of at least three resolvable peaks in the spectra with a height of at least twice the baseline values or when a mass could not be assigned due to overlap with a stronger signal $\pm$ 2 Daltons from the mass analyzed.

N-Terminal Amino Acid Sequence Analysis: Samples of both corn and microbial Cry3Bb1 protein (10 g total protein) were diluted with 2X Laemmli buffer, heated to 100°C for three minutes, loaded onto a 4 20% polyacrylamide gel, and run at 150V for approximately 1.3 hours under reducing conditions (SOP PB-EQP-005-01). The separated proteins were electro-transferred to a PVDF membrane for 1.1 hours at a constant 200mA. The bands were visualized by brief Ponceau S reagent staining. The bands in the 74kDa region expected for the Cry3Bb1 protein were excised as well as a band in the 22Kda region. N-terminal analysis of these bands were done by 15 cycles of an automated Edman degradation (Applied Biosystems 494 Procise Sequencing System with a 140C microgradient using SOP BR-EQ-0265-01). Calibration of the PTH-amino acid standards was done to assist in reading the output of chromatography. A - lactoglobulin standard was also included for 15 cycles to insure the degradtion reactions were proceeding as assumed.

Immunoblot Analysis: Samples of corn and microbial Cry3Bb1 proteins were subjected to SDS-PAGE (SOP PB-EQP-005-01) then electrotransferred onto 0.45 m nitrocellulose membranes for from 1 to 1.5 hours at 200mA. The membranes were then blocked with 10% NFDM in PBST for 45 minutes prior to reacting with either a 1:500 dilution of polyclonal rabbit antibody (lot 6199830B) for 45 minutes or a 1:300 dilution of mouse monoclonal IgG (lot 6199915) overnight at 4°C. Any bound IgG was detected by either 1:7500 dilution of goat-anti rabbit HRP conjugate or a 1:5000 dilution of goat anti-mouse IgG HRP conjugate and Neutravidin HRP (for the

440

biotinylated molecular weight markers), respectively. Any immunoreactive bands were visualized by enhanced chemiluminescence and exposed to Hyperfilm ECL high performance chemiluminescence film.

Molecular Weight and Purity Analysis: Samples of microbial and corn Cry3Bb1 proteins were prepared in 2X Laemmli buffer to a final concentration of 0.25 g/ l. Molecular weight markers were included and the Cry3Bb1 samples were loaded at 1, 2 and 3 g of protein per lane. Electrophoresis was performed according to SOP PB-EQP-005-01, fixed in 40% (v/v) methanol with 7% (v/v) acetic acid for 30 minutes stained with collodial brilliant blue G for one hour then destained with 25% (v/v) methanol. The gel was then examined by scanning densitometry with a Bio-Rad GS-710 densitometer.

Glycosylation Analysis: Corn and microbial Cry3Bb1.11098 proteins were diluted in 2x Laemmli buffer, loaded onto a 4 20% polyacrylamide gel with a transferrin glycoprotein positive control sample (20 g/ l) and prestained molecular weight markers included and subjected to SDS-PAGE (SOP PB-EQP-005-01). The proteins were elctrotransferred onto a 0.45 m PVDF membrane where the carbohydrate detection was performed. The PVDF membrane was first shaken in PBS then transferred to an oxidizing solution containing 100mM sodium acetate with 10mM sodium metaperiodate, pH 5.5 and incubated for 20 minutes in the dark. The oxidizing solution was removed and the membrane was rinsed in PBS three times before being transferred to a 100mM sodium acetate buffer with 25nM biotin hydrazide, pH 5.5 and incubated for 40 minutes. The membrane was then blocked with 5% NFDM (w/v) in PBS prior to being reacted with streptavidin-HRP conjugate to detect carbohydrate moieties that would bind the conjugate through the biotin/streptavidin reaction. The positive carbohydrate reaction was monitored by chemiluminescence and exposure to Hyperfilm ECL high performance chemiluminescence film.

Protein Assay: Protein concentrations were determined by a Bio-Rad colorimetric dye-binding assay based on the Bradford method (SOP GEN-PRO-015-00). The concentrations were estimated by absorbance values comparison to a standard curve.

Amino Acid Composition: Aliquots of both microbial and corn Cry3Bb1.11098 protein were assessed for amino acid composition on a Hitachi L-8800 Amino Acid Analyzer. Included in the tests were a NIST BSA sample and each Cry3Bb1 sample was spiked with a NIST amino acid calibration control internal standard. Vapor phase acid hydrolysis (6N Hydrochloric acid with 1% (v/v) phenol) was performed at 150°C for approximately 90 minutes. Samples were cooled, evaporated, reconstituted with hydrozylate buffer, loaded onto the analyzer and the individual amino acids were detected by post-column ninhydrin derivatization.

Storage Stability: SDS-PAGE analysis was compared at the initiation and completion of each phase of this study to determine if significant degradation had occurred over the study. The extent of degradation over the study was done by comparison with samples held for 29 days at specific storage temperatures (4,-20 and -80°C). Samples were run on the same SDS-PAGE gel

and compared by scanning densitometry.

Insect Bioassay: Aliquots of both corn and microbial Cry3Bb1.11098 protein were given to the Monsanto Entomology Laboratory of the Ecological Safety Technology Center. Bioassays were performed on Colorado potato beetle (*Leptinotarsa decemlineata*) according to SOP BR-ME-0044-02. Test substance was incorporated into a defined diet at several concentrations (approximately 5.73, 2.86, 1.43, 0.72,0.36 and 0.18 g/ml for microbial and 9.62, 4.81, 2.40, 1.20, 0.60 and 0.30 g/ml for corn Cry3Bb1) and fed to 16 larvae per dose. Larvae were scored for survival on day 7 and the data analyzed by SAS system software for LC_{50} values with corresponding 95% confidence intervals using the SAS probit procedure. The bioactivity between the two preparations was compared by overlapping confidence intervals.

RESULTS AND DISCUSSION

MALDI-TOF analysis of the microbial and corn Cry3B1.11098(Q349R) proteins yielded an agreement of from 42 to 50 predicted amino acid fragments. MALDI-TOF results, while an indirect means for establishing identity between two proteins, can identify a protein if 10 to 15 fragments can be discerned. Some differences were seen between the two Cry3Bb1 preparations and the theoretical sequence (table 1). The N-terminus of the microbial form lacked the terminal methionine which is commonly cleaved in expressed proteins. The corn form was apparently not only lacking the terminal methionine but the N-terminal alanine residue was acetylated as indicated by a 42 Dalton greater weight. Other molecular weight differences were accounted for by the oxidation reduction reaction associated with the MALDI-TOF procedure such as the presence of carboxymethyl cysteine which increased the weight by 58 Daltons or the oxidation of methionine and tryptophan to yield an increase of 16 or 32 Daltons, respectively. The matches and overlap of the MALDI-TOF results are given in figure 2 attached to this document.

The N-terminal amino acid sequence analysis intended to confirm the sequence of the first 15 amino acids in the two Cry3Bb1 forms. The determinations were flawed in that unequivocal determinations were not possible due to the presence of multiple residues in most cycles. However, by comparison to the expected sequences several different start sites for N-terminal sequencing could be detected. In the *E. coli* Cry3Bb1, the sequence started at both position 2 and 32. In the corn Cry3Bb1, three different starts were detected at position 19, 25 and 36 (figure 3). Since all these forms were found at the 75kDa band it is probable that these forms resulted from either protease activity during purification or possibly during expression in plant or *E. coli*. The lack of the N-terminus in the corn Cry3Bb1 can be explained by the recalcitrance of the acetylated alanine to the Edman degradation chemistry.

The immunoblot analysis gave similar positive band patterns that indicated the Cry3Bb1 protein produced in both corn and *E. coli* had essentially the same electrophoretic mobility (figure 4 attached). The electroblotted membrane also demonstrated immunorecognition by both a polyclonal rabbit anti-Cry3Bb1 antiserum and a mouse monoclonal anti-Cry3Bb1.11231 antibody. The positive bands themselves were sometimes rather broad but no series of distinct bands could be discerned from the quality of the photographs provided. The authors did,

however, indicate that a region of positive bands from about 74 to 66kDa was seen in the membrane. The rabbit polyclonal treated membrane also had a positively staining band in the 220kDa region of the MON863 corn samples. This 220kDa band was also excised for N-terminal AA sequencing.

The molecular weight and purity analyses for the corn and microbial extracts indicate that the microbially produced samples were of higher purity in Cry3Bb1 proteins, nearly twofold, compared to the corn extracts (table2 attached). By comparison with the immunoblots, it was determined that the bands from 74 to 66kDa represented the Cry3Bb1 protein. There was an apparent discrepancy between the apparent molecular weight in the microbial (72kDa) versus the corn (74.6kDa) form of the Cry3Bb1 protein. The authors attribute this to an artifact of the gel molecular weight markers and calculation phenomenon. The range of molecular weight values from 66 to 74 kDa agrees with the results seen in the MALDI-TOF analysis where N-terminal truncation was evident along with some other modifications such as acetylation and removal of the terminal methionine. The purity of the two preparations for Cry3Bb1 were 92.6% and 53.9% for microbial and corn, respectively. Total protein concentrations for the two extracts were determined as 0.58 mg/ml and 0.46 mg/ml for microbial and corn extracts, respectively, by colormetric assays.

The amino acid compositional analysis gave good correlation for the theoretical mole percentages and those of the *E. coli* produced Cry3Bb1 but not as well for the corn extract. This was attributed to the lower level of Cry3Bb1 enrichment in the corn extracts.

The glycosylation analysis for the Cry3Bb1 extracts gave no positive carbohydrate staining regions for either the microbial or corn samples in the expected regions for Cry3Bb1 protein (figure 6 attached). The corn extracts did have positive staining smears with molecular weights well above 100kDa and below 66kDa. There was also a positive carbohydrate staining band slightly above the expected Cry3Bb1 region. A typical SDS-PAGE gel was included for comparison next to the carbohydrate stained one and it confirmed that the region where the major Cry3Bb1 bands were found in the area distinctly showing negative results for carbohydrate staining.

The SDS-PAGE gel data on storage stability presented Coomassie Blue stained bands with samples of corn and microbial Cry3Bb1 protein stored at 4°, -20° and -80°C for 29 days. The authors claim that the decreased band intensity at 4°C (figure 7 compared to the initial values found in figure 5) indicates that the Cry3Bb1 protein degrades at this temperature. The molecular weight of the Cry3Bb1 protein stored at 4°C shifts to the 66kDa form as opposed to the 74kDa form which predominates in the -20° and -80°C samples. There appears to no significant decrease in the quantity and quality of the Cry3Bb1 stored at -20°or -80°C.

The results of the bioassays for the two Cry3Bb1extracts against Colorado potato beetle larvae (table 5 attached) indicate that there was a dose/response in all tests and the LC_{50} values were similar and had overlapping 95% confidence intervals. There was a total of 112 insects tested in

each replicate and each Cry3Bb1 type was tested in two replicate. The pooled LC_{50} value for *E. coli* produced Cry3Bb1 was 0.76 g/ml (95%CI= 0.57-092) and the Cry3Bb1 from corn's LC_{50} was 0.63 g/ml (95%CI=0.48-0.77). The concentration values used in these bioassays was corrected for the apparent Cry3Bb1 values based on the results of the purity determinations found in table 2. The overlapping 95% confidence intervals indicate that the two preparations have very similar bioactivities.

THE ATTACHED APPENDICES CONTAIN FIFRA CONFIDENTIAL INFORMATION

Reg. # 524-528

Volume 2 of 4

Page ___ is not included in this copy.

Pages 1-15 through 456 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist

Secondary Reviewer: John L. Kough, Ph.D., Biologist

STUDY TYPE:	Acute Oral Toxicity (885.3050)
MRID NO:	455382-02
TEST MATERIAL:	Cry3Bb1 Protein (Cry3Bb1.11098)(Q349R)
PROJECT NO:	SLI Study No. 3044.856; Monsanto Study No. SB-2001-085, MSL 17382
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Springborn Laboratories, Inc. (SLI) Ohio Research Center, Spencerville, OH 45887
TITLE OF REPORT:	An Acute Oral Toxicity Study in Mice with *E. coli*-Produced Cry3Bb1.11098 (Q349R) Protein - Amended Final Report
AUTHOR(S):	Kimberly L. Bonnette, M.S., LATG (SLI); Paul D. Pyla, B.S. (Monsanto)
STUDY COMPLETED:	July 9, 2001
CONCLUSION:	There did not appear to be significant adverse affects to animals resulting from exposure to Cry3Bb1.11098 at dose amounts of 300, 900 & 2700 mg/kg body weight. Observations included some minor clinical affects and a relatively insignificant lack of weight gain in two animals, however, these do not appear to be related to exposure to the test substance, because these occurred in the various test groups. Based upon the data contained in this submission, the LD_{50} for Cry3Bb1.11098 is greater than 3200 mg/kg body weight in mice.
CLASSIFICATION:	ACCEPTABLE. TOXICITY CATEGORY III
GOOD LABORATORY PRACTICE:	This study was conducted in accordance with GLP guidelines.

Note: This study supercedes MRID# 449043-06

I. STUDY DESIGN

Test Material: *E. coli*-produced Cry3Bb1.11098 (Q349R) @ 300 mg/kg (Lot No. 6962478); *E. coli*-produced Cry3Bb1.11098 (Q349R) @ 900 mg/kg (Lot No. 6962478); *E. coli*-produced Cry3Bb1.11098 @ 2700 mg/kg (Lot No. 6962478)

Protein Control - BSA (Lot# B38089)

Vehicle Control (Lot# 6839197A)

Test Animals: One hundred (50 male and 50 female) Crl:CD1®(ICR)BR(VAF/Plus®) mice obtained from Charles River Laboratories, Portage, MI and arriving April 10, 2001 were used after a five day acclimation. The animals were identified by metal ear tags and housed individually in stainless steel cages. All animals received PMI certified rodent meal #5002 and water *ad libitum* except prior to dosing.

Methods:

1. Three groups of ten male and ten female (nulliparous, non-pregnant) mice seven weeks old were dosed orally with target doses of: 300, 900, & 2700 mg/kg body weight of Cry3Bb1.11098 protein at a dosing volume of approximately 33.3 ml/kg in two equal separate doses, administered 4 hours apart (i.e., 2 doses of 33.3 ml/kg each). The males weighed between 25.6 and 32.1 gm and the females weighed between 22.4 and 26.1 gm on the day of dosing
2. Similarly, ten male and ten female protein control animals were dosed with BSA @ 2700 mg/kg body weight at the same dosing volume and method (i.e., 2 doses) as described above.
3. Ten male and ten female vehicle control mice were administered vehicle control at the same dosing volume (one dose). All animals were provided food and water ad libitum after dosing.
4. Checks for mortality and moribundity were recorded twice daily, except on day of necropsy. Clinical observations for toxicity were recorded after dosing and daily thereafter.
5. Body weights were recorded prior to the start of the study and on days 7 and 14. Food consumption was measured on days 0, 7 and 14.
6. Following the 14 day observation period, each animal was euthanized with excess CO_2 and subjected to examination by gross necropsy.

II. RESULTS

1. Mortality - There was no mortality through the 14 day study.
2. Clinical Observations - One animal (A1072/M) dosed with 300 mg/kg test substance had a raised area on its abdomen that progressed to a scab beginning on Day 4, clearing on Day 13. No other significant clinical observations were made during the study.
3. Body Weight - All male animals except one displayed an overall weight gain during the study. All except three female animals displayed overall weight gain. Animal #A1166F (2700 mg/kg BSA) did not display any weight gain, animal # A1125F (300mg/kg test substance) and animal #A1079M (2700mg/kg test substance) lost 0.1 g at the last weighing and animal #A1148F (900 mg/kg test

substance) lost 0.4 g over the course of the study. None of these observations appear to be significant, nor does this specifically relate to the test substance since one animal was in the BSA control group and there was no dose response evident.

4 Gross Necropsy: Examination of the animals by gross necropsy revealed some minor abnormalities. Two animals (A1144F and A1150F) in the protein control group displayed reddened lungs. Periovarian cysts were also observed in ten animals total, with observations in each of the test groups (5 animals in the BSA control and one or two in each of the other groups). Therefore, this abnormality do not appear to be related to exposure to the test substance.

III. DISCUSSION

There did not appear to be significant adverse affects to animals resulting from exposure to Cry3Bb1 at dose amounts of 300, 900 & 2700 mg/kg body weight. The minor clinical observations and the relatively insignificant lack of weight gain in two animals do not appear to be related to exposure to the test substance because these occurred in the various test groups. Based upon the data contained in this submission, the LD_{50} for Cry3Bb1.11098 is greater than 3200 mg/kg body weight in mice.

CLASSIFICATION: ACCEPTABLE, TOXICITY CATEGORY III

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist

Secondary Reviewer: John L. Kough, Ph.D., Biologist

STUDY TYPE:	*In vitro* Digestibility
MRID NO:	455382-03
TEST MATERIAL:	Cry3Bb1.11098 and Cry3Bb1.11231 Proteins
PROJECT NO:	MSL Number: 17291; Study Number: 01-01-39-13
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Assessment of the *in vitro* digestibility of Cry3Bb1 Protein purified from corn event MON863 and Cry3Bb1 Protein purified from *E. coli*
AUTHOR(S):	John N. Leach, Ronald E. Hileman, Ph.D., and James D. Astwood, Ph.D.
STUDY COMPLETED:	July 13, 2001
CONCLUSION:	The tests performed in this study show that the Cry3Bb1 proteins are degraded in simulated gastric fluid. Incubation of corn-produced and *E. coli*-produced Cry3Bb1 protein in SGF results in the loss of detectable protein by the 15 second observation point, as detected by SDS-PAGE.
CLASSIFICATION:	ACCEPTABLE. Cry3Bb1 protein does not appear to be stable to digestion when exposed to simulated gastric fluid
GOOD LABORATORY PRACTICE:	This study was conducted in accordance with Good Laboratory Practice guidelines with four exceptions as described in the submission. These exceptions should not have a significant impact on the outcome of the study.

I. STUDY DESIGN

Test Material:

1. The test substances were: 1) corn grain containing MON 863 (LOT# 6957088) at a concentration of 0.46 mg/ml total protein and a purity of 53.9%. 2) *E. coli* produced Cry3Bb1 (lot# 6952478) at a concentration of 0.65 mg/ml with a purity of 97.5%.
2. There were no control or reference substances used.
3. Hemoglobin as used as reference protein and molecular weight standards were used for SDS-PAGE.

4/61

Methods:

Test Treatments:

1. Simulated gastric fluids were prepared and confirmed to be functionally active on the day of use. SGF was measured in accordance with SOP BR-ME-0460-01 (not submitted) which relates to the amount of pepsin activity to the rate of hemoglobin digestion. The assay determines the amount of pepsin activity by change in the solution absorbance. Digestion is directly related to increased absorbance due to the amount of amino acids/small fragments present in TCA protein precipitated solutions.
2. Timed incubations began with the addition of Cry3Bb protein to individual incubation tubes in a shaking 37° C waterbath. [zero incubation time trials were quenched prior to addition of the test substance].
3. Test treatments were prepared by addition of corn-produced or *E. coli*-produced Cry3Bb1 protein to SGF with incubation times of 0, 15, 30 seconds and 1, 2, 4, 8, 15, 30 and 60 minutes. SGF digestions were quenched by addition of 0.53M sodium carbonate, dilution with 5X Laemmli buffer, then heating to 100°C for 5 minutes.
4. Reference treatments were prepared by adding either corn-produced or *E. coli* produced Cry3Bb1 protein to tubes containing SGF w/o pepsin for incubation times of 0 and 60 minutes.
5. Proteins were separated and visualized on 10→20% tricine gels (SDS-PAGE) and staining with colloidal blue dye staining solution (SOP BtC-PRO-026-01).

II. RESULTS

Effectiveness of the Test Systems:

The proteolytic activity of SGF prepared for corn-produced protein was 18,883 U/ml (where 1U = $\Delta 0.001\ A_{280\ nm}\ min^{-1}$ at 37 °C) and 18, 317 for *E. coli*-produced protein, which, according to the registrant, was within the requirements of SOP Br-ME-0460-01 (not submitted).

Digestibility of Cry3Bb1 Proteins in SGF:

Figures 1 shows that Cry3Bb1 protein in corn grain was degraded to a "small transient peptide fragment" (approx. 3 kDa) by the 15 second timepoint. A transient fragment was claimed to be present for 15 minutes according to the report. However, the gel figure in the report makes it difficult to verify this finding (Figure 2). *E. coli* produced protein was also degraded to a claimed "transient fragment" within 15 seconds in SGF (Figure 4) and degraded below the limit of detection after 2 minutes (figure 5). Again, the attached figures make it difficult to verify the result. There does appear to be a cloudy area in the figures at the indicated area which is

462

not found after the 1 minute sample time.

Limit of Detection:
Figures 3 & 6 show the apparent level of detection in this study. For the corn-produced protein, as little as 17 ng of undegraded protein is visible and for the *E. coli* produced protein, as little as 10 ng of protein is detectable.

III. DISCUSSION

Protein stability in digestive fluids is one characteristic for food allergenic proteins which has been described. Although all proteins which have been identified as food allergens are stable to digestion, not all proteins which are stable to digestion are known to be food allergens. The tests performed in this study show that the Cry3Bb1 proteins are not stable to digestion in simulated gastric fluid. Incubation of corn-produced and *E. coli*-produced Cry3Bb1 protein in SGF results in the loss of detectable protein by the 15 second observation point, as detected by SDS-PAGE.

CLASSIFICATION: ACCEPTABLE. The Cry3Bb1 proteins do not appear to be stable when incubated in SGF.

Reg. # 524-528

Volume 2 of 4

Page ___ is not included in this copy.

Pages 469 through 469 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD

Secondary Reviewer: Chris Wozniak, Ph.D, Plant Pathologist, BPPD

STUDY TYPE:	Product Characterization
MRID NO:	455382-09
CHEMICAL NO:	006484
TEST MATERIAL:	NPTII protein in MON 863 corn
STUDY NO:	01-01-39-36; MSL-17300
SPONSOR:	Monsanto Co., St. Louis, MO
TESTING FACILITY:	Monsanto Co., St. Louis, MO
TITLE OF REPORT:	Immuno-Detectability of NPTII Protein in the Grain of Insect Protected Corn Event MON 863 After Heat Treatment
AUTHOR:	Gyula Holleschak, Ronald E. Hileman, James D. Astwood
STUDY COMPLETED:	July 20, 2001
CONCLUSION:	The immunoblot shows that extraction of the MON863 corn grain spiked with NPTII yielded an immunoreactive band that comigrated with the *E. coli* produced NPTII. The blot also showed that, regardless of the extraction buffer used, the heat treatment effectively removed any immunoreactive bands from the samples. The results suggest that, even if detectable levels of NPTII were present in MON863 corn grain, the heat treatment would remove them. Unfortunately, the use of a mouse monoclonal antibody limits the ability of this data to be extrapolated. A heat treatment significantly above the 95.8°C used for sample preparation for SDS-PAGE destroyed the epitope(s) recognized by the anti-NPTII antibody used.
CLASSIFICATION:	ACCEPTABLE.

STUDY DESIGN This study is intended to provide data to support the effects of heat treatment on the immunorecognition of the NPTII protein with implications about another aspect of its potential allergenicity.

GLP STATEMENT This study was performed in compliance with the requirements of 40 CFR Part 160.

Test material: Grain from corn hybrid event MON 863 (MON 863 grain; LIMS 00ZMGRO002934) and the control corn hybrid RX 670 (control grain; LIMS 00ZMGRO002937). Both samples of corn were obtained from plants grown in the field under production plan 00-01-39-18 The presence or absence of the NPTII protein was confirmed by PCR reaction for the presence of the *cry3Bb1* gene. NPTII protein (lot 6340505) with a

molecular weight of approximately 29kDa, produced in and purified from *E. coli* was also included in the study as a reference substance. The NPTII protein was purified to greater than 90% purity by chromatography and stored in 100mM sodium carbonate buffer at -80°C until needed.

Test methods

Heat Treatment: To simulate the type of heating typically used in the industrial preparation of corn flour for use in corn flakes or salty snacks, a temperature of 204°C for 30 minutes was selected. This is near the temperature used for the deep-fat frying (188-210°C) and lower than that used for preparation of corn flour for flaking (288-302°C), however, it is probably longer than typical cooking time.

Extraction of NPTII from **MON863 corn**: After the described heat treatment, the MON863 corn samples were extracted by homogenization with either PBST or Laemmli buffer. The 1g tissue to 10 ml buffer ratio was employed and the samples were held at a setting of 5.5 on the hand held Ultr-Turrax T8 homogenizer for approximately 10 seconds. The homogenates were clarified by an unspecified centrifugation for 20 minutes with removal of the supernatant. Extracts done in PBST were diluted with 5X Laemmli buffer and all extracts were stored at -80°C until analysis.

SDS-PAGE and Immunoblots: Samples were heated to 95.8°C for 3 minutes prior to loading on a 4 20% polyacrylamide gradient NOVEX minigel following SOP PB-EQP-005-01. The gels were run at 170V for 1.5hours and electrophoretically transferred to a 0.45 m nitrocellulose. The membranes were blocked for non-specific binding with 10% (w/v) non-fat dried milk (NFDM) in PBST overnight at 4°C. The membranes were then reacted for 60 minutes at RT with a 1:500 dilution of anti-NPTII IgG monoclonal antibody (lot 5372482) in PBST with 5% (w/v) NFDM. The bound mouse antibody was detected by probing with a 1:5000 dilution of goat anti-mouse horseradish peroxidase conjugate. Immunoreactive bands were visualized by the Amersham Pharmacia enhanced chemiluminescence detection system and exposed to high performance chemiluminescence film.

RESULTS AND DISCUSSION

An unanticipated result of the initial trials was that the NPTII protein was undetectable in the MON863 corn grain matrix. This was also confirmed in another immunoassay format where ELISA showed no detectable NPTII (<0.076 ppm). Therefore, the rest of the results shown were done with MON863 corn spiked with NPTII protein produced in *E. coli* prior to any heat treatment.

An immunoblot of the results is attached as figure 1. The blot shows that extraction of the MON863 corn grain spiked with NPTII yielded an immunoreactive band that comigrated with the *E. coli* produced NPTII. The corn samples were apparently spiked with *E. coli* produced NPTII at a level that yielded approximately 50ng per lane. The level of detection for the immunoblot assay format was suggested by the fact that the NPTII alone sample loaded with

0.5ng gave a barely discernable band. The blot also showed that, regardless of the extraction buffer used, the heat treatment effectively removed any immunoreactive bands from the samples.

The results suggest that, even if detectable levels of NPTII were present in MON863 corn grain, the heat treatment would remove them. Unfortunately, the use of a mouse monoclonal antibody limits the ability of this data to be extrapolated. A heat treatment significantly above the 95.8°C used for sample preparation for SDS-PAGE destroyed the epitope(s) recognized by the anti-NPTII antibody used.

Reg. # 524-528
Volume 2 of 4

Page 473 is not included in this copy.

Pages ___ through ___ are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD

Secondary Reviewer: Chris Wozniak, Ph.D., Plant Pathologist, BPPD

STUDY TYPE:	*In vitro* digestibility in simulated intestinal fluid
MRID NO:	455770-02
CHEMICAL NO:	006484
TEST MATERIAL:	Cry3Bb1.11098 (Q349R) protein
STUDY NO:	01-01-62-11; MSL 17530
SPONSOR:	Monsanto Co., St. Louis, MO
TESTING FACILITY:	Monsanto Co., St. Louis, MO
TITLE OF REPORT:	Assessment of the *in vitro* digestibility of the Cry3Bb1.11098(Q349R) protein in simulated intestinal fluid
AUTHOR:	Ronald E. Hileman, John N. Leach & James Astwood
STUDY COMPLETED:	December 14, 2001
CONCLUSION:	Simulated intestinal fluid activity was verified to be present and at a level deemed acceptable by SOP GEN-PRO-058-01. The gels provided indicate that the Cry3Bb1.11098 (Q349R) protein is present as a single band at 74 kDa which rapidly degraded to two bands of 68 and 57kDa at the first assay time point of 1 minute. The subsequent samples (from 5 minute to 24 hours) all gave a single 57 kDa band which did not appear to decrease in intensity. This lack of degration by intestinal fluids is similar to the majority of Cry proteins which are resistant to the action of trypsin.
CLASSIFICATION:	ACCEPTABLE.

STUDY DESIGN

To determine the stability of the Cry3Bb1.1098 variant protein to trypsin degradation in simulated intestinal fluids.

GLP STATEMENT: The study was claimed as GLP according to 40 CFR Part 160 except for an SOP for the densitometer. Equipment maintenance and calibration for the densitometer was done and is archived with the study and a draft SOP was followed. The spectrophotometer equipment records do not include an SOP or maintenance and calibration records.

Test material: Cry3Bb1.11098 (Q349R) protein was produced by fermentation in *E. coli* (lot 6962478). The *E. coli* contained a plasmid pET24d(+)/25097 which coded for the same variant of the Cry3Bb1 protein found in MON 863 corn. There were no reference or control substances in this study. However, the test system did include experimental controls showing stability of the Cry3Bb1 variant in buffer lacking the pancreatin and an internal standards for molecular weight in the SDS-PAGE and acceptance of a level of pancreatin activity against hemoglobin prior to use of the enzyme solution.

474

Test methods

Enzyme Reagents: The simulated intestinal fluid (SIF) was prepared according to SOP GEN-PRO-058-01 (not included) and was based on the method published in the US Pharmacopeia (1995). The activity of the SIF was determined both before and after the test was performed. SIF proteolytic activity was determined by trichloroacetic acid (TCA) soluble resorufin labeled peptides released from resorufin labeled casein. After 15 min. SIF incubation the reaction was quenched with TCA, centrifuged to remove insoluble material and the absorbance (574 nm) of the resulting supernatant relates directly to proteolytic activity.

SIF Reaction with Cry3Bb1.11098: Solutions of SIF and Cry3Bb1.11098 were incubated at 37°C in a shaking water bath. The timed incubations were started by addition of the test substance to individual tubes with the SIF test system. The scheduled incubation times were T0 (0 min), T1 (1min), T2 (5min); T3 (15 min), T4 (30 min), T5 (1 hr), T6 (2 hr), T7 (4hr), T8 (8 hr), T9 (16 hr) and T10 (24 hr). The SIF reaction was quenched by the addition of Laemmli sample buffer[1] and heating to 100°C for approximately 5 minutes. The zero time point was quenched and similarly heated prior to addition of the test substance. Control samples (at 0 min and 24 hrs) were included to ascertain the stability of the test substance in SIF buffer without added enzymes. An additional control was for spontaneous background due to the SIF itself with added phosphate buffer (10mM phosphate, pH 7.0) to replace the test substance volume. Both these controls were quenched with Laemmli buffer and heated like the test samples.

SDS-PAGE and immunoblots: The enzymatically digested samples were loaded into the gel lanes at 2.0 ng/µl calculated from the pretreated protein concentration values. The total amount loaded was 10ng total protein per lane. Pre-stained molecular weight markers (Amersham Life Sciences) were included to provide visual landmarks on the gels and provide an acceptance criterion. The NOVEX® 10-20% polyacrylamide gradients tricine buffered precast minigels (Invitrogen, Carlsbad, CA) were run at 125V for approximately 100 minutes (dye front near the bottom of the gel) according to the protocol BtC-PRO-026-01 (not provided).

The minigels were electroblotted onto NOVEX 0.45 µm pore size nitrocellulose membranes (Invitrogen, Carlsbad, CA) in transfer buffer[2] at 25V for 90 minutes in a NOVEX Xcell II bolt module. The transferred blot membranes were blocked with PBST (1mM KH_2PO_4, 100mM Na_2HPO_4, 137mM NaCl, 2.7mM KCl, pH 7.4; although indicated, no Tween 20 was mentioned in the formulation) with added 5% (w/v) nonfat dried milk (NFDM) to block the membranes for nonspecific binding of antibodies. The membranes were then incubated with rabbit polyclonal anti-Cry3Bb1 antibody (lot 6199830B) diluted 1:5000 in PBST with 1% NFDM for 1 hour. Rabbit polyclonal anti-Cry3Bb1 antibody not specifically bound to the target proteins were removed by repeated washing with PBST. Bound specific antibody was detected by one hour

---

[1]Laemmli sample buffer=62.5mM Tris-HCl, pH6.8, 5% (v/v) β-mercaptoethanol, 2%(w/v) SDS, 10% (v/v) glycerol and 0.005% (w/v) bromophenol blue.

[2]Transfer buffer=24mM Tris, 192 mM glycine, 20% (v/v) methanol.

incubation with goat anti-rabbit IgG horseradish peroxidase conjugate diluted 1:7500 in PBST with 1%(w/v) NFDM. Non bound conjugate was removed from the membrane by repeated washes with PBST. The immunoreactive bands were visualized with the enhanced chemiluminescence detection systems (ECL Amersham Pharmacia) and exposed to Hyperfilm ECL high performance chemiluminescence film.

Specific antibody: The rabbit polyclonal antibody used for specific detection of the Cry3Bb1 protein was generated by repeated immunization with the wild type Cry3Bb1.11037 protein. There are seven amino acid differences between the wild type and that expressed in corn MON863 or isolated from *E. coli* containing pET24d(+)/25097. No information was provided to characterize the reactivity if this anti-Cry3Bb1 antibody to the variant Cry3Bb1 protein but it was assumed by the author to be cross-reactive with the variant protein since the wild type and varaint only differ by seven amino acids.

RESULTS AND DISCUSSION

SIF activity was verified to be 13,749 Units/ml prior to the assay and 12,795 Units/ml after the test (Absorbance at 574nm of TCA soluble resorufin labeled peptides) indicating that the simulated intestinal enzyme activity was present and at a level deemed acceptable by SOP GEN-PRO-058-01.

The gels provided indicate that the Cry3Bb1.11098 protein is present as a single band at 74 kDa which rapidly degraded to two bands of 68 and 57kDa at the first assay time point of 1 minute. The subsequent samples (from 5 minute to 24 hours) all gave a single 57 kDa band which did not appear to decrease in intensity. This lack of degration by intestinal fluids is similar to the majority of Cry proteins which are resistant to the action of trypsin.

Classification: ACCEPTABLE.

Reg. # 524-528
Volume 2 of 4

Page ___ is not included in this copy.

Pages 479 through 496 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist

Secondary Reviewer: John L. Kough, Ph.D., Biologist

STUDY TYPE:	Product Characterization (885.1100)
MRID NO:	454240-01
TEST MATERIAL:	Cry3Bb-Protected Corn (Corn Root Worm-Protected)
PROJECT NO:	Study 99-01-39-22; MSL-17181
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Amended Report for MSL-16559: *B.t.* Cry3Bb1.11098 and NPTII Protein Levels in Sample Tissue Collected from Corn Event MON 863 Grown in 1999 Field Trials
AUTHOR(S):	Yelena A. Dudin, Bich-Phoung Tonnu, Lee D. Albee, and Ronald P. Lirette
STUDY COMPLETED:	April 10, 2001
CONCLUSION:	The protein titer data provided shows the ranges of Cry3Bb1 protein in various parts of the plant, as well as geographical variation. Overall, based upon the ranges provided, there appears to be significant variation between the samples analyzed on different days post-planting and at different sites.
CLASSIFICATION:	ACCEPTABLE
GOOD LABORATORY PRACTICE:	This study was conducted in accordance with Good Laboratory Practice guidelines 40CFR part 160.

I. STUDY DESIGN

Test Material:

Test Substance:	Corn rootworm (*Diabrotica virgifera virgifera*, CRW) protected corn event MON 863. The originally transformed plant (R0) was crossed to corn line MTCY10412 to produce F1 plants. The F1 plants were self-pollinated to produce F2 plants. F2 progeny were crossed to corn line A1 and this event was identified as MON 863.
Control:	Non-modified corn line MON 846 which is genetically representative of the test substances. The genetic background for these plants was progeny of a MTCY10412 x A1 cross.
Reference Substances:	Pure protein standards of Cry3Bb1 and NPTII were used in each ELISA assay. Cry3Bb1.11231 (Lot# 631812) was purified from *B. thuringiensis* (Hileman et al., 1999). Lyophilized standard protein powder (Lot# 6307780) was dissolved in 50 mM sodium bicarbonate containing 0.05%

(v/v) Tween 20, pH 9.6) for a densitometry corrected concentration of approximately 0.271 mg/ml. Aliquots were stored at -80 °C.

The neomycin phosphotransferase II gene (*nptII*) was cloned and transferred to *E. coli* for fermentation and purification of pure NPTII protein (Berberich et al., 1993). Lyophilized standard protein powder (Lot# 4821020) was dissolved in 50 mM sodium bicarbonate for a concentration of approximately 1.15 mg/ml. Aliquots were stored at -80 °C.

Methods:

A. Generation of Plant Samples

1. Field Design: The test events and control lines were planted at four varied corn-growing U.S. locations during the 1999 season. The sites were located at Van Horne, Iowa (Benton Co.), Richland, Iowa (Jefferson Co.), York, Nebraska (York Co.), and Monmouth, IL (Warren Co.).

   Young leaf, mature root (forage) and grain samples were collected from plants at the four field sites. Additional samples collected included leaf: whole plants and roots from three U.S. field sites; silk and pollen tissues collected as a composite sample from one U.S. site; pollen from three field sites in Argentina (Production Plan # 99-04-39-01) - Fontezuela in Pergamino county, Salto in Salto county and Rojas in Rojas county. Corresponding control tissues (MON 846) were collected from each of the field sites.

2. Young Leaf: the first young leaf (YL-1) sample was collected approximately 21 days after planting (~V-4 stage) at four field sites (four replications/site). The number of MON 863 leaves collected per plot ranged between 37 and 50. The number of control leaves collected ranged from 20-32 because of a lower number of seeds planted for the control line. Only one replicated plot from each field site was analyzed by ELISA.

3. Overseason Leaf Samples: the young leaf sampling was repeated at approximately 2 week intervals until pollination at three field sites. Two additional YL samplings (YL-2 and YL-3) were collected at three field sites at approximately 35 and 49 days post-planting, respectively. Each time point included 15 non-systematically selected plants.

4. Overseason Whole Plant: at each collection point, two whole plants (above ground parts) were collected for each test event and control line at multiple locations. Overseason whole plant samples (OSWP-1 and OSWP-2) were collected at ~35 and ~ 49 days post-planting, respectively. The first two collections of OSWP-1 and 2 correspond to the YL-2 and YL-3 sampling points respectively. OSWP-3 and 4 were samples collected at the early dent stage (~ 90 days post-planting) and

senescent plant stage (~126 days post-planting). OSWP-1, 2 & 4 were collected at three U.S. field sites a one replicate. OSWP-3 had 4 replicates, but only one analyzed.

5. Overseason Root: at each collection time point, the root mass from two overseason whole plants was collected, rinsed to remove soil, composited, placed in a labeled bag and frozen on dry ice. The overseason root samples (OSR) were collected at three locations as one replicate per site with the exception of mature root samples at the forage stage (OSR-3) which were collected at four field sites as four replicates per site. Only one replicate of OSR-3 was analyzed from each field site.

6. Silks: silk tissue was collected at approximately 58 days post-planting (~50% pollen shed). Each test and control sample was a composite of five plants from the Monmouth IL site.

7. Pollen: a composite sample from the Monmouth, IL, site was collected over a 7 day period beginning at 60 days post-planting. Additional pollen samples were collected from the Argentina sites and added to the study via an amended study protocol.

8. Grain: all self-pollinated ears of each test event and control line were hand harvested at approximately 35% moisture content and grain composited. The grain (dried to below 15% moisture) was shipped to Monsanto and stored at ambient temperature.

B. ELISA Analytical Methods

1. Extraction of Protein from Corn Tissues/Cry 3Bb1 and NPTII ELISA: corn tissue, except for pollen, was ground to a fine powder on dry ice in a blender or vertical cutter mixer (Production Plan 99-01-39-08 - not submitted). Each specific tissue was extracted with the appropriate buffer[1] and homogenized in a Polytron with two bursts of approx. 17.5K rpm for 30 seconds each. Insoluble material was removed by centrifugation at ~ 20,000 x g for 10 minutes at 4 °C or by used of a Serum Filter System (Fisher Scientific). The supernatant was removed and stored frozen at approximately -80° C.
For Cry3Bb1 and NPTII, a DAS-ELISA (double antibody sandwich ELISA) was developed and validated to quantitate the levels of the proteins in genetically modified corn tissues (Dudin et al., 1999 and Bhakta et al., 1999, respectively). The ELISA validation summary is shown in Appendix 2 (attached). The Cry3Bb1 and NPTII protein levels were measured by ELISA according to the appropriate

---

[1] PBST=137mM NaCl, 2.7mM KCl, 10mM Phosphate, 0.05%(v/v) Tween 20, for grain samples; adding 0.1% BSA for pollen, silk, leaf and root samples. TBA= 100mM Trizma, 10mM sodium borate, 5mM $MgCl_2$, 0.2% (w/v) L-ascorbic acid, 0.05% Twen 20, pH 7.5, for forage and whole plant extractions.

SOP (listed in Appendix but not submitted). Cry3Bb1 and NPTII protein levels were measured by analyzing extracts in a validated sandwich ELISA using purified rabbit antisera and HRP-conjugated antibodies. TMB (3,3'5,5'-tetramethylbenzidine) was used as a substrate for color development in both assays. Quantitation of the Cry3Bb1 or NPTII protein levels was accomplished by interpolation from a Cry3Bb1 or NPTII protein standard curve with a range of ~ 1 to 64 ng/ml for each assay.

2. Control of Bias: The accuracy of the ELISA system was evaluated and each method optimized to minimize assay bias during assay validation (Production Plans: 99-01-39-08 & 99-04-39-01 - not submitted). Accuracy was defined by two components: recovery of the standard protein spiked at several levels and extraction efficiency (Appendix 2). All reported protein levels were corrected for assay bias:

$$\text{method bias} = \frac{(\%\ \text{extraction efficiency})}{100} \times \frac{(\%\ \text{spike and recovery})}{100}$$

3. Data Reduction and Statistical Analyses: The absorbance of each reaction was measured by dual wavelength at 450 nm and 650 nm using a Molecular Devices SPECTRAmax Plus photometer. Standard protein concentrations and the corresponding absorbance were plotted by SOFTmass PRO, version 2.4.1 using a 4-parameter curve fit. The concentrations of Cry3Bb1 or NPTII proteins in corn tissue extracts were transformed to microgram protein per gram fresh weight of tissue using the tissue:volume rations and corrected for assay bias. Microsoft Excel (V. 7) was used to calculate the mean protein level and standard deviation.

II. RESULTS

1. Test and Control Substance Characterization: the identity of each sample was confirmed by the study director. Production Plan 99-01-39-08 was used to characterize and positively id the samples. Because of limited tissue samples from the Argentina Production Plan (99-04-39-01), pollen tissue samples were not confirmed by molecular analyses. However, chain of custody information was available. Plant tissue samples were analyzed within the time frame of these proteins' stability demonstrated in other studies.

2. Plant Samples Analyzed: protein values were reported as micrograms of protein per gram of fresh tissue weight. Young leaf, forage, mature root and grain samples were analyzed using extracts from samples collected at U.S. field sites. The results are shown in Table 1 (attached). Young leaf samples were collected at the V-4 stage (~ 21 days) and the level of protein averaged 81 µg/g fwt. Cry3Bb1 protein in grain averaged 70 µg/g fwt. Cry3Bb1 protein levels in mature root and forage averaged 41 and 39 µg/g fwt respectively. Finally, protein levels in pollen averaged 62 µg/g

46

fwt (one U.S. site and four Argentina sites) and in silk averaged 10 μg/g fwt (single site).

For NPTII, the protein levels in young leaf and forage tissues averaged 0.98 and 0.19 μg/g fwt respectively. NPTII levels in grain were below the LOD (<0.76 μg/g) for all samples analyzed.

Site to site variation is shown in Table 1 for both Cry3Bb1 and NPTII proteins. Also, as shown in Table 2, the levels of Cry3Bb1 protein declined over time in each of the tissues examined (leaf, whole plant, root).

Finally, Cry3Bb1 and NPTII protein levels in the non-transgenic control line (MON 846) were below the level of detection as expected.

III. DISCUSSION

The protein titer data provided shows the ranges of Cry3Bb1 protein in various parts of the plant, as well as geographical variation. Overall, based upon the ranges provided, there appears to be some variation between the samples analyzed on different days post-planting and at different sites.

CLASSIFICATION: ACCEPTABLE

Reg. # 524-528

Volume 2 of 4

Page ___ is not included in this copy.

Pages 464 through 490 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist MTW

Secondary Reviewer: John L. Kough, Ph.D., Biologist JLK APR 25 2001

STUDY TYPE:	Acute Oral Toxicity (885.3050)
MRID NO:	449043-06
TEST MATERIAL:	Cry3Bb1 Protein (Cry3Bb1.11098)
PROJECT NO:	MSE-N Study Number: MSE-N 98035; Monsanto Study Number: ML-98-141; MSL Number: 16215
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO [Metabolism and Safety Evaluation-Nested (MSE-N); Protein Characterization and Safety Center (PCSC)]
TITLE OF REPORT:	Acute Oral Toxicity Study *B.t.* Protein 11098 in Mice
AUTHOR(S):	Charles L. Bechtel, B.S., B.A.
STUDY COMPLETED:	August 5, 1999
CONCLUSION:	There did not appear to be significant adverse affects to animals dosed with Cry3Bb1 at corrected dose amounts of 38.7, 419, or 2980 mg/kg bodyweight. Two animals died during the study - animal #'s 98035M3-007 and 98035F3-004 in the 2980 mg/kg treatment group. These deaths appeared to be result of trauma from dosing rather than from the test substance. In addition, although, there was some minor weight loss and minor abnormal observations at gross necropsy, these occurred in both test and control groups and therefore do not appear to be Cry3Bb1.1098 protein exposure related. Based upon the data provided, the LD_{50} for Cry3Bb1.1098 is greater than 2980 mg/kg bodyweight in mice.
CLASSIFICATION:	ACCEPTABLE, TOXICITY CATEGORY III
GOOD LABORATORY PRACTICE:	This study was conducted in accordance with Good Laboratory Practice guidelines with minor exceptions: 1) the protein control is a marketed product and is characterized by its labeling; 2) egg receipt and incubation to the first instar larvae stage for the insect bioassay portion of the analytical procedures was not conducted in accordance with the GLPs; 3) documentation for the characterization of the test material (Study 98-01-39-05) confirmed the identity of the test material; 4) SDS-Page analysis was performed to reevaluate the molecular weights fo the test substance in July, 1999. The resulting value was a more accurate assessment of the molecular weights than previously reported. None of these issues should have had any significant

impact on the results described.

I. STUDY DESIGN

Test Material: Cry3Bb1.11098 protein produced in *B.t.* strain EG11098 (lot# 6312803) (92.3% purity).

Protein Control - BSA (Lot# 16H9317 - Sigma).

Test Animals: One hundred (50 male and 50 female) Crl:CD1®(ICR)BR(VAF/Plus®) mice were obtained from Charles River Laboratories, Portage, MI.

Methods:

1. Three groups of ten male and ten female mice were dosed orally with target doses of: 40, 400 or 4000 mg/kg bodyweight of Cry3Bb1.11098 protein at a dosing volume of 33.3 ml/kg. [Note: because of the viscosity of the dosing material, the dose concentration required for the 4000 mg/kg treatment was too thick to flow through the needle. Therefore, the dose was divided into two 33.3 ml/kg doses at 2000 mg/kg each. The other dose levels were also divided into two doses.]
2. Ten male and ten female protein control animals were dosed with BSA @ 4000 mg/kg bodyweight at the same dosing volume and method (i.e., 2 doses) as described above.
3. Ten male and ten female vehicle control mice were administered deionized water at the same dosing volume (one dose). All animals were provided food and water ad libitum after dosing.
4. Checks for mortality and moribundity were recorded twice daily, except on day of necropsy. Clinical observations for toxicity were recorded after dosing and daily thereafter.
5. Bodyweights were recorded prior to the start of the study and on days 7 and 14. Food consumption was measured post-dosing on days 7 and 14.
6. Following the 14 day observation period, each animal was euthanized with excess CO_2 and subjected to examination by gross necropsy.

II. RESULTS

1. Mortality - There were two unscheduled deaths (animal #'s 98035M3-007 and 98035F3-004) in the animals treated with 2980 mg/kg.
2. Clinical Observations - One animal (#98035M3-007) was observed to be hypoactive on Day 1 of the observation. This animal died on Day 2 of the study. Animal 98035M1-006 showed focal loss of hair (38.7 mg/kg dose group). No other abnormal clinical signs were

observed.

3. Body Weight - Each of the male animals displayed an overall weight gain. Two of the female animals displayed a slight loss between days 7 and 14, but an overall weight gain (Table 1). The registrant indicates that there was a statistically significant reduction ($p \leq 0.05$) in the mean body weight of females in the 400 mg/kg dose group on day 14. The mean for the 40 mg/kg group was 26.98; for the 400 mg/kg group was 26.39; and for the 4000 mg/kg group was 27.0.

Table 1. Animal Weight Loss

Test Group	Animal #	Sex	Weight Change
Cry3B2.11098 @ 40 mg/kg	98035F1-010	F	Lost 0.1 g between Day 7 and Day 14
Cry3B2.11098 @ 400 mg/kg	98035F2-010	F	Lost 0.1 g between Day 7 and Day 14

The small weight loss in two animals does not appear to be significant, nor treatment related. As well, the relatively small difference between the mean body weight of the 400 mg/kg group, while significant, does not appear to be related to the test substance as the group treated with a higher dose showed the highest mean body weight.

4. Food Consumption: There was no difference in the food consumption among the different test groups.

5. Gross Necropsy: Examination of the animals by gross necropsy revealed some minor abnormalities. However, such affected animals were male and female, and part of both the test and control groups. Therefore, these effects do not appear to result from exposure to the test substance. The following animals displayed abnormalities:

Animal #	Group	Abnormality
98035M1-006	Cry3B2.11098 @ 400 mg/kg	Enlarged spleen (2.6 x 0.7 cm) and enlarged/prominent thymus - bilateral, moderate
98035M3-007 (unscheduled death - Day 1)	Cry3B2.11098 @ 4000mg/kg	Abnormal brain color, diffuse cortex; abnormal lung color, diffuse lobes; abnormal small intestine color, multifocal areas; abnormal stomach color, diffuse fundus.
98035FV1-004	Water Vehicle Control	Right thyroid lost during necropsy; urinary bladder incomplete- portion lost during necropsy

98035FV1-007	Water Vehicle Control	Bilateral cyst (0.3 cm) on ovary - filled with clear fluid
98035FV1-008	Water Vehicle Control	Caudate liver lobes lost during necropsy
98035FV1-009	Water Vehicle Control	Small (atrophy) right kidney - marked
98035FV2-006	BSA 33.33 mg/kg	Cyst (0.3 cm) on right kidney - filled with clear fluid
98035F1-001	40 mg/kg Cry3B2.11098	Incomplete pituitary, damaged during necropsy
98035F1-003	40 mg/kg Cry3B2.11098	Kidney cyst (right serosa - 0.1 cm, clear); small (atrophy) left kidney-moderate; calculus (0.3 cm) on urinary bladder
98035FV1-005	40 mg/kg Cry3B2.11098	Lymph Node, submax. - enlarged/prominent (left 0.6 x 0.4 cm)
98035F1-006	40 mg/kg Cry3B2.11098	Cyst on right kidney (serosa) - 0.1 cm filled with clear fluid
98035F30-002	4000 mg/kg Cry3B2.11098	Cyst on left ovary (0.4 cm) - filled with clear fluid; enlarged/dilated/distended uterus - bilateral horns (0.3 cm) - filled with clear fluid
98035F3-004 (unscheduled death - Day 2)	4000 mg/kg Cry3B2.11098	Bilateral corneal opacity of eyes; lung perforation - right caudal; abnormal color
98035F3-007	4000 mg/kg Cry3B2.11098	Cyst on left ovary (0.4 cm) - filled with clear fluid
98035F3-008	4000 mg/kg Cry3B2.11098	Enlarged/dilated/distended uterus with bilateral horns (0.3 cm) - filled with clear fluid

III. DISCUSSION

Although two animals died prematurely, there did not appear to be significant adverse affects to animals resulting from exposure to Cry3Bb1 at corrected dose amounts of 38.7, 419, or 2980 mg/kg bodyweight. The two animals which died prematurely (#'s 98035M3-007 and 98035F3-004 in the 2980 mg/kg treatment group) both showed potential adverse to the dosing procedure, rather than to the test substance itself. Animal 98035F3-004 showed a lung perforation which indicates trauma during dosing. Animal 98035M3-007 showed various abnormalities including purple/black/red color in the stomach, intestine and lung which also indicates trauma likely resulting from the dosing. In addition, the 33 ml/kg (even as a split dose) is near the limits of volume tolerance for dosing, further implicating physical trauma as the cause for the abnormalities and deaths described.

Otherwise, there was some minor weight loss in a few animals and some minor abnormal

observations via gross necropsy, but in each case, the abnormality occurred in both test and control groups and therefore do not appear to be Cry3Bb1.11098 protein exposure related. Based upon the data provided, the LD_{50} for Cry3Bb1.11098 is greater than 2980 mg/kg bodyweight in mice.

CLASSIFICATION: ACCEPTABLE, TOXICITY CATEGORY III

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist MTW

Secondary Reviewer: John L. Kough, Ph.D., Biologist JLK APR 25 2001

STUDY TYPE:	Acute Oral Toxicity (885.3050)
MRID NO:	449043-05
TEST MATERIAL:	Cry3Bb1 Protein (Cry3Bb1.11231)
PROJECT NO:	MSE-N Study Number: MSE-N 98036; Monsanto Study Number: ML-98-142; MSL Number: 16216
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO [Metabolism and Safety Evaluation-Nested (MSE-N); Protein Characterization and Safety Center (PCSC)]
TITLE OF REPORT:	Acute Oral Toxicity Study of *B.t.* Protein 11231 in Mice
AUTHOR(S):	Charles L. Bechtel, B.S., B.A.
STUDY COMPLETED:	August 5, 1999
CONCLUSION:	There were no apparent adverse effects identified in mice dosed orally with 36, 396 and 3780 mg/kg bodyweight of Cry3Bb1.11231 protein. There was some minor weight loss in a few animals and some minor abnormal observations via gross necropsy, but these occurred in both the test and control groups and therefore do not appear to be Cry3Bb1.11231 protein exposure related. Based upon the data provided, the LD_{50} for Cry3Bb1.11231 is greater than 3780 mg/kg bodyweight in mice. ACCEPTABLE. TOXICITY CATEGORY III
CLASSIFICATION: GOOD LABORATORY PRACTICE:	This study was conducted in accordance with Good Laboratory Practice guidelines with minor exceptions: 1) the protein control is a marketed product and is characterized by its labeling; 2) egg receipt and incubation to the first instar larvae stage for the insect bioassay portion of the analytical procedures was not conducted in accordance with the GLPs; 3) documentation for the characterization of the test material (Study 98-01-39-05) confirmed the identity of the test material; 4) SDS-Page analysis was performed to reevaluate the molecular weights fo the test substance in July, 1999. The resulting value was a more accurate assessment of the molecular weights than previously reported. None of these issues should have had any significant impact on the results described.

I. STUDY DESIGN

Test Material: Cry3Bb1.11231 protein produced in *B.t.* strain EG11231 (lot# 6312812) (96% purity).

Protein Control - Bovine Serum Albumin (BSA; Lot# 16H9317 - Sigma).

Test Animals: One hundred (50 male and 50 female) Crl:CD1®(ICR)BR(VAF/Plus®) mice were obtained from Charles River Laboratories, Portage, MI.

Methods:

1. Three groups of ten male and ten female mice were dosed orally with: 40, 400 or 4000 mg/kg bodyweight of Cry3Bb1.11231 protein at a dosing volume of 33.3 ml/kg. [Note: because of the viscosity of the dosing material, the dose concentration required for the 4000 mg/kg treatment was too thick to flow through the needle. Therefore, the highest dose was divided into two 33.3 ml/kg doses at 2000 mg/kg each. The other dose levels were also divided into two doses to mimic the highest dose regime].
2. The dose amounts of the test substance were confirmed by SDS-PAGE gels with densitometry (to account for Cry3Bb1 purity) and by incorporation into Colorado potato beetle diet for bioassay.
3. Ten male and ten female protein control animals were dosed with BSA @ 4000 mg/kg bodyweight at the same dosing volume and method (i.e., 2 doses) as described above.
4. Ten male and ten female vehicle control mice were administered deionized water at the same dosing volume (one dose).
5. Checks for mortality and moribundity were recorded twice daily, until the day of necropsy when only one observation was performed. Clinical observations for toxicity were recorded after dosing and daily thereafter.
6. Bodyweights were recorded prior to the start of the study and on days 7 and 14. Food consumption was measured post-dosing on days 7 and 14.
7. Following the 14 day observation period, each animal was euthanized and subjected to examination by gross necropsy.

II. RESULTS

1. Mortality - There were no unscheduled deaths through the 14 day observation period.
2. Clinical Observations -No abnormal clinical signs were observed.
3. Body Weight - The submission states that "all animals gained weight throughout the study with no statistically significant differences between groups". However, the individual animal data shows that several animals in both the control and test groups lost weight

through the observation period:

Test Group	Animal #	Sex	Weight Change
Water Vehicle Control	98036FV1-006	F	no overall weight change
Cry3Bb1.11231 @ 400 mg/kg	98036F2-002	F	no overall weight change
Cry3Bb1.11231 @, 400 mg/kg	98036F2-006	F	overall weight loss (-0.3 g)
Cry3Bb1.11231 @ 400 mg/kg	98036F2-008	F	overall weight loss (-0.1g)

The lack of weight gain or the loss of weight in some of the animals does not appear to be the result exposure to the test substance since there was no discernable dose response. Animals in the vehicle control groups, as well as those dosed with the test substance both displayed weight loss, which was minor is each case.

4. Food Consumption: There was no difference in the food consumption among the different test groups.
5. Gross Necropsy: Examination of some of the animals by gross necropsy revealed some minor abnormalities. However, such affected animals were part of both the test and control groups and therefore do not appear to result from exposure to the test substance. The following animals displayed abnormalities:

Animal #	Group	Abnormality
98036MV1-002	Water-Vehicle Control	Right thyroid lost during necropsy
98036-M1-002	40 mg/kg Cry3Bb1.11231	cyst on kidney (0.1 cm); kidney filled with clear fluid
98036FV1-002	Water-Vehicle Control	cyst on ovary; ovary filled with clear fluid; cyst on uterus; uterus filled with clear fluid; bilateral horns on uterus
98036FV1-004	Water-Vehicle Control	enlarged, dilated, distended uterus; bilateral horns; uterus filled with clear fluid
98036FV2-003	BSA Control	enlarged, dilated, distended uterus; bilateral horns; uterus filled with clear fluid
98036FV2-009	BSA Control	enlarged, dilated, distended uterus; bilateral horns; uterus filled with clear fluid

98036F1-002	40 mg/kg Cry3Bb1.11231	cyst on ovary (0.3 cm); ovary filled with clear fluid
98036F1-003	40 mg/kg Cry3Bb1.11231	enlarged, dilated, distended uterus; bilateral horns; uterus filled with clear fluid
98036F1-005	40 mg/kg Cry3Bb1.11231	enlarged, dilated, distended uterus; bilateral horns; uterus filled with clear fluid
98036F1-007	40 mg/kg Cry3Bb1.11231	cyst on ovary (0.3 cm); ovary filled with clear fluid
98036F1-010	40 mg/kg Cry3Bb1.11231	enlarged, dilated, distended uterus; bilateral horns; uterus filled with clear fluid
98036F2-009	400 mg/kg Cry3Bb1.11231	enlarged, dilated, distended uterus; bilateral horns; uterus filled with clear fluid
98036F3-001	4000 mg/kg Cry3Bb1.11231	cyst on ovary (0.4 cm); ovary filled with clear fluid
98036F3-003	4000 mg/kg Cry3Bb1.11231	moderate atrophy/small ovaries

III. DISCUSSION

There were no apparent adverse effects identified in mice dosed orally with 36, 396 and 3780 mg/kg bodyweight of Cry3Bb1.11231 protein. There was some minor weight loss in a few animals and some minor abnormal observations via gross necropsy. Because these abnormalities occurred in both the test and control groups they do not appear to be Cry3Bb1.11231 protein exposure related. Based upon the data provided, the LD_{50} for Cry3Bb1.11231 is greater than 3780 mg/kg bodyweight in mice.

CLASSIFICATION: ACCEPTABLE, TOXICITY CATEGORY III

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist MTW

Secondary Reviewer: John L. Kough, Ph.D., Biologist JLK APR 25 2001

STUDY TYPE:	*In vitro* Digestibility
MRID NO:	449043-07
TEST MATERIAL:	Cry3Bb1.11098 and Cry3Bb1.11231 Proteins
PROJECT NO:	Study Number: 98-01-39-02
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Assessment of the *in vitro* digestibility of *B.t.* Protein 11098 and *B.t.* Protein 11231 Utilizing Mammalian Digestive Fate Models
AUTHOR(S):	John N. Leach, Ronald E. Hileman, Ph.D., John W. Martin, B.A., Richard S. Thoma, M.S. and James D. Astwood, Ph.D.
STUDY COMPLETED:	July 22, 1999
CONCLUSION:	The tests performed in this study show that the Cry3Bb1 proteins are not stable to digestion in simulated gastric fluid. Incubation of Cry3Bb1.11098 and Cry3Bb1.11231 in SGF results in the loss of detectable protein by the 30 and 15 second observation points, respectively, as detected by SDS-PAGE. Insect bioassay data indicated that the protein loss insecticidal activity within 2 minutes of incubation in SGF. Incubation in the SIF resulted in a ~59 kDa digestion product that retained its insecticidal activity after at least 30 minutes incubation.
CLASSIFICATION:	ACCEPTABLE. Neither Cry3Bb1.11098 nor Cry3Bb1.11231 appear to be stable to digestion in simulated gastric fluid for more than 30 seconds. However, there appears to be a discrepancy between the results discussion and figures 2 & 4. The Cry3Bb1.11098 protein appeared to be significantly digested within 15 seconds and completely digested in less than 30 seconds based upon the figures provided. This differs somewhat with the discussion provided by the registrant which indicates that the protein was digested to undetectable levels between 30 and 60 seconds. These do represent drastically different digestion times, but the discrepancy should be addressed given that the submitted gel data do not verify the claims.
GOOD LABORATORY PRACTICE:	This study was conducted in accordance with Good Laboratory Practice guidelines with four exceptions as described in the

500

submission. These exceptions should not have a significant impact on the outcome of the study.

I. STUDY DESIGN

Test Material:

1. The test substances were: Cry3Bb.11098 protein (lot# 6312803) and Cry3Bb1.11231 protein (lot# 6312812) which were isolated from transformed *Bacillus thuringiensis* strains EG11098 and EG11231 respectively.
2. The reference substance for the simulated gastric fluid (SGF) assay was hemoglobin. The reference substance for the simulated intestinal fluid (SIF) assay was resorufin-labeled casein.
3. The control substances were prepared by using purified water (vehicle) instead of the Cry3Bb proteins.

The recovery of the test substances was demonstrated by trials which served as reference treatments. These reference treatments were prepared in SGF without pepsin or SIF without pancreatin.

Methods:

Test Treatments:

1. Simulated gastric and intestinal fluids were prepared and confirmed to be functionally active on the day of use. SGF was measured in accordance with SOP No. GEN-PRO-057-01 (not submitted) which relates to the amount of pepsin activity to the rate of hemoglobin digestion. SIF activity was measured in accordance with SOP No. GEN-PRO-058-01 (not submitted) which relates to the amount of proteolytic activity to the rate of resorufin released from resorufin-labeled casein. Both assays determine the amount of digestion by comparison to the change in absorbance. Digestion is directly related to increased absorbance due to the amount of small fragments present in TCA protein precipitated solutions.
2. The rate of Cry3Bb digestion in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was examined in two sets of digestion trials (based upon volume), small scale and large scale. The large scale digestions were conducted for use in insect bioassays.
3. Timed incubations began with the addition of Cry3Bb protein to individual incubation tubes in a shaking 37° C waterbath. [zero incubation time trials were quenched prior to addition of the test substance].
4. SGF digestions were quenched by addition of sodium carbonate and SIF digestions were quenched by addition of 1X Laemmli buffer.
5. Small scale SGF trials were further diluted in 5X Laemmli protein

sample loading buffer, then heated to 100 °C for 5 min. Small scale SIF trials were diluted with Laemmli sample buffer followed by heating to 100 °C for 5 min.

6. Large scale SGF trials were placed in dry ice-methanol baths until storage at -15 °C or colder. Large scale SIF trials were diluted in purified water followed by freezing in a dry-ice methanol bath, then stored at -15° C.

Test Treatments:

Treatment	Incubation Times
SGF	0, 15, 30 s and 1, 2, 4, 8, 15, 30, 60, and 120 min
SIF	0, 1, 5, 15, 30 min and 1, 2, 4, 8, 16, and 24 hr

Control and Reference Treatments:

1. Control treatments were prepared replacing the test substance with purified water vehicle in the SGF or SIF test system. Reference samples were placed in the SIF and SGF fluids without the pancreatin and pepsin respectively. Samples were collected at 0 and 120 min for SGF and 0 and 24 hr for SIF small scale trials. For large scale trials, the 0 min time point was used for both SGF and SIF.

SDS-PAGE Analysis:

1. *SDS-PAGE/Colloidal Brilliant Blue G Staining:*
   SDS-PAGE analysis was performed according to SOP No. BtC-PRO-026-01 (not submitted). Previous work identified the level of detection for Cry3Bb1.11098 and Cry3Bb1.11231 proteins using Brilliant Blue G staining to be 1 ng. Tricine gels and running buffers were used, which provide good resolution of peptides ≥ 2 kDa.. After separation of the proteins by electrophoresis, proteins were visualized by staining with Colloidal Brilliant Blue G concentrate. After electrophoresis, gels were fixed (methanol/acetic acid) for 30 minutes followed by overnight staining. Gels were then washed with 25% methanol solution, then photographed.

2. *SDS-PAGE/Immunoblot Analysis:*
   The limit of detection for this method was determined to be approximately 0.2 ng for the upper protein band (~67 kDa) and 1 ng for the lower protein band (~59 kDa). Proteins were separated by electrophoresis and transferred to 0.45μm PVDF membrane. The membrane was incubated in blocking solution (5% NFDM [nonfat

502

dried milk] dissolved in TBST [TBST = Tris-buffered saline with Tween 20]). Protein specific monoclonal antibody was added at 1:5000 (antibody in 1% NFDM in TBST) and incubated (usually overnight at 4 °C). Membranes were washed in TBST and protein-bound antibody was detected using anti-rabbit IgG conjugated to horseradish peroxidase. The membrane was incubated with the secondary antibody (1:7500 dilution in 1% (w/v) NFDM dissolved in TBST) for at least 1 hour at room temperature, followed by TBST washing. Secondary antibody bound to the membrane was visualized by ECL (chemiluminescence) on X-Ray film.

Functional Assay:
The functional assay was an insect bioassay performed according to SOP No. BR-ME-0044-02 (not submitted) to evaluate the functional activity of the Cry3Bb1 proteins. An agar-based diet into which the digestion trials were added was used. Colorado potato beetle (CPB) larvae were placed onto wells containing the diet. Protein functional activity was determined by comparing the number of surviving insects and mean insect weight to a standard dose curve and other trials. Large-scale digestion trials were modified to maintain the functionality of the proteins. The Laemmli sample buffer and heating steps were eliminated.

N-terminal Amino Acid Sequence Analysis:
SDS-PAGE analysis of the Cry3Bb1 proteins identified minor small molecular weight bands which were analyzed by N-terminal sequencing to determine if they were Cry3Bb1 protein digestion products Searle SOP No. 400.324 (not submitted).

For preparation of a protein digestion product with a high enough amount of resulting peptide fragments protein for analysis, the experimental volumes, concentrations and pepsin to protein ratio were changed. A series of incubation trials were used to generate the same fragments as previously identified. SDS-PAGE gels were run followed by electro-transfer to 0.45 µm pore size PVDF membrane in CAPS transfer buffer (10 mM CAPS pH 11, 10% (v/v) methanol). 10.5µg of total protein was loaded into each of five lanes. Proteins were visualized by staining the membrane with Ponceau S.

II. RESULTS

Effectiveness of the Test Systems:
The proteolytic activity of SGF prepared for small scale digestions was 16,733 U/ml (where 1U = $\Delta 0.001\ A_{280\ nm}\ min^{-1}$ at 37 °C) which, according to the registrant, was within the requirements of SOP No. GEN-PRO-057-01

(not submitted). The SGF prepared for the large scale digestions had an activity of 15,683 U/ml, which was not within the SOP requirements (SOP acceptability range for SGF not provided). However, this preparation was accepted by the registrant anyway as they believe that there was not any impact on the study based upon the following:

i) if the activity units are argued to be only valid to two significant figures, the low activity rounds up to the acceptable range.
ii) the same lot of pepsin was used to ensure comparable results between the small-scale and large-scale digestions.
iii) the variability within the activity assay is broad. One standard deviation is approximately 25% of the mean.
iv) the SGF which fell outside the accept criteria, was close to the activities of earlier preparations with the same lot of pepsin.
v) the acceptance criteria established in the SOP was based on only a few lots of pepsin and did not include data from the lot being used.
vi) the particular lot of pepsin used to prepared SGF had been observed to consistently produce low activities within the acceptable range
vii) validation experiments for the SGF model demonstrated that protein digestion rates are not sensitive to small variations in pepsin concentration.

The SIF proteolytic activity of SIF was 11,581 U/ml (1U=Δ0.001 A574nm min-1 at 37 °C) for the small scale digestions and 10,111 U/ml for large scale digestions. Each of these activities are within the SOP requirements according to the submission (data not provided).

Digestibility of Cry3Bb1 Proteins in SGF/Insect Bioassay:

Figures 2 & 4 show the results of staining and immunoblot analysis for the small scale digestion of Cry3Bb1.11098 protein samples incubated in SGF. The Cry3Bb1.11098 protein was significantly digested within 15 seconds and apparently completely digested in less than 30 seconds based upon the figures provided. This differs somewhat with the discussion provided by the registrant which indicates that the protein was digested to undetectable levels between 30 and 60 seconds. These do represent drastically different digestion times, but the discrepancy should be addressed given that the submitted gels do not verify the claims.

Figures 3 & 5 show the results of staining and immunoblot analysis for the small scale digestion of Cry3Bb1.11231 protein samples incubated in SGF.

The Cry3Bb1.11231 protein was apparently completely digested by the 15 second timepoint based upon the figures provided. This interpretation agrees with that provided by the registrant.

Further, the registrant indicates that there was a low molecular weight protein fragment (~ 3 kDa) present in SGF digestions as a faint band in the SDS-PAGE stained gels (Figures 2 & 3) up to the 4 min digestion. Increased sample load and gel analysis indicated that this band was a doublet. No sequence data could be obtained for these doublet bands for Cry3Bb1.11098 when examined by N-terminal sequencing. For the Cry3Bb1.11231 doublets, no sequence data was obtained for the upper doublet, but analysis of the lower doublet indicated that it originated from the Cry3Bb1.11231 protein beginning at aa 563:

LQRYRVRIKYAST(T)(N)	- determined
LQRYRVRIRYASTTN	- expected

Insect bioassay analysis indicated that the proteins subjected to large scale SGF digestions lost insecticidal activity within 2 minutes (Figure 1, graph A-D).

Digestibility of Cry3Bb1 Proteins in SIF/Insect Bioassay:
Incubation of the Cry3Bb1 proteins in SIF appears to generate a stable ~59 kDa fragment of Cry3Bb1 which is present through the 24 hour timepoint (Figures 6 & 7). Insect bioassay of SIF-digested proteins indicated that insecticidal activity remained even after 30 minutes of SIF incubation (Figure 1). These results indicate that the ~59 kDa protein is biologically active (Figure 1, graph E-H).

Test Substance Stability:
The test substance reference samples (incubated in the absence of enzyme) were stable throughout the experiment as shown on Figures 2-7. These results indicate the enzymatic activity of the SGF or SIF alone is responsible for the breakdown of the Cry3Bb1 proteins. No effect of solution pH was evident.

Comparison of Large-Scale and Small-Scale Digestions:
Large and small-scale SGF digestions were compared by SDS-PAGE/Colloidal Blue staining to ensure that the results obtained by each were comparable. Figures 8 & 9 show the results of this testing and indicate that based upon the figures provided, the small and large-scale digestions provided similar results for the Cry3Bb1.11231 protein. However, there appears to be a small difference in the digestion times for the

Cry3Bb1.11098 protein in small and large-scale digestions. This protein had took slightly longer for complete digestion in the large-scale digestion. However, this difference does not appear to be significant enough to affect the insect bioassay results. Therefore, the results of the insect bioassay should be applicable to both sizes of digestions.

Figure 10 shows the results of SIF small and large-scale comparisons. There was some difference in the digestion times for the small and large-scale digestions. However, each produced the same ~59 kDa protein and therefore appear the insect bioassay data is applicable to both.

III. DISCUSSION

Protein stability in simulated digestive fluids, specifically SGF, is one characteristic for food allergenic proteins which has been described. Although all proteins which have been identified as food allergens are stable to digestion, not all proteins which are stable to digestion are known to be food allergens. The tests performed in this study show that the Cry3Bb1 proteins are not stable to digestion in simulated gastric fluid. Incubation of Cry3Bb1.11098 and Cry3Bb1.11231 in SGF results in the loss of detectable protein by the 30 and 15 second observation point, respectively, as detected by SDS-PAGE. Insect bioassay data indicated that the protein loss insecticidal activity within 2 minutes of incubation in SGF. Incubation in the SIF resulted in a ~59 kDa digestion product that retained its insecticidal activity after at least 30 minutes incubation. These results will be combined with amino acid similarity and heat stability data to determine the potential allergenicity of this protein.

CLASSIFICATION: ACCEPTABLE. Cry3Bb1.11098 and Cry3Bb1.11231 do not appear to be stable to digestion in simulated gastric fluid. However, there appears to be a discrepancy between the results discussion and figures 2 & 4. The Cry3Bb1.11098 protein appeared to be significantly digested within 15 seconds and completely digested in less than 30 seconds based upon the figures provided. This differs somewhat with the discussion provided by the registrant which indicates that the protein was digested to undetectable levels between 30 and 60 seconds. These do represent drastically different digestion times, but the discrepancy should be addressed given that the submitted gel data do not verify the claims.

Reg. # 524-528
Volume 2 of 4

Page ___ is not included in this copy.

Pages 509 through 516 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist MTW

Secondary Reviewer: John L. Kough, Ph.D., Biologist JLK APR 25 2001

STUDY TYPE:	Product Characterization (885.1100)
MRID NO:	449043-08
TEST MATERIAL:	Cry3Bb1 Protein
PROJECT NO:	MSL # 15870; Study Number 99-640E
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Bioinformatics Analysis of *B.t.* Protein 11098 and *B.t.* 11231 Sequences Utilizing Toxin and Public Domain Genetic Databases
AUTHOR(S):	Ronald E. Hileman, Ph.D. and James D. Astwood, Ph.D.
STUDY COMPLETED:	July 28, 1999
CONCLUSION:	Several amino acid database comparison tools were employed to compare the amino acid sequence of Cry3Bb1.11098 and Cry3Bb1.11231 to known protein toxins. The TOXIN4 database was compiled to allow for comparison of Cry3Bb1.11098 and Cry3Bb1.11231 to these known toxin proteins. All of the protein similarities identified were to insecticidal proteins, with no similarity to proteins known to be toxic to humans and/or animals. Based upon this data, it does not appear that Cry3Bb1.11098 nor Cry3Bb1.11231 share significant structural, biological or immunological similarity with known protein toxins other than those affecting insects.
CLASSIFICATION:	ACCEPTABLE
GOOD LABORATORY PRACTICE:	No statement is provided regarding Good Laboratory Practice guidelines, but this type of study would not be subject to those guidelines

I. STUDY DESIGN

Test Material: Amino acid sequence of *Bacillus thuringiensis* Cry3Bb1 proteins as expressed in corn. The wild-type sequence of Cry3Bb1 is GenBank Accession number M89794. The two variants are Cry3Bb1.11098 and Cry3Bb1.11231 which differ by 5 and 4 amino acids from wild-type respectively (full sequences are found in Appendix 1) and have a codon corresponding to alanine inserted at position 2 of the Cry3Bb1 protein to facilitate cloning of the Cry3Bb1 gene (created an *Nco*I site).

Methods: The purpose of this study was to determine if there is any amino acid similarity between the Cry3Bb1 proteins and the amino acid sequence of known protein toxins.

1. Toxin database preparation:
This database was constructed using public domain databases (GenBank and EMBL GenPept version 108, PIR and NRL3D version 56 and SwissProt version 36). Protein sequences were retrieved using the STRINGSEARCH function (keyword = toxin) of GCG (version 9.1). The DATASET function was used to compile the individual toxin sequences into a database named TOXIN4 (4677 sequences).

2. Toxin database search for sequences similar to Cry3Bb1.11098 and Cry3Bb1.11231 proteins:
The SHUFFLE function of GCG (version 10.0) was used to a randomized sequence for the Cry3Bb1.11098 and Cry3Bb1.11231 proteins and this sequence was used to test for amino acid bias (negative control) in a similarity search of the parent Cry3Bb1 parent sequences against TOXIN4. TOXIN4 was searched using FASTA. Where sequence similarities were observed between the Cry3Bb1.11098 and Cry3Bb1.11231 proteins and toxins, the complete data file of TOXIN4 was retrieved. For the similar sequences identified, the scientific literature was consulted to verify that the similarity of "toxin" as either a true toxin or an accidental (irrelevant) protein sequence retrieved by the STRINGSEARCH program.

3. Public domain genetic database search for sequences similar to Cry3Bb1.11098 and Cry3Bb1.11231 proteins:
The Cry3Bb1.11098 and Cry3Bb1.11231 protein sequences were compared to all known proteins in the public domain databases using the ALLPEPTIDES database. Sequence similarity was assessed using the FASTA program of GCG.

II. RESULTS

1. Toxin sequence database (TOXIN4):
The keyword "toxin" was used to identify and retrieve amino acid sequences (4677 - although some may be duplicates since STRINGSEARCH may retrieve multiple entries of the same toxin). The sequences were assembled into a DATASET named TOXIN4.

2. Cry3Bb1.11098 and Cry3Bb1.11231 protein sequences are similar to insecticidal toxins:
The majority of sequences which shared similarity with Cry3Bb1.11098 and Cry3Bb1.11231 were B.t. δ-endotoxins. For each protein, 169 protein sequences which shared similarity were identified and 162 of these (96%) were B.t. δ-endotoxins. The other similarities where to mosquitocidal

520

proteins Cbm71 and Cbm72 (accession numbers Q45882, JC6033, X94146 and X99478, O05102 respectively). The Cbm71 sequence similarity occurs over a 579 aa overlap where there is ~26% similarity. This similarity has been previously identified (Crickmore et al., 1998), but according to the registrant, the protein is not immunologically related to any of the Cry toxins (no data provided). In the revised nomenclature, this protein is identified as Cry16Aa1. The Cbm72 protein (produced in *Clostridium bifermentans*) sequence also shares sequence similarity of about 27% over a 638 aa overlap with both Cry3Bb1 proteins. However, this protein does not show significant toxicity to mosquito larvae according to the registrant. Under the revised nomenclature, this protein is identified as Cry17Aa1. Additional similarity was identified with a *Caenorhabditis elegans* protein and a *Vibrio cholerae* toxin transport protein (not an actual toxin).The similarity with the *C. elegans* protein was about 24% over a 226 aa overlap with Cry3Bb1.11098 and a 230 aa with Cry3Bb1.11231. The *V. cholerae* protein showed about a 27% similarity over a 143 amino acid overlap with the Cry3Bb1 proteins. There was no significant homology to Cry3Bb1.11098 nor Cry3Bb1.11231 identified using the SHUFFLE function

3. *Public Domain Genetic Database Search for Sequences* Similar to Cry2Ab2 Protein:
Sequence similarity of the Cry3Bb1 proteins to proteins found in current public domain protein databases was determined. The majority of the sequence similarity to Cry3Bb1.11098 and Cry3Bb1.11231. Additional proteins identified were the *C. bifermentans* protein (described above) and a *Bacillus popilliae* parasporal crystal protein (Accession No. A62580, X99049 and Q45358). This similarity has been previously described (Zhang, et al., 1997) and is now identified as Cry18Aa1.

III. DISCUSSION

Several amino acid database comparison tools were employed to compare the amino acid sequence of Cry3Bb1.11098 and Cry3Bb1.11231 to known protein toxins. The TOXIN4 database was compiled to allow for comparison of Cry3Bb1.11098 and Cry3Bb1.11231 to these known toxin proteins. All of the protein similarities identified were to insecticidal proteins, with no similarity to proteins known to be toxic to humans and/or animals. Based upon this data, it does not appear that Cry3Bb1.11098 nor Cry3Bb1.11231 share significant structural, biological or immunological similarity with known protein toxins other than those affecting insects.

CLASSIFICATION: ACCEPTABLE

Reg. # 524-528

Volume 2 of 4

Page 527 is not included in this copy.

Pages ___ through ___ are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist MTW APR 25 2001

Secondary Reviewer: John L. Kough, Ph.D., Biologist JLK

STUDY TYPE:	Product Characterization (885.1100)
MRID NO:	449043-09
TEST MATERIAL:	Cry3Bb Protein
PROJECT NO:	MSL# 15873
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Bioinformatics Analysis of *B.t.* Protein 11098 and *B.t.* Protein 11231 Sequences Utilizing an Allergen Database
AUTHOR(S):	Ronald E. Hileman, Ph.D., and James D. Astwood, Ph.D.
STUDY COMPLETED:	July 30, 1999
CONCLUSION:	Several amino acid database comparison tools were employed to compare the amino acid sequence of Cry3Bb to known protein allergens and gliadins. The UPDATE2 database was compiled to allow for comparison of Cry3Bb1.11098 and Cry3Bb1.11231 to these proteins. The level of similarity identified does not indicate significant similarity to any of the proteins or gliadins contained in the database. In addition, no contiguous stretch of 8 identical amino acids was identified in either the FASTA or IDENTITYSEARCH algorithms suggesting a lack of immunological similarity. Based upon this data, it does not appear that Cry3Bb1 shares significant structural, biological or immunological similarity with known protein allergens or gliadins.
CLASSIFICATION:	ACCEPTABLE
GOOD LABORATORY PRACTICE:	No GLP statement is contained in this study. However, because it involved computer sequence comparisons only, the study would not be subject to GLP standards.

I. STUDY DESIGN

Test Material: Amino acid sequence of Cry3Bb1.11098 and Cry3Bb1.11231 protein as expressed in corn. Cry3Bb1.11098 has 5 amino acid changes from wild-type: D165G, H231R, S311L, N313T and E317K. Cry3Bb1.11231 has four amino acid changes from wild-type: H231R, S311L, N313T and E317K. In

addition to these changes, a codon corresponding to alanine was inserted at position 2 of the Cry3Bb1 protein to facilitate cloning of the Cry3Bb1 gene (created an *Nco*I site). The complete amino acid sequences of these two proteins are found in Appendix 1.

Methods: The purpose of this study was to determine if there is any amino acid similarity between the Cry3Bb1 proteins and the amino acid sequence of known protein allergens. Structural similarities were determined using FASTA and immunologically relevant similarities were determined using IDENTITYSEARCH. A sequence containing 8 or more linearly contiguous amino acid identities are considered relevant and would suggest further examination of relatedness.

1. Allergen and gliadin database preparation:
This database was constructed using public domain databases (GenBank and EMBL GenPept version 108, PIR and NRL3D version 56 and SwissProt version 36). A preliminary list (AllPeptides_121.strings) of 804 sequences was compiled using the keyword "allergen" within the STRINGSEARCH function of GCG. The preliminary list included duplicate sequences and non-allergens. A modified list (AllPeptides_121_ver1.strings) was compared to a previous allergen database (Metcalfe et al., 1996) and sequences not found in the previous database were merged to create a new list of 829 sequences (AllPeptides_121_ver2.strings). Duplicate sequences were identified and removed using a FASTA search on each sequences against the version 2 list. The file containing no duplicate sequences (AllPeptides_121_ver3.strings) contained 487 unique allergen and gliadin protein sequences. This list was used for a preliminary allergen database (UPDATE2) using DATASET of the GCG software.

The allergen database was finalized by merging 80 additional allergen sequences. These sequences were identified by comparison to UPDATE2 sequences to the publicly available list (available at: **ftp://biobase.dk/resources/pub/who-iuis/allergen.list**) and by searching the Internet PubMed and Entrez search engines. In some cases where the amino acid sequence was not available, the DNA sequence was translated using TRANSLATE (GCG Software). The 80 additional sequences were appended using the APPEND command of DATASET (GCG) creating a database of 567 unique allergen and gliadin protein sequences.

2. Allergen database search for sequences similar to Cry3Bb protein:
The SHUFFLE function of GCG (version 10.0) was used to a randomized sequence for both Cry3Bb1.11098 and Cry3Bb1.11231 test proteins and this sequence was used to test for amino acid bias in a similarity search of the Cry3Bb proteins against UPDATE2. This function served as a negative

524

control. The allergen and gliadin database was searched using FASTA. A significant similarity result was defined as sequence identity of at least eight contiguous amino acids.

3. Allergen database epitope sequence comparisons to Cry3Bb protein sequence using IDENTITYSEARCH:
An algorithm was developed to identify whether a linearly contiguous match of 8 amino acids existed between the test protein and sequences within the allergen database (UPDATE2). This algorithm compares the test protein with each protein in the allergen database using a sliding window of eight amino acids. The search attempts to identify 8 or more contiguous amino acids. An epitope of 8 amino acids was chosen to represent the smallest likely immunologically significant IgE binding epitope (Metcalfe et al., 1996).

II. RESULTS

1. Allergen and gliadin database (UPDATE2):
A database search using the STRINGSEARCH function of GCG and internet searches identified a database containing 567 unique allergen and gliadin protein sequences (UPDATE2; Appendix 2).

2. Cry3Bb1.11098 and Cry3Bb1.11231 protein sequences are not similar to allergens and gliadins:
The similarity search comparing the Cry3Bb protein sequences to the allergen/gliadin database (UPDATE2) revealed no sequences with significant similarity. A FASTA alignment was also preformed to identify structural similarity and immunologically relevant (i.e., 8 contiguous amino acid identity) similarity to protein within UPDATE2.

The best sequence similarity to Cry3Bb1.11098 and Cry3Bb1.11231 was a 65 amino acid overlap to *Aspergillus fumigatus* protein rAsp f 9. This protein shares 33.8% identical residues and 56.9% similar residues in the overlap. This similarity does not indicate significant biological or structural similarity. The complete FASTA output is shown in Attachments 3 and 4. The negative control sequences used in the SHUFFLE analysis also showed no significant similarities.

3. Cry3Bb1.11098 and Cry3Bb1.11231 protein sequences do not contain immunologically significant allergen epitopes:
As described above, the FASTA alignment did not identify a contiguous 8 amino acid stretch of identical amino acids shared between Cry3Bb1.11098 and Cry3Bb1.11231 and known protein allergens and gliadins. This likely shows a lack of immunological similarity with known protein allergens and

gliadins. In addition to the FASTA alignment, an IDENTITYSEARCH algorithm was used in an attempt to identify at least 8 contiguous identical amino acids. The results of this are shown in Appendix 5 and indicated a lack of similarity to known allergens and gliadins.

III. DISCUSSION

Several amino acid database comparison tools were employed to compare the amino acid sequences of Cry3Bb1.11098 and Cry3Bb1.11231 to known protein allergens and gliadins. The UPDATE2 database was compiled to allow for comparison of Cry3Bb1 to these proteins. The level of similarity does not indicate significant similarity to any of the proteins or gliadins contained in the database. In addition, no contiguous stretch of 8 identical amino acids was identified in either the FASTA or IDENTITYSEARCH algorithms suggesting a lack of immunological similarity. Based upon this data, it does not appear that Cry3Bb1.11098 nor Cry3Bb1.11231 share significant structural, biological or immunological similarity with known protein allergens or gliadins.

CLASSIFICATION: ACCEPTABLE

Reg. # 524-528

Volume 2 of 4

Page ___ is not included in this copy.

Pages 527 through 550 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist MTW

Secondary Reviewer: John L. Kough, Ph.D., Biologist JLK

STUDY TYPE:	Validated Detection Method - Cry3Bb1 Protein in Corn
MRID NO:	
TEST MATERIAL:	Cry3Bb1 Protein
PROJECT NO:	99-640E-1
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Validated Method for Extraction and Direct ELISA Analysis of Cry3Bb1 in Corn Grain
AUTHOR(S):	David Kolwyck, Bich-Phuong Tonnu, Yelena A. Dudin, Tesa Ploesser and Karen Gustafson
STUDY COMPLETED:	April 7, 2001
CONCLUSION:	The extraction and detection method as described for Cry3Bb1 protein appears to be adequate for analysis of Cry3Bb1 protein in corn grain. However, this method must be validated by both an independent laboratory and the EPA Biological and Economic Analysis Division laboratory before it can be considered a valid method.
CLASSIFICATION:	ACCEPTABLE, pending validation as described.
GOOD LABORATORY PRACTICE:	This is a detection method and is not subject to Good Laboratory Practice standards.

I. STUDY DESIGN

Methods:

A. Sample Preparation:
   1. Confirm identity of sample.
   2. Homogenize grain sample with dry ice using a vertical cutter mixer (sample size should be at least 15-20 g).
   3. Place processed sample in plastic bag and label. Once dry ice dissipates, samples should be placed at -80 °C.
   4. Rinse equipment with hot water and clean with appropriate detergent to clean equipment. Rinse again with hot water followed by acetone. Air dry.

B. Extraction of Cry3Bb1 Protein:
   1. Place processed frozen grain sample on dry ice until weighing.

2. Weigh enough sample for desired volume of extract. Record weight and buffer volume [for grain sample: use 1:100 tissue to buffer (PBST) ratio with approximately 0.1g ± 0.05 g].
3. Place extraction vessel on dry ice or at -80 °C until use.
4. Place extraction vessel on wet ice and add extraction buffer.
5. Homogenize sample for 60 seconds by immersing tip of blade ≥½" into extraction mixture and homogenize at 17, 500 rpm
6. Place extraction vessel on wet ice until use (< 4hours).

C. Preparation and Storage of Extracts:
1. Clean equipment to insure removal of potential contaminants.
2. Remove particulate matter from the extract by centrifugation (10' @ 9,700 x g, 4-8 °C) - this step can be repeated if pellet does not form. Carefully withdraw supernatant.
3. Aliquot samples into labeled tubes and store at -80 °C.

D. ELISA Analysis of Cry3Bb1 Protein in Corn Grain:
1. Protein-A purified polyclonal rabbit anti-Cry3Bb1 IgG is prepared in carbonate/bicarbonate buffer and placed into 96-well microtiter plates - overnight at ~ 4 °C.
2. After washing coated plates, samples are added and plates are incubated for 1 hour at 37 °C.
3. Plates are again washed and color development initiated with TMB.
4. The enzymatic reaction is terminated with phosphoric acid after about 10 minutes.
5. Absorbance is determined compared to a 7-point Cry3Bb1 reference curve ranging from 1 to 64 ng/ml.
6. Absorbance reading and protein concentration are fitted with a 4-parameter regression model.

II. RESULTS

A. Assay Acceptance Criteria

Criteria	Value/Range
Mean absorbance of buffer blank: $(L_1-L_2) + 3$ SD	- PBST/0.1% BSA: ≤0.094
Mean absorbance of highest standard (minus blank): $L_1-L_2 + 3$ SD	- PBST/0.1% BSA: ≥0.0950
% error of back-calculated concentration of standards	- 1 ng/ml standard: ≤20% - 2-64 ng/ml standards: ≤15%
R^2 of the standard curve	≥0.98

557

Positive QC (± 3 SD)	Lot specific criteria
Negative QC (+ 3 SD)	Lot specific criteria

B. Sample Acceptance Criteria:

Criteria	Value
% CV of quantifiable triplicate sample wells (based on concentration, value given as absorbance with blank subtracted)	
range of standards ≥1 and ≤ 2 ng/ml	20%
range of standards ≥2 and ≤ 64 ng/ml	15%

C. Performance Parameters:

Parameter	Value/Range		
LOD (negative extract tissue + 3 SD) - non-transgenic or negative extracts were used neat	**Tissue**	**ng/ml**	**µg/g fwt**
	corn grain	0.96	0.096
Range of quantitation	1-64 ng/ml		
Method for curve fit (SOFTmax PRO software version 2.4.1)	4-parameter		

D. Fresh Weight Expression Level:

This calculation is used to determine the µg/g fwt (fresh weight) expression level prior to correction for method bias. After this value is determined, the correction factor is used to determine the actual expression level in the grain.

µg/g fwt = (ng/ml of extract SOFTmax PRO)(DF*)(T:B ratio)/1000

* DF = dilution factor used before samples were loaded onto plate

E. Correction for Method Bias:

All expression data generated for a sample is corrected for method bias by dividing the µg/g fwt value by the appropriate correction factor (the bias correction factor is determined during ELISA validation).

Grain Type	Spike & Recovery	Extraction Efficiency	Bias Correction Factor (Spike & Recovery)(Extraction Efficiency)
Corn Grain	87.7%	70.9%	0.62

F. ELISA Validation Summary:

1. Accuracy

Tissue Type	Tissue to Buffer Ratio	Extraction Variability[1]	Extraction Efficiency[2]	Spike and Recovey[3]	Method Bias Correction Factor[4]
Corn Grain	1:100	21.1	87.7	70.9	0.62

2. Precision

- Standard Curve Precision Range[5]: 4.4-14.6% CV
- Intra-Assay Variability[5]: 6.1% CV
- Inter-Assay Variability[5]: 8..4% CV

3. Range of Quantitation

1.0 - 64 ng/ml

4. Sensitivity

Tissue Type	Dilution or Max Sample Volume (µl/well)	LOD (ng/ml)[6]	LOD (µg/g fwt)[7]
Corn Grain	Neat/(50)	0.96	0.096

554

1. Extraction variability was assessed by determining % CV (standard deviation divided by average and multiplied by 100%) of the Cry3Bb1 protein levels based on fourteen extractions of the same sample of corn grain.
2. Extraction efficiency for each tissue type was determined by extracting three replicates and re-extracting the same tissue several successive times.
3. Spike and recovery: grain from control corn plants were extracted with assay buffer spiked with known quantities of Cry3Bb1 protein at three concentrations spanning the range of quantitation. Each spike was performed in three replicates.
4. Method Bias Correction Factor: more specifically, mean recovery of the standard protein from spiked control tissues and extraction efficiency for transgenic tissues comprised the method bias which was calculated from the combined measurement of these two parameters.
5. The intra-assay precision was assessed by determining the %CV of the protein level in a particular sample within a single assay in triplicate wells. The inter-assay was assessed by measuring the concentration within a single assay in triplicate wells. The inter-assay precision was assessed by measuring the concentration of a positive sample in 10 ELISAs. Intra- and inter-assay variability were calculated using a one way analysis of variance (ANOVA).
6. The limit of detection (LOD) was calculated using the non-transgenic corn grain sample extracts. The LOD was calculated at the maximum sample loading volume of 50 µl/well as follows: LOD=mean (ng/ml) + 3 standard deviations.
7. LOD (µg/g fwt): the ng/ml values were converted to µg/g fwt by multiplying the LOD (ng/ml) by the tissue weight to buffer volume ratio (g/ml) and dividing by a conversion factor of 1000 ng/µg.

III. DISCUSSION

The extraction and detection methods described in this submission appear to be adequate to analyze Cry3Bb1 protein in corn samples. The detection method described uses Cry3Bb-specific antisera for direct ELISA analysis. In addition, the acceptance criteria for test results appear to be appropriate. However, this method must be validated by both an independent laboratory and by the EPA Biological and Economic Analysis Division laboratory before it can be considered a valid method for detection of Cry3Bb1.

CLASSIFICATION: ACCEPTABLE (pending validation as described above)

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist MTW APR 25 2001

Secondary Reviewer: John L. Kough, Ph.D., Biologist JLK

STUDY TYPE:	Validated Detection Method for Cry2Ab2 in Corn Grain
MRID NO:	
TEST MATERIAL:	Cry2Ab2 Protein
PROJECT NO:	99-819E-2
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Validated Method for Extraction and Direct ELISA Analysis of Cry2Ab2 Protein in Corn Grain
AUTHOR(S):	David Kolwyck, Karen Gustafson and Terry B. Stone
STUDY COMPLETED:	April 13, 2001
CONCLUSION:	The methods described appear to adequate for extraction and detection of Cry2Ab2 protein in corn grain samples. In addition, the acceptance criteria for the tests appear to be appropriate. However, this method must be validated by both an independent laboratory and by the EPA Biological and Economic Analysis Division laboratory before it can be considered a valid method for detection of Cry2Ab2.
CLASSIFICATION:	ACCEPTABLE, pending validation as described.
GOOD LABORATORY PRACTICE:	This is a detection method and is not subject to Good Laboratory Practice standards.

I. STUDY DESIGN

Methods:

A. Sample Preparation:
   1. Confirm identity of sample.
   2. Homogenize grain sample with dry ice using a Waring blender.
   3. Place processed sample in plastic bag and label. Once dry ice dissipates, samples should be placed at -80 °C.
   4. Rinse equipment with hot water and clean with appropriate detergent to clean equipment.

B. Extraction of Cry2Ab2 Protein:
   1. Place processed frozen grain sample on dry ice until weighing.

2. Weigh enough sample for desired volume of extract. Tissue to buffer ratio should be 1:100 (use approximately 0.20 g ± 0.05 g of processed grain material).
3. Place extraction vessel on dry ice or at -80 °C until use.
4. Inspect blade and make sure it rotates freely, then immerse blade in ethanol and run generator at maximum speed for 15-20 seconds.
5. Rinse generator with mQ water to remove ethanol.
6. Immerse blade in mQ water and run generator at maximum speed for 15-20 seconds.
7. Place extraction vessel on wet ice and add extraction buffer.
8. Homogenize sample for 60 seconds by immersing tip of blade ≥½" into extraction mixture and run generator twice for 30-35 seconds @ 17,500 rpm.
9. Place extraction vessel on wet ice until use (< 4hours).

C. Preparation and Storage of Extracts:
1. Clean equipment to insure removal of potential contaminants.
2. Remove particulate matter from the extract by centrifugation (10' @ 10,000 rpm, 4-8 °C). If pellet does not form, this step may be repeated 2x. Carefully withdraw supernatant.
3. Aliquot samples into labeled tubes and store at -80 °C.

D. ELISA Analysis of Cry3Bb1 Protein in Corn Grain:
1. Cry2Ab2-specific antibody is prepared in carbonate/bicarbonate buffer and placed into 96-well microtiter plates - overnight at ~ 4 °C.
2. After washing coated plates, samples are added and plates are incubated for 1 hour at 37 °C.
3. Plates are again washed and color development initiated with TMB.
4. The enzymatic reaction is terminated with phosphoric acid after about 10 minutes.
5. Absorbance is determined compared to a 7-point Cry3Bb1 reference curve ranging from 0.16 to 10 ng/ml.
6. Absorbance reading and protein concentration are fitted with a 4-parameter regression model.

II. RESULTS

A. Evaluation Criteria

Specification	Value/Range
OD of buffer blank	<0.13
OD of highest standard	> 0.90
Range for the standard (Cry2Ab2)	0.16-10 ng/ml

557

% C.V. for triplicate sample wells: for sample OD<OD of Std #6 for sample OD>OD of Std#6	 <25% <15%
Method for curve fit (SOFTmax PRO software)	4-parameter
R^2 of the standard curve	>0.98
Positive QC sample at pre-dilution (± 3 SD)	Lot specific criteria
Negative QC sample at pre-dilution (± 3 SD)	Lot specific criteria

- sample ODs below the OD of the lowest standard should be considered unreliable. Repeat the sample by decreasing the dilution or loading at a higher volume. Sample ODs above the OD of the highest standard should be considered unreliable. Repeat sample by increasing the dilution or loading at a lower volume.

B. Fresh Weight Expression Level:

This calculation is used to determine the µg/g fwt expression level prior to correction for method bias. After this value is determined, the bias correction factor is used to determine the actual expression level in the cottonseed:

µg/g fwt = (ng/ml of extract SOFTmax PRO)(DF*)(T:B ratio)/1000

* DF = dilution factor used before samples were loaded onto plate

C. Correction for Method Bias:

All expression data generated for a sample is corrected for method bias by dividing the µg/g fwt value by the appropriate correction factor (the bias correction factor is determined during ELISA validation).

- Bias correction factor = (extraction efficiency)(spike and recovery)

D. Cry2Ab2 ELISA Validation Summary

1. <u>Accuracy</u>

Tissue Type	Tissue to Buffer Ratio	Extraction Efficiency[1]	Spike and Recovey[2]	Correction Factor
Corn Grain	1:100	65%	103%	0.66

554

2. Precision

- Standard Curve Precision Range[3]: 2.0-9.91% C.V.
- Intra-Assay Variability[3]: ≤4.89% C.V.
- Inter-Assay Variability[4]: ≤20.9% C.V.

3. Range of Quantitation

0.32-20 ng/ml

4. Sensitivity

Tissue Type	Dilution	LOD (μg/g fwt)[5]
Corn Grain	1:8	0.44

1. Extraction efficiency for corn grain was determined by extracting two replicates per day over three different days, where the last extraction employed harsh buffer (such as 2M Urea/TBA).
2. To evaluate the analytical accuracy of the ELISA, extracts prepared from seed tissue of non-transgenic corn plants were spiked with known quantities of protein at three concentrations spanning the range of the standard curve.
3. An intra-assay precision range was generated for the Cry2Ab2 protein concentration in the assay's standard curve. The precision profile of the assay was generated by calculating the C.V. of each concentration in the assay's standard curve over ten individual assays using one-way analysis of variance. The intra-assay precision is the mean of the intra-assay precision values generated for each of the protein standards in the standard curve.
4. The inter-assay precision was assessed by determining the average C.V., using ANOVA of the amount of protein reported by the ELISA for the positive control sample among ≥10 independent trials.
5. The limit of detection was calculated as the mean value of protein reported by ELISA in non-transgenic corn grain plus three standard deviations. transgenic tissues comprised the method bias which was calculated from the combined measurement of these two parameters.

III. DISCUSSION

The extraction and detection methods described in this submission appear to be adequate to analyze Cry2AB2 protein in corn samples. The detection method described uses Cry2Ab2-specific antisera for direct ELISA analysis. In addition, the acceptance criteria for test results appear to be appropriate. However, this method must be validated by both an independent laboratory and by the

EPA Biologics and Economic Analysis Division laboratory before it can be considered a valid method for detection of Cry2Ab2.

CLASSIFICATION: ACCEPTABLE (pending validation as described above)

562

EPA Biological and Economic Analysis Division laboratory before it can be considered a valid method for detection of Cry2Ab2.

CLASSIFICATION: ACCEPTABLE (pending validation as described above)

561

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist MTW APR 25 2001

Secondary Reviewer: John L. Kough, Ph.D., Biologist JLK

STUDY TYPE:	Validated Detection Method for Cry2Ab2 in Cottonseed
MRID NO:	
TEST MATERIAL:	Cry2Ab2 Protein
PROJECT NO:	99-819E-1
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Validated Method for Extraction and Direct ELISA Analysis of Cry2Ab2 in Cottonseed
AUTHOR(S):	David Kolwyck and Karen Gustafson
STUDY COMPLETED:	April 13, 2001
CONCLUSION:	The Cry2Ab2 extraction and detection methods for use with cottonseed appear to be adequate. The methods provided include acceptance criteria for test results which appear to be appropriate. However, this method must be validated by both an independent laboratory and the EPA Biological and Economic Analysis Division laboratory before it can be considered a validated method.
CLASSIFICATION:	ACCEPTABLE, pending validation as described.
GOOD LABORATORY PRACTICE:	This is a detection method and is not subject to Good Laboratory Practice standards.

I. STUDY DESIGN

Methods:

A. Acid Delinting of Cottonseed:

1. Confirm identity of sample.
2. Place fuzzy seeds into 250 ml plastic beaker with holes. Place beaker with seeds into 500 ml beaker with sufficient acid to cover seeds making sure the seeds are completely wetted by the acid.
3. Mix the seeds and acid using a stainless steel spatula for 30 seconds. Repeat until all fiber coating is dissolved.
4. Remove beaker with seeds from acid and place under running water to rinse for 2 minutes.
5. Place beaker with seeds in saturated baking soda solution. Stir seeds occasionally with spatula and let soak until bubbling stops.

6. Remove and place beaker with seeds under running water for at least 30 seconds.
7. Place beaker with seeds into 50% ethyl alcohol solution. Scoop out any immature seeds that float to the top of the solution and discard.
8. Place beaker with remaining seeds under running water and rinse for one minute.
9. Empty seeds onto a flat mesh or wire screen tray and spread out for even drying.
10. Place trays into dryer (40 °C) overnight or until dry.
11. Rinse all containers, equipment and work surfaces with bicarbonate solution and then water.
12. Store acid delinted seeds in appropriately labeled container under ambient conditions for up to 3 months (or 4 °C for long-term storage).

B. Cottonseed Grinding
1. Homogenize desired amount of acid delinted cottonseed sample with dry ice using Waring blender.
2. Collect a processed sample sufficient for analysis and place into plastic bag and label. Place remaining homogenized sample back into original bag.
3. Processed cottonseed samples containing dry ice should remain outside freezer until dry ice dissipates. Then store at -80 °C until extraction.
4. Rinse and clean equipment.

C. Extraction of Cry2Ab2 Protein from Cottonseed
1. Place processed cottonseed on dry ice until weighing.
2. Weigh adequate amount of processed cottonseed for desired volume of extract. Use a tissue to buffer ratio of 1:60 and weigh out 0.20 g ± 0.05 g of processed cottonseed
3. Place extraction vessel on dry ice or at -80 °C until beginning extractions.

D. Initial Blade Inspection and Cleaning
1. Inspect blade to make sure that it rotates freely.
2. Immerse blade in ethanol and run generator for 15-20 seconds at max speed.
3. Rinse generator with mQ water to remove ethanol.
4. Immerse blade in mQ water and run generator for 15-20 seconds at max speed.

E. Sample Extraction
1. Place extraction vessel on wet ice and add appropriate volume of extraction buffer.
2. Homogenize ground cottonseed using Brinkmann Polytron generator. Mix twice for 10-30 seconds at 17,500 rpm.
3. Place extraction vessel on wet ice (< 4 hours).

F. Blade Cleaning
1. Rinse blade in mQ water.
2. Immerse blade in mQ water and run generator at maximum speed for 15-20 seconds.
3. Check blade for cleanliness.

4. Immerse blade in ethanol and run generator at maximum speed for 15-20 seconds.
5. If cleaning between extractions of non-replicate samples, repeat steps 1-3 before processing next sample.

G. Preparation and Storage of Cottonseed Extracts
1. Remove particulate matter from extract by centrifugation (10', 10,000 x g @ 4-8 °C). Remove supernatant.
2. Aliquot sample into labeled tubes. If sample is to be analyzed immediately, then keep on wet ice until analysis. If sample will not be analyzed immediately, store at -80 °C.

H. ELISA Analysis of Cry2Ab2 Protein in Cottonseed
1. Cry2Ab2-specific polyclonal rabbit antibody is prepared in carbonate/bicarbonate buffer and dispensed onto 96-well microtiter plates and coated overnight at ~ 4 °C - each ELISA is done in triplicate.
2. The plates are washed and the incubation step includes simultaneous addition of standards, quality control samples and unknowns with rabbit anti-Cry2Ab2 polyclonal antibody conjugated to horseradish peroxidase - 1 hour @ 37 °C.
3. Plates are washed and developed with TMB for ~ 10 minutes at room temperature.
4. The enzymatic reaction is terminated by addition of phosphoric acid and absorbance readings are determined .
5. The concentration of Cry2Ab2 is determined by reference to a 7-point standard curve ranging from 0.78-50 ng/ml.
6. Absorbance readings and protein concentrations are fitted with a four-parameter regression model.

II. RESULTS

A. Evaluation Criteria - the following criteria are used to evaluate the performance of the assay. Any assay that does not meet these criteria is not considered reliable.

Specification	Value/Range
OD of buffer blank	<0.080
OD of highest standard	0.935-2.098
Range for the standard (Cry2Ab2)	0.78-50 ng/ml
% C.V. for triplicate sample wells	<10%
Method for curve fit (SOFTmax PRO software)	4-parameter

564

R^2 of the standard curve	>0.95
Positive QC sample at pre-dilution (± 3 SD)	Lot specific criteria
Negative QC sample at pre-dilution	Lot specific criteria

- sample ODs below the OD of the lowest standard should be considered unreliable. Repeat the sample by decreasing the dilution or loading at a higher volume. Sample ODs above the OD of the highest standard should be considered unreliable. Repeat sample by increasing the dilution or loading at a lower volume.

B. Fresh Weight Expression Level:

this calculation is used to determine the µg/g fwt expression level prior to correction for method bias. After this value is determined, the bias correction factor is used to determine the actual expression level in the cottonseed:

µg/g fwt = (ng/ml of extract SOFTmax PRO)(DF*)(T:B ratio)/1000

* DF = dilution factor used before samples were loaded onto plate

C. Correction for Method Bias:

All expression data generated for a sample is corrected for method bias by dividing the µg/g fwt value by the appropriate correction factor (the bias correction factor is determined during ELISA validation).

Grain Type	**Spike & Recovery**	**Extraction Efficiency**	**Bias Correction Factor (Spike & Recovery)(Extraction Efficiency)**
Cotton seed	99.27	71.43	0.71

- Bias correction factor = (extraction efficiency)(spike and recovery)

F. ELISA Validation Summary:

1. <u>Accuracy</u>

565

Tissue Type	Tissue to Buffer Ratio	Extraction Efficiency[1]	Spike and Recovey[2]	Method Bias Correction Factor
Cotton seed	1:100	71.4%	99.3%	0.71

2. Precision

- Standard Curve Precision Range[3]: 7.5-14.4% C.V.
- Intra-Assay Variability[3]: ≤11.0% C.V.
- Inter-Assay Variability[4]: ≤15.0% C.V.

3. Range of Quantitation

1.56-100 ng/ml

4. Sensitivity

Tissue Type	Dilution	LOD (µg/g fwt)[5]
Cottonseed	1:100	3.35

1. Extraction efficiency for cottonseed was determined by extracting two replicates per day, over three different days, where the last extraction employed harsh buffer, such as 2M Urea/TBA buffer
2. To evaluate the analytical accuracy of the ELISA, extracts prepared from seed tissue of non-transgenic cotton plants were spiked with known quantities of protein at three concentrations spanning the range of the standard curve.
3. An intra-assay precision range was generated for the Cry2Ab2 protein concentration in the assay's standard curve. The precision profile of the assay was generated by calculating the %C.V. of each concentration in the assay's standard curve over ten individual assays using one-way analysis of variance (ANOVA). The intra-assay precision is the mean of the intra-assay precision values generated for each of the protein standards in the standard curve.
4. The inter-assay precision was assessed by determining the average %C.V., using ANOVA, of the amount of protein reported by the ELISA for the positive control sample among ≥10 independent trials.
5. The limit of detection was calculated as the mean value of protein reported by ELISA in non-transgenic cottonseed plus three standard deviations.

566

III. DISCUSSION

This submission provides both an extraction and ELISA detection method for Cry2Ab2 protein in cottonseed. The information provided appears to describe adequate methods for analysis of Cry2Ab2 protein in cottonseed. In addition the acceptance criteria for test results also appear to be adequate. However, this detection method must be validated by an independent laboratory, as well as the EPA Biological and Economic Analysis Division laboratory before it can be considered a validated method.

CLASSIFICATION: ACCEPTABLE, pending validation as described.

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist MTW APR 25 2001

Secondary Reviewer: John L. Kough, Ph.D., Biologist JLK

STUDY TYPE:	Product Characterization - Protein Equivalence (885.1100)
MRID NO:	451568-03
TEST MATERIAL:	Cry3Bb1 Protein (Event MON 863)
PROJECT NO:	99-01-39-31, MSL-16596
SPONSOR:	Monsanto Company, St. Louis, MO
TESTING FACILITY:	Monsanto Company, St. Louis, MO
TITLE OF REPORT:	Assessment of the Physicochemical Equivalence of Cry3Bb1.11098 and NPTII Proteins in Corn Event MON 863 to Microbial Sources
AUTHOR(S):	Gyula Holleschak, B.S., Ronald E. Hileman, Ph.D. and James D. Astwood, Ph.D.
STUDY COMPLETED:	April 26, 2000
CONCLUSION:	Based upon the data provided, it appears that both the Cry3Bb1.11098 and NPTII proteins produced in event MON 863 have equivalent molecular weights and antigenic properties with these same proteins produced in *B.t.* and *E. coli* respectively.
CLASSIFICATION:	ACCEPTABLE
GOOD LABORATORY PRACTICE:	This study was conducted in accordance with Good Laboratory Practice guidelines .

I. STUDY DESIGN

Test Materials and Methods:

A. *Equivalence Assessment of Bacterial-Produced and Plant-Produced Cry3Bb1.11098 and Cry3Bb1.11231 Proteins*:

Materials:

- Test Material - Root extract from event Mon 863 which contained both cry3Bb1.1098 and NPTII proteins. Roots were obtained from plants grown in the field. (NOTE: data for event MON 862 is also included in this study, but the line was dropped by the registrant for what they indicate to be "business decisions". Although the data is contained herein and reviewed, event MON 862 will not be considered as part of the overall assessment of Cry3Bb1.11098).

568

- Control Materials - Root extracts from non-transgenic corn lines MON 846 and MON 847 were used for antibody evaluation. Roots were also obtained from plants grown in the field.
- Reference Materials - Cry3Bb1.11098 protein (Lot# 6312803) produced in *B.t.* strain EG1098 and Cry3Bb1.11098 protein (lot# 6321003) expressed in event 96.040; NPTII protein expressed in *E. coli* (Lot# 6340505).

Methods:

A. Bridging Equivalence of Cry3Bb1.11098 Protein Produced in IPC Events MON 862 and MON 863 to Cry3Bb1.11098 Protein from IPC Event 96.040 and *B.t.*

1. Extraction of Cry3Bb1.11098 Protein from Root Tissue of IPC Event MON 862 and MON 863: Root samples of IPC events MON 862 and MON 863, as well as control lines MON 846 and 847 were homogenized separately in 15 ml tubes with extraction buffer (50 mM sodium borate, pH 9.7, 1 mM EDTA, 2.5 mM DTT, 0.5 mM PMSF, 1 mM benzamidine) at a 1:5 ratio (1 g tissue:5 ml buffer). An Omni 2000 homogenizer was used at high speed for 1 minute. The samples were clarified by centrifugation at 14,500 x g for 20 min and stored as 100µl aliquots at -80 °C.
2. SDS-PAGE: Aliquots of purified protein was diluted or concentrated as necessary and added to an equal amount of 2x Laemmli buffer, heated to 100 °C (~ 3 min) and loaded onto the NOVEX 4→20% polyacrylamide gels (SOP PB-EQP-005-01). Proteins were transferred to 0.45 µm nitrocellulose membrane and membranes were blocked with 5% NFDM (non-fat dry milk) in PBST. The blot was then incubated for 45 minutes at 25 °C with anti-Cry3Bb1 wild type IgG polyclonal antibody (Lot# 783) at a 1:5000 dilution. The membrane was probed using a 1:7500 dilution of goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Sigma # 1-9169, Lot# 77H4807). Immunoreactive bands were visualized with the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech).

B. Bridging Equivalence of NPTII Protein Produced in IPC Events MON 862 and MON 863 to NPTII Protein Produced by *E. coli*:

1. Extraction of NPTII Protein from Plant Tissues: Root samples of MON 846, MON 847, MON 862 and MON 863 were separately homogenized in extraction buffer [25 mM sodium phosphate, pH 7.00, 2.5 mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 0.05% N-lauroyl sarcosine and 0.05% triton X-100] - tissue to buffer ratio of 1 g root tissue/5 ml buffer. Samples were homogenized with the Omni hand-held homogenizer at high speed for ~ 1 min, clarified by

569

centrifugation for ~ 20 min (14,500 x g) and stored as 100 µl aliquots at -80 °C.

2. SDS-PAGE: NOVEX 4→20% polyacrylamide gels were used. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in 1x TBST for 45 minutes at room temperature. The membrane was incubated overnight at 4 °C with an anti-NPTII protein monoclonal antibody (Lot# 6340503) at 1:300 dilution in PBST containing 1% NFDM. Mouse IgG bound to the protein was probed with ~1:5000 dilution of goat anti-mouse secondary antibody conjugated to horseradish peroxidase in 1x TBST with 1% NFDM for 45 min. Immunoreactive bands were visualized with the ECL system.

II. RESULTS

A. Equivalence Assessment of Bacterial-Produced and Plant-Produced Cry3Bb1.1 1098 Protein:

Figure 1 shows the Western blot results comparing bacterial-produced and plant-produced Cry3Bb1 protein. Lanes 2-4 represent protein from *B.t.* (Lanes 3 & 4 spiked with non-transformed MON 846 & 847 respectively) and lanes 8 & 9 represent MON 862 & 863 respectively. Each of these protein bands appear co-migrate and therefore to be of the same molecular weight (~ 66 kDa). The 59 kDa band is also present (although the quality of the gel figure provided does not show this band in all lanes as expected). There are no protein bands visible in the negative control lanes (5 & 6) which both indicate the specificity of the antibodies and the lack of the Cry3Bb1 protein in these lines. The report also indicates that a larger (74 kDa) band is also present in lanes 8 & 9 (MON 862 & 863). This is apparently the full-length form of the Cry3Bb1.1098 protein which is apparently proteolytically process to the 66 kDa fragment which is usually observed.

B. Equivalence of NPTII Protein Produced in MON 862 & 863 to NPTII Protein Produced in *E. coli*:

Figure 2 shows the results of the comparison of the bacterial-produced and plant-produced NPTII protein. Comparison of lanes 2-4 (*E. coli*-produced) to lanes 7 & 8 show that the proteins co-migrated at the same rate and appear to share the same molecular weight. A lack of a signal in the control lanes (lanes 5 & 6) indicate that the antisera used in this study is specific for NPTII protein.

III. DISCUSSION

The results of this study indicated that the plant-produced Cry3Bb1 and NPTII proteins comigrated in SDS-PAGE gels with their respective bacterial-produced proteins. The plant-produced and

570

bacterial-produced proteins appear to share the equivalent molecular weights (based on gel comigration) and have similar immunoreactivity with antibodies specific for each protein. These same antibodies to Cry3Bb1 and NPTII did not produce a signal in the negative controls. Based upon this data, it appears that the molecular weights and antigenic properties of the plant-produced and bacterial-produced proteins are equivalent.

CLASSIFICATION: ACCEPTABLE

571

Reg. # 524-528

Volume 2 of 4

Page ___ is not included in this copy.

Pages 512 through 591 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

✓ FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.