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

OFFICE OF
PREVENTION, PESTICIDES, AND
TOXIC SUBSTANCES

AUG 24 2009

MEMORANDUM

Decision: 405910, 399686, 392086, 403859
DP Barcode: 363723, 363726, 363605, 359742

SUBJECT: Review of Monsanto's Corn Rootworm Monitoring Reports from 2005-2007 for Susceptibility to Cry3Bb1 and Revised Corn Rootworm Resistance Monitoring Plan for MON 88017, MON 88017 x MON 810, and MON 89034 x MON 88017 (EPA Reg. Nos. 524-528, 524-545, 524-551, 524-552, and 524-576; MRIDs 473547-01, 475231-01, and 469491-01)

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

FROM: Jeannette Martinez, Ecologist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

PEER REVIEW: Alan Reynolds, Entomologist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

Actions Requested:

BPPD[1] has been asked to review resistance monitoring data from 2005-2007 for *Diabrotica virgifera virgifera* LeConte (Western corn rootworm, WCR), which was submitted by Monsanto for Bt protected corn registrations expressing Cry3Bb1 as a condition of registration (MRIDs 469491-01, 473894-01, and 475231-01) as well as its revised monitoring plans for MON 88017, MON 88017 x MON 810 (MRID 473547-01), and MON 89034 x 88017. BPPD has summarized Monsanto's reports in this review and provides the Agency's conclusions and recommendations.

[1] The use of BPPD in this review refers to the BPPD IRM team consisting of Jeannette Martinez and Alan Reynolds.

CONCLUSIONS AND RECOMMENDATIONS

BPPD has identified some areas of concern with the revised monitoring plans for MON 88017 and its stacked products (MON 88017 x MON 810 and MON 89034 x MON 88017).

1. Monsanto's proposed response to unexpected pest damage is delayed (a 2 year reaction time) and too conservative. Specifically, the same growers would have to submit a report of unexpected pest damage in two consecutive years before Monsanto would collect CRW samples. Furthermore, monitoring for resistance is proposed to occur in an unfavorable manner with the biology of CRW. Specifically, Monsanto proposed to collect suspect/potentially resistant CRW ½ mile away from the nearest MON 88017 fields. BPPD notes that this type of collection will not serve the monitoring program well: the development of CRW resistance will likely be a localized event, and so by sampling ½ mile away from the nearest 88017 field, resistance may likely go undetected until crop failure occurs. Furthermore, while CRW have the ability to be very mobile, it is more common to observe local dispersal within or among adjacent fields. Monitoring for unexpected damage should ideally reveal the occurrence of localized resistance (or hot spots) before resistance spreads. Given this information, *BPPD recommends that Monsanto respond to reports of unexpected pest damage during the same growing season when the report has been filed or no later than July of the following planting season, and that monitoring samples for potentially resistant insects will be taken from within and adjacent fields where the pest damage has occurred.*

2. Monsanto further stated that if a refuge should not be available (i.e. flooding) to compare root damage of Bt and non-Bt plants, then the frequency of MON 88017 corn plants with one or more nodes destroyed should not exceed 50% of the sampled plants, or a Monsanto investigation would be triggered. *BPPD would like Monsanto to further clarify the total sample size of damaged roots that would be collected and inspected for the hopefully rare circumstance when there is no refuge in place but also for cases when refugia are available.* A limited sample size may be less informative than a larger sample size; *BPPD recommends that Monsanto determine in advance what sample size is needed to have enough power to detect a significant difference (>50%) in node injury (≥1).*

3. Monsanto reported in the revised monitoring report that baseline susceptibility information has been established by Siegfried et al. (2005) for WCR using bioassay techniques. *BPPD recommends strongly that Monsanto consider the use of sublethal seedling assays (SSA) to establish baseline susceptibility data and monitor for resistance of CRW.* Nowatzki et al. (2007) tested the sensitivity of SSA side-by-side with diet bioassay. They found that the SSA measuring survival and developmental shifts in age structure of larval populations was able to detect changes in susceptibility of CRW at a much finer scale than the diet bioassay. Diet bioassay endpoints (LC_{50} and EC_{50}) were relatively insensitive to detecting shifts in susceptibility, while the SSA was most sensitive to changes in susceptibility when selected individuals were present at ≤25%.

BPPD concludes and recommends the following regarding the monitoring reports covering the time period from 2005-2008:

4. *BPPD has reviewed the CRW monitoring data for Cry3Bb1 as expressed in MON 88017 and MON 88017 x MON 810, which was submitted by Monsanto for the corn growing seasons 2005-2007 and agrees with Monsanto's main conclusion that corn rootworm remain susceptible to Cry3Bb1 across its range in the US.*

5. BPPD notes that in 2006 Monsanto may have used the population samples from DM Crop Research Group, Inc. that were also provided to Dow and Syngenta for their resistance monitoring analyses. *BPPD recommends that sampling for resistance monitoring occur in such a manner that the focus be on areas where Monsanto's products are sold and have high adoption levels (acreage) by growers.*

6. Maintaining consistency in methodology and the environment facilitates with the interpretation of results and is an important aspect of any research experiment. Over the course of the last three years, Monsanto has used two different labs to conduct the rearing of corn rootworm insects (Crop Characteristics in 2005; Custom BioProducts in 2006 and 2007). *BPPD is hopeful that Monsanto will continue to collaborate with the latest lab in order to reduce environmental and methodological variation, and so that monitoring results can be viewed in a historical context.*

7. Monsanto stated that 170.8 $\mu g/cm^2$ might represent a putative diagnostic concentration when mortality plus stunting was considered and provided a range for the average mass of survivors in Bt treatments as well as controls. *BPPD notes that the 95% confidence intervals for average mass of survivors in Bt treatments and control treatments should not overlap should 170.8 $\mu g/cm^2$ be chosen as a putative diagnostic concentration.* This information was not provided in the 2007 monitoring report.

I. BACKGROUND

Corn rootworm (*CRW*) are among the most serious economic insect pests of corn in the United States (Levine & Oloumi-Sadeghi, 1991). Western corn rootworm (*Diabrotica virgifera virgifera*, WCR) are found from Mexico to the U.S. Corn Belt and Canada and are widely distributed; Northern corn rootworm (*D. barberi*, NCR) are found in the Midwest of the U.S and have a localized distribution. Together, they are the most prevalent *Diabrotica* pests in the US. The Mexican corn rootworm (*D. virgifera zeae*) has a distribution from Central America to the southern U.S. and is a sporadic problem in central Texas and southern Oklahoma. In 2003, the first transgenic (Bt) corn was registered to control CRW. Today, there are currently three registered Bt toxins available from different registrants to control target pest damage, all of which do not express a high dose against CRW. Simulation modeling supports that pests are at greater risk of evolving resistance to Bt crops when toxins are less than high dose (Tabashnik et al., 2004).

In February 2003, the Agency approved Monsanto's registration of *Bacillus thuringiensis* (Bt) Cry3Bb1 protein and the genetic material (Vector ZMIR13L) necessary for its production. Corn expressing the Cry 3Bb1 protein, designated event MON 863 (YieldGard Rootworm, EPA Reg. No. 525-528) by Monsanto, was the first Bt corn product registered to protect crops against corn rootworm (CRW) species.

In December 2005, MON 88017 and MON 88017xMON810 were approved for registration. MON 88017xMON 810 expresses Cry3Bb1 and Cry1Ab and is targeted against corn rootworm (CRW) larvae (Cry3Bb) and European corn borer (ECB)/stalk boring lepidopteran larvae (Cry1Ab). The product was created by conventional breeding in which MON 88017 (EPA Reg. No. 524-LLR) was crossed with MON 810 (YieldGard, EPA Reg. No. 524-489). The Cry3Bb1 toxin expressed in MON 88017 is the same as expressed in MON 863. The Cry3Bb1 protein produced in MON 88017 and MON 863 is a variant of the wild-type Cry3Bb1 protein from Bt subspecies *kumamotoensis*, whereas the Cry1Ab toxin originated from Bt subspecies *kurstaki*. When compared by amino acid sequencing, the Cry3Bb1 protein expressed in MON 88017 has been reported to be 99.8% similar to the Cry3Bb1 protein expressed in MON 863. The primary difference between the hybrids is that MON 88017 also expresses a gene for resistance to glyphosate (Roundup) based herbicides.

MON 89034 x MON 88017 is a plant-incorporated protectant that was registered for commercial use on June 10, 2008. MON 89034 expresses the Bt-derived insecticidal proteins Cry1A.105 and Cry2Ab2. The Cry1A.105 toxin is a "chimeric" protein containing domains I and II and the C-terminal from Cry1Ac and domain III from Cry1Fa (domain III). The Cry2Ab2 protein is exactly the same as that currently expressed in Monsanto's Bollgard II cotton.

II. PROPOSED MONITORING PLANS FOR MON 88017, MON 88017 X MON 810 (MRID 473547-01), AND MON 89034 X MON 88017 (no MRID assigned)

Monsanto states that monitoring for CRW resistance will consist of two main parts, namely: 1) monitoring for unexpected field damage by growers, extension agents, consultants, and company

agronomists, and 2) monitoring for resistance through targeted population sampling and testing. Monitoring for unexpected damage will reveal the occurrence of localized resistance (or hot spots) before resistance will have spread. Resistance monitoring through targeted field sampling should reveal changes in susceptibility of geographically representative populations.

Population sampling will focus on the WCR species, which will serve as a worst-case surrogate for Northern corn rootworm and Mexican corn rootworm. Because of their widespread distribution and abundance but similarity in life cycles compared to NCR and MCR, it is much more likely that resistance due to exposure to MON 88017 would evolve in WCR first.

Monsanto proposed to focus its geographic sampling in areas where MON 863 and MON 88017 adoption has been highest and selection pressure is greatest. These areas are 1) Eastern Illinois and Western Indiana, 2) Western Illinois, Iowa, and Missouri, and 3) Nebraska and Kansas. The breakdown into these three regions has been determined based on the three WCR biotypes found in the U.S.: soybean, wild type, and organophosphate resistant Western corn rootworm. Monsanto's proposed to target between 4-6 populations, but no less than three, in these areas with different biotypes. Also, not all states specifically listed above may be represented by the sample collection. Actual sample sites are decided by DM Crop Research Group based on beetle abundance and environmental conditions. Monsanto stated that based on a periodic review of MON 88017 sales information sample areas may need to be modified and that the Agency would be informed of such changes.

Since Cry3Bb1 as expressed in MON 88017 is not high dose, it will not control CRW at the same level as registered Lepidoptera protected Bt corn products. Hence, this requires a different approach to discern between unexpected pest damage and damage caused by CRW due to non-high dose control. Monsanto reported that the 2002 SAP Advisory Panel suggested consideration of the following factors specifically related to CRW resistance monitoring:

- CRW survival and some degree of root damage are expected in fields planted with MON 88017
- A single corn root system supports numerous rootworm larvae. Therefore, the effect of resistant individuals on the overall root structure will not be easy to detect unless the resistant individuals represent a significant proportion of the population on that root system.
- Root damage caused by corn rootworm larvae feeding is not readily visible; plants must be dug up and roots washed to assess damage.
- Above-ground symptoms of root damage, such as lodging, often have causes other than larval feeding (e.g. high winds in combination with high soil moisture content).
- Environmental factors can be significant determinants of the amount of damage caused by rootworm larvae.

Monsanto stated that Performance Inquiries of unexpected damage in MON 88017 fields would trigger an investigation. The root damage in the Bt field must be found greater than the root damage in the refuge corn (all else being equal), which has to be greater than 1.0 on a scale of 0-3. When a comparable refuge is not available (i.e. refuge flooded), then the following shall serve as guidelines:

- Average root damage in the MON 88017 field is >1.5 on the 0-3 nodal injury scale (Oleson et al. 2005).
- The frequency of MON 88017 corn plants with one or more nodes destroyed exceeds 50% of the sampled plants.

Monsanto further stated that if the above conditions were met and complaints of unexpected pest damage were received from the same growers in two consecutive years that Monsanto would attempt to collect CRW populations from the fields the following year. Collections would be undertaken when CRW flight is at its peak and approximately ½ mile away from the nearest MON 88017 fields.

In 2005, Monsanto began collecting baseline susceptibility information from 12 CRW populations from CO, IA, IL, KS, NE, MN, NY, and PA (Siegfried et al., 2005) and found little variation in susceptibility among populations at different geographic locations. The data indicate that bioassays can be used to develop a monitoring program. Monsanto proposed to collect between 1500-2500 CRW adults per population during the peak flight season (July throughout August) from non-Bt pumpkin and corn fields. No more than one population will be collected per county. BPPD will be provided with date of collection, crop type, location, and GPS coordinates. Custom BioProducts, Inc. in Maxwell, IA, will subsequently mate collected adults and rear their offspring in the lab as well as conduct bioassays.

Monsanto's report stated that population susceptibility will be assessed using a diet-based bioassays approach as described by Siegfried et al. (2005). The dose-response curve will be determined for each population and compared to historical data from populations in the same regions. A diagnostic concentration for Cry3Bb1 should be established using baseline susceptibility and annual monitoring data as well as other historical information for WCR and Cry3Bb1. Once a diagnostic concentration has been established, approximately 400 neonates (4 replicates total) will be bioassayed at the assumed discriminating concentration.

Unexpected survivors at the discriminating dose will be reared to adults and mated amongst themselves or single-pair mated with individuals from a susceptible lab colony if numbers are low. The resulting progeny will once more be exposed to the diagnostic concentration bioassays to determine heritability of survival on a Cry3Bb1-containing diet. If heritability is confirmed, survivors will be placed on MON 88017 corn plants to assess whether level of resistance is enough to cause severe root damage. Whether an increase in susceptibility has occurred will be assessed with a discriminating concentration bioassay when such dose has been established. Until then, either of the following criteria will serve to confirm resistance, however, Monsanto stated that their working definition of resistance will be refined based upon continued research and experience:

- The LC_{50} of the standard bioassay exceeds the 95% confidence interval of the mean historical LC_{50} for susceptible pests according to the baseline measurements
- Over 50% of Cry3Bb1 expressing plants have $\geq$1 root nodes destroyed by suspected resistant populations under controlled lab conditions

Monsanto proposed a 2.5 year time frame from the moment of initial detection of CRW resistance to MON 88017 to the actual implementation of an appropriate remediation plan. This is the same time frame that has been proposed for Lepidopteran resistance in Bt corn. MON 88017 does not express Cry3Bb1 at high dose and hence, resistance is likely additive and potentially dominant. ABSTC (2003) demonstrated in their report that if resistance is additive and dominant, the resistance allele frequency needs to be detected at 0.03 and 0.002, respectively, to allow detection 2.5 years before the population has become resistant. The sample size needed is 1000 insects in order to detect a particular allele frequency with 80% or 95% confidence when resistance is incomplete or dominant. Failure to detect resistance with 1000 genomes suggests that the resistance allele frequency is less than 0.001. The upper 80% and 95% confidence limits of this estimate (0.001) are 0.0016 and 0.003, respectively, which is in agreement with the allele frequencies (0.03 and 0.002) that need to be detected for a 2.5 year reaction time before a population has become resistant.

Monsanto's proposed remedial action plan and steps are more suitable to situations where field resistance is detected through product performance monitoring and subsequently confirmed in the lab. These steps may include:

- Confirm that resistance is heritable;
- Confirm field resistance;
- Use crosses to determine the nature of resistance;
- Estimate the r-allele frequency in the original population;
- Determine whether the r-allele frequency is increasing by analyzing field collections;
- Sample from the site in subsequent years where the resistant allele(s) was originally collected and determine if resistance is still detectable;
- Determine the geographic distribution of the r-allele by analyzing field collections in subsequent years from sites surrounding the site where the resistant allele was originally collected;
- If the r-allele frequency is determined to be increasing or spreading, design an appropriate remedial action plan based on the knowledge of the genetics and level of resistance it confers in the field; and
- The remedial action plan will be similar to that previously described by MON 863.

Monsanto concluded that "The various steps outlined in this insect resistance monitoring plan will increase the likelihood that any potential resistance to CRW from plantings of MON 88017, MON 88017 x MON 810, and MON 89034 x MON 88017 will be detected at an early enough stage to allow confirmation and characterization of resistance, so that the economic impact to growers is minimized".

III. BPPD'S REVIEW OF MONITORING PLANS

Overall, the revised monitoring plans for MON 88017 and its stacked products (MON 88017 x MON 810 and MON 89034 x MON 88017) are similar to the revised monitoring plan for MON 863. BPPD has identified some areas of concern and will focus its discussion on those items.

Monsanto has also tried to address some of BPPD's concerns with the revised monitoring plan for MON 863 (BPPD 2004) in their revised monitoring plan for MON 88017 and its stacks.

Under the Performance Inquiry section of the revised monitoring plan, Monsanto's proposed response to unexpected pest damage is not proactive and aggressive enough. 1) The proposed response to unexpected pest damage is delayed and too conservative. Specifically, the same growers would have to submit a report of unexpected pest damage in two consecutive years before Monsanto would collect CRW samples. 2) Monitoring for resistance is proposed to occur in an unfavorable manner with CRW biology. Specifically, Monsanto proposed to collect putative resistant CRW ½ mile away from the nearest MON 88017 fields. BPPD notes that this type of collection is not supported by the pest's biology and will not serve the monitoring program well. The development of CRW resistance will likely be a localized event; hence, by sampling ½ mile away from the nearest 88017 field, resistance may likely go undetected until crop failure occurs. Furthermore, while CRW have the ability to be very mobile, it is more common to observe local dispersal within or among adjacent fields. Monitoring for unexpected damage should ideally reveal the occurrence of localized resistance (or hot spots) before resistance spreads. Given this information, *BPPD recommends that Monsanto respond to reports of unexpected pest damage during the same growing season when the report has been filed or no later than July of the following planting season, and that monitoring samples for potentially resistant insects will be taken from within and adjacent fields where the pest damage has occurred.* Monsanto further stated that if a refuge should not be available (i.e. due to flooding) to compare root damage of Bt and non-Bt plants, then the frequency of MON 88017 corn plants with one or more nodes destroyed should not exceed 50% of the sampled plants, or a Monsanto investigation would be triggered. *BPPD would like Monsanto to further clarify the total sample size of damaged roots that would be collected and inspected for the hopefully rare case that there is no refuge in place and also for cases when refugia are available.* A limited sample size may be less informative than a larger sample size; *BPPD recommends that Monsanto determine in advance what sample size is needed to have enough power to detect a significant difference (>50%) in node injury (≥1).*

With respect to the geographic sampling/monitoring program in place to screen for MON 88017 resistance genes in CRW populations, Monsanto stated that it would focus its sampling efforts in areas of highest MON 88017 adoption (likely greatest selection pressure) and the three regions that have been determined based on the corn rootworm biotypes: soybean (eastern IL, western IN), wild type (western IL, IA, and MO), and organophosphate resistant (NE, KS). Monsanto stated that it would occasionally reassess the sampling regions based on sales information and inform the Agency of such changes. *BPPD notes that it is acceptable to reassess the sampling regions should new sales information result in a different geographic distribution of MON 88017 and as long as the Agency is informed during the same year that collections are planned.*

Monsanto reports in the revised monitoring report that baseline susceptibility information has been established by Siegfried et al. (2005) for WCR using bioassay techniques. *BPPD recommends that Monsanto also explore the sublethal seedling assay (SSA) as described by Nowatzki et al. (2007) as a tool to monitor for WCR susceptibility to Cry3Bb1 as expressed in MON 88017 (and MON 863) and its stacked products.* BPPD will discuss this method (SSA) in more detail in the 'monitoring results' section of BPPD's review.

Monsanto stated that it would conduct an F1 screen with unexpected survivors at the discriminating dose using a Cry3Bb1-containing diet in order to determine heritability of survival. If heritability is confirmed, survivors will be placed on MON 88017 corn plants to assess whether level of resistance is enough to cause severe root damage. Monsanto further stated that an increase in susceptibility would be assessed with a discriminating concentration bioassay when such dose has been established. *BPPD recommends that Monsanto explore the Sublethal Seedling Assay methodology in addition to bioassays since this new tool has shown to be more sensitive at detecting shifts in CRW susceptibility to Bt proteins (Nowatzki et al., 2007).*

IV. MONITORING RESULTS (2005) (MRID 469491-01)

As a condition of registration, Monsanto is required to conduct annual resistance monitoring for corn rootworm by field collecting the target pest from different geographic locations. Using a larval diet bioassay, Monsanto assessed the potential changes in target pest susceptibility to Cry3Bb1 as expressed in MON 88017 corn.

Methodology:
Monsanto collaborators collected samples from fifteen adult western and eight northern corn rootworm populations at 23 distinct locations in the US Corn Belt. Crop Characteristics in Farmington, MN maintained the adult collections and eggs collected from ovipositing females using standard rearing procedures. Eggs were allowed to undergo diapause in a refrigerated environment and upon completion of diapause were sent to the University of Nebraska for bioassay analysis.

The bioassay protocol used was developed by Monsanto Company (Pleau et al., 2002). Prior to hatching, the eggs were washed with an anti-fungicidal solution to minimize microbial contamination. Neonates were transferred into 96-well plates containing artificial diet developed for CRW and overlaid with solutions containing Cry3Bb1 at five different concentrations. The overlay solutions were prepared from purified crystal Cry3Bb1 protein, diluted in 0.05% Triton X-100, placed in wells containing the diet (10 µL/well), and allowed to dry for 1 hour before neonates were placed into wells. Larvae were allowed to feed for four to seven days depending on when microbial contamination set in.

There were between 1-4 replications of each treatment for each population depending on the number of neonates available. Probit analysis was applied to the mortality data, and results were expressed as LC_{50}s; non-linear regression was applied to % growth inhibition data (relative to control), and results were expressed as EC_{50}s.

Results and Discussion:
For WCR, LC_{50} values ranged from 0.31 µg/cm^2 to 4.59 µg/cm^2. Monsanto stated that this 14.8 fold difference in susceptibility was reported to be similar to baseline variability observed in Lepidopteran pests of corn and variation observed in collections obtained from 2002-2004 by the University of Nebraska researchers. The EC_{50} values ranged from 0.29-2.64 µg/cm^2 and overlap with the LC_{50} range reported; this range is not as wide as the range obtained for mortality data.

For NCR, LC_{50} values ranged from 0.42 µg/cm^2 to 1.36 µg/cm^2. The EC_{50} values ranged from 0.25-1.28 µg/cm^2 and, as was seen with WCR, overlap with the LC_{50} range reported.

Sublethal effects of Cry3Bb1 of WCR and NCR were not as apparent as was seen with lepidopteran active toxins where growth inhibition concentrations (EC_{50} values) were below mortality concentrations (LC_{50} values).

Monsanto concluded that the susceptibility of WCR and NCR to Cry3Bb1 appeared to be similar. The variations in susceptibility (see Table 1) were ascribed to natural variation in responses of the populations tested.

Table 1. Susceptibility of WCR and NCR exposed to Cry3Bb1 as estimated by EC_{50} and LC_{50} values (2005 Data)

Population	Target Pest	Sample Size (N)	Mean *EC_{50}* (95% CI) (µg/cm^2)	Mean *LC_{50}* (95% CI) (µg/cm^2)
New Castle, DE	WCR	417	1.07 (0.9-1.3)	3.34 (2.4-4.5)
Rice Co., MN		413	1.06 (0.9-1.2)	4.59 (0.8-14.9)
Rice Co., WI		58	0.87 (0.6-1.2)	1.11 (0.1-2.5)
Scott Co., IA		71	0.29 (0.1-0.5)	0.48 (0.1-1.0)
Henry Co., IL		344	1.90 (1.5-2.5)	3.20 (0.3-9.3)
LaSalle Co., IL		106	0.42 (0.4-0.5)	0.31 (0.02-0.8)
Story Co., IL		169	0.74 (0.5-1.0)	1.27 (0.2-3.1)
Whiteside Co., IL		292	0.88 (0.7-1.2)	1.11 (0.3-2.3)
Seward Co., NE		80	2.64 (0.6-8.5)	3.30 (0.7-6.6)
Range			*0.29-2.64*	*0.31-4.59*
Franklin Co., IA	NCR	52	0.79 (0.76-0.8)	0.94 (0.3-1.6)
Fillmore Co., MN		119	0.69 (0.1-1.6)	1.23 (0.1-3.5)
Freeborn Co., MN		88	1.28 (1.1-1.5)	1.36 (0.4-2.6)
Kandiyohi Co., MN		40	0.75 (0.7-0.8)	0.85 (0.5-1.3)
Rice Co., MN		155	0.25 (0.1-0.4)	0.42 (0.1-0.9)
Range			*0.25-1.28*	*0.42-1.36*

Modified from MRID 469491-01

V. MONITORING RESULTS (2006)

Objective:
Determine susceptibility of field collected neonate WCR from geographically distinct populations to Bt Cry3Bb1 protein using bioassays.

Methodology:
In July and August of 2006, DM Crop Research Group (Granger, IA) collected samples from 15 distinct WCR populations from Nebraska, Iowa, and Illinois. Eggs from each colony were sent to Custom BioProducts in Maxwell, IA, where the dose-response bioassays were conducted with Cry3Bb1 protein. The bioassay protocol followed was a combination of Pleau et al. (2002) and Siegfried et al. (2005).

As in 2005, the Cry3Bb1 protein used for bioassays was obtained as a solution (4.0 g/mL) from Monsanto. The solution was diluted with 0.1% Triton-X 100 to obtain a series of concentrations (0.56, 1.67, 5.0, 15.0, and 45.0 µg of Cry3Bb1/cm^2) against which newly hatched neonates were tested according to procedures outlined in Section III of this review (methodology). However, dose response bioassays were conducted with 4 to 6 replicates for each population.

Statistical analysis was conducted by Dr. Siegfried and colleagues at the University of Nebraska as outlined in Section III of this review.

Results and Discussion:
Fourteen of the 15 colonies collected in 2006 produced viable eggs and were maintained as separate colonies. The population sample collected from Livingston Co. (IL) produced <0.5% viable eggs and could not be bioassayed.

Fungal contamination in the wells required that bioassays were shortened to four days. Dose-responses from the insects were not affected and contamination and control mortality were reduced by reading the test results during the shortened time frame.

The sample collections came from geographic areas where historically WCR infestations and the adoption of Cry3Bb1 are high and where the risk for resistance evolution is the highest. The assay results indicated that WCR remained susceptible to Cry3Bb1 in these high risk areas. The observed mean EC_{50} values ranged from 0.64 µg/cm^2 to 1.88 µg/cm^2; these EC_{50} values were similar to the 2005 results. The observed mean LC_{50} values ranged from 1.43 µg/cm^2 to 22.22 µg/cm^2; this range was higher than the 2005 results. Monsanto believed that this increase was artificial and due to the shorter assay time. While shorter assay durations were able to measure effects on development, mortality typically was underestimated. Some larvae in that sample survived exposure to Cry3Bb1 without growing but would have died if the bioassay had been run for a longer period of time. Monsanto noted that the mean EC_{50} value for the LaSalle County sample was 1.21 µg/cm^2 and comparable to other populations, while the mean LC_{50} was 22.22 µg/cm^2 and greater than the mean LC_{50} value for other populations.

Monsanto ascribed the slight difference in susceptibility among populations tested to natural variation in response only and concluded that WCR remained susceptible to Cry3bb1 throughout its range.

Table 2. Susceptibility of WCR exposed to Cry3Bb1 as estimated by EC_{50} and LC_{50} values (2006 Data)

Population	Replicates	Mean EC_{50} (95% CI) ($\mu g/cm^2$)	Mean LC_{50} (95% CI) ($\mu g/cm^2$)
York Co., NE	5	1.58 (1.0-2.4)	9.73 (4.4-21.7)
Seward Co., NE	6	1.27 (0.9-1.7)	9.27 (3.3-22.4)
Sarpy Co., NE	6	1.32 (0.9-1.8)	7.06 (2.2-18.4)
Filmore Co., NE	6	1.25 (0.5-2.5)	10.21 (4.7-22.1)
Clay Co., NE	6	0.78 (0.5-1.1)	3.19 (1.2-6.4)
Scott Co., IA	6	1.67 (1.2-2.3)	6.62 (4.5-8.5)
Polk Co., IA	5	0.83 (0.7-1.0)	1.43 (0.7-2.4)
Franklin Co., IA	6	1.78 (1.1-2.7)	9.87 (3.5-36.1)
Pottawattamie Co., IA	6	1.02 (0.3-2.1)	7.20 (4.6-11.4)
Hardin Co., IA	4	0.64 (0.4-0.9)	5.32 (1.3-17.8)
Henry Co., IL	6	1.88 (0.5-6.5)	5.58 (0.6-22.0)
Bureau Co., IL	6	0.92 (0.6-1.2)	6.83 (3.2-14.3)
Whiteside Co., IL	6	1.45 (1.0-2.1)	5.07 (2.0-11.2)
LaSalle Co., IL	6	1.21 (1.0-1.5)	22.22 (12.9-47.1)
Range		*0.64-1.88*	*1.43-22.22*

Modified from MRID 473894-01

VI. MONITORING RESULTS (2007)

Objective:
Continue ongoing monitoring of susceptibility to Bt Cry3Bb1 protein in geographically distinct populations of WCR by contrasting susceptibility to lab strain as well as data from previous years.

Methodology:
DM Crop Research Group made field collections; Custom Bio Products maintained the colonies, and the University of Nebraska conducted the bioassays. The materials and methods remained the same in 2007; however, the form of Cry3Bb1 used was soluble rather than crystalline which was the form of Cry3Bb1 used in previous years. The duration of bioassays was four days (*no explanation was provided as to why the switch to soluble protein occurred*); the maximum concentration that could be used in the concentration-response assays was 170.8 µg of Cry3Bb1/cm^2 of diet.

The University of Nebraska performed statistical analyses using SAS and Probit Analysis to determine EC_{50}, EC_{95}, LC_{50}, and LC_{90} values as well as goodness of fit for each population.

Results and Discussion:
Table 3 shows that, much like in previous years, the LC_{50} values (50.18-289.25 $\mu g/cm^2$) were more variable than the EC_{50} values (14.20-33.46 $\mu g/cm^2$). Higher LC_{50}'s were an artifact of the assay system because larvae were capable of surviving for some time without feeding. EC values were primarily used to make inferences about CRW field susceptibility.

The EC_{50} values represented a 2.4 fold difference in CRW susceptibility which was representative of the difference in susceptibility (2.6 fold) found in populations collected during

the 2006 corn growing season. The actual values, however, were higher because Monsanto switched from a crystalline Cry3Bb1 preparation used in the bioassays to a soluble form of Cry3Bb1. Hence, the magnitude of EC values was a result of the protein standard rather than a change in susceptibility.

Monsanto stated that 170.8 µg/cm^2 might represent a putative diagnostic concentration when mortality plus stunting was considered. At this concentration, % mortality ranged from 46.72 – 80.56 and average mass of survivors across replicates ranged from 0.06 mg - 0.13 mg. As a reference, the average mass of survivors in the control treatments across all populations was 0.35 mg.

Monsanto concluded that WCR remained susceptible to Cry3Bb1 across its range in the US.

Table 3. Susceptibility of WCR exposed to Cry3Bb1 as estimated by EC_{50}/EC_{90} and LC_{50}/LC_{90} values (2007 Data)

Population	Sample Size (N)	Mean EC_{50} (95% CI) (µg/cm^2)	Mean EC_{90} (95% CI) (µg/cm^2)	Mean LC_{50} (95% CI) (µg/cm^2)	Mean LC_{90} (95% CI) (µg/cm^2)	Slope	Chi^2 Prob
Clinton Co., IA	6	15.66 (12.2-19.0)	177.53* (94.4-312.7)	85.37 (60.6-127.4)	842.77* (423.9-3011)	1.289	0.52
Hamilton Co., IA	10	33.46 (27.2-40.8)	463.47* (233.9-831.5)	90.03 (69.2-117.0)	596.90* (372.4-1324)	1.560	0.17
Hardin Co., IA	6	16.45 (11.0-21.8)	310.79* (121.7-721.5)	106.78 (74.8-176.1)	1434.95* (611.8-7550)	1.136	0.26
Howard Co., IA	6	20.80 (15.4-26.7)	257.23* (113.7-520.2)	64.76 (26.2-137.9)	495.77* (200.3-16,492)	1.450	0.78
Scott Co., IA	6	15.46 (11.2-19.6)	507.30* (223.4-1082)	63.79 (44.2-98.2)	1224.78* (508.1-7059)	0.999	0.35
Story Co., IA	7	19.90 (13.4-26.7)	356.98* (131.6-855.6)	83.10 (59.0-117.7)	686.99* (376.6-312.7)	1.397	0.35
DeKalb Co., IL	6	18.55 (10.0-27.4)	764.31* (184.9-2755)	158.19 (96.9-378.7)	3028.30* (885.5-85041)	1.000	0.09
Henry Co., IL	6	16.24 (8.16-27.22)	82.49 (28.2-206.9)	50.18 (18.2-125.0)	461.03* (163.6-44990)	1.331	0.91
Lee Co., IL	6	26.08 (18.35-35.10)	438.35* (160.0-1034)	225.47* (122.3-846.5)	7548.61* (1545-53,519)	0.840	0.15
McLean Co., IL	6	17.93 (15.29-20.57)	312.39* (196.2-479.9)	87.62 (62.4-125.4)	**652.86*** (357.9-2053)	1.469	**0.01**
Wolford Co., IL	7	28.65 (23.29-34.70)	375.74* (194.8-661.9)	154.3 (94.5-356.0)	897.3* (377.1-29,475)	1.676	0.73
Buffalo Co., NE	6	27.30 (17.79-38.71)	683.20* (197.5-1978)	289.25* (145.0-1509)	7039.76* (1399-1E+6)	0.924	0.82
Clay Co., NE	6	17.04 (14.82-19.25)	299.46* (200.0-436.8)	115.31 (87.3-168.5)	892.5* (476.3-2676)	1.442	0.09
Seward Co., NE	6	14.20 (10.1-18.00)	165.22 (78.2-324.1)	64.22 (45.4-89.8)	532.69* (301.4-1475.6)	1.395	0.11
Monsanto Lab	5	12.91 (10.21-15.42)	53.61 (31.1-85.6)	22.29 (15.8-28.9)	**97.03** (72.4-148.8)	2.006	**0.01**
Range		*12.91-33.46*	*53.61-764.31*	*22.29-289.25*	*97.03-1E+6*		

Modified from MRID 475231-01

*LC_{50} values above the highest concentration tested (170.8 µg/cm^2) are extrapolated estimates.

Bolded LC_{95} values represent values were goodness of fit is best.

VII. BPPD REVIEW OF MONITORING DATA (2005-2007)

BPPD has reviewed the CRW monitoring data for Cry3Bb1 as expressed in MON 88017 and MON 88017 x MON 810, which was submitted by Monsanto for the corn growing seasons 2005-2007 and agrees with Monsanto's main conclusion that corn rootworm remain susceptible to Cry3Bb1 across its range in the US.

BPPD notes that in 2006 Monsanto may have used the population samples from DM Crop Research Group, Inc. that were also provided to Dow and Syngenta for their resistance monitoring analyses. *BPPD recommends that sampling for resistance monitoring occur in such a manner that the focus be on areas where Monsanto's products are sold and have high adoption levels (acreage) by growers*

The variability and inflation in EC_{50} and LC_{50} values (Table 3) appear to be an artifact due to switching the form of Cry3Bb1 protein used for the 2007 bioassays; however the magnitude of the ranges for EC values from 2006 (2.9 fold difference) compared to 2007 (2.6 fold difference) have remained similar. The inclusion of a susceptible lab strain in 2007 as a point of reference is very helpful and allows Monsanto and BPPD to discern between anomalies in the data due to changes in procedures and decreased CRW susceptibility in the field. For this purpose Monsanto should continue to include susceptible lab strains as a reference in future bioassays.

The inclusion of 'mortality plus stunting' in the 2007 report as a measure to assess susceptibility to Cry3Bb1 is useful. It is evident from the 2007 data that LC_{50} and extrapolated LC_{95} values from diet bioassays are not meaningful in determining a precise dose response for corn rootworm populations to Bt crystal proteins. For this particular pest and toxin, measuring sublethal effects (i.e. mortality plus stunting) is more sensitive at assessing onsets of deleterious effects rather than % mortality measures.

Maintaining consistency in methodology and the environment facilitates with the interpretation of results and is an important aspect of any research experiment. Over the course of the last three years, Monsanto has used two different labs to conduct the rearing of corn rootworm insects (Crop Characteristics in 2005; Custom BioProducts in 2006 and 2007). *BPPD is hopeful that Monsanto will continue to collaborate with the latest lab in order to reduce environmental and methodological variation as much as possible and so that results can be viewed in a historical context.* Furthermore, for the 2005 results no mention was made with respect to the range of concentrations that corn rootworm samples were exposed to; for 2006, a range was provided but not all concentrations were reported. For 2007, Monsanto reported all bioassay concentrations used to the Agency. However, the report lacked slopes and Chi^2 probability results for the EC_{90} probit analysis. On the other hand, Monsanto reported slope and Chi^2 probabilities for the LC_{90} values, which the report dismissed because LC_{50} values were more variable and an artifact of the diet bioassay system.

Monsanto stated that 170.8 $\mu g/cm^2$ might represent a putative diagnostic concentration when mortality plus stunting was considered and provided a range for the average mass of survivors in Bt treatments as well as controls. *BPPD notes that the 95% confidence intervals for average*

mass of survivors in Bt treatments and control treatments should not overlap if 170.8 μg/cm^2 is chosen as a putative diagnostic concentration. This information was not provided with the 2007 monitoring report.

BPPD recommends that Monsanto also consider the use of sublethal seedling assays (SSA) to establish baseline susceptibility data and monitor for resistance of CRW. A diagnostic concentration for CRW will probably not work like one for Lepidopteran. This is why the SSA is more important. Nowatzki et al. (2007) tested the sensitivity of SSA side-by-side with diet bioassay. They found that the SSA measuring survival and age structure of larval populations in three potential instar groups was able to detect shifts in susceptibility of CRW at a much smaller scale than the diet bioassay, which measured mortality and growth inhibition responses. Diet bioassay endpoints (LC_{50} and EC_{50}) were relatively insensitive to detecting shifts in susceptibility (treatments were 0%, 5%, 25%, and 50% selected individuals mixed into susceptible population samples of CRW), while the SSA was most sensitive to changes in susceptibility when selected individuals were present at ≤25%. Nowatzki et al. (2007) stated that the SSA may be a more sensitive tool to measure shifts in susceptibility than the bioassay because it uses the increased sensitivity of a sublethal measure (developmental shifts of larvae into three instar stages/cohorts). Nowatzki et al. (2007) used the quantile-quantile analysis and conducted pair-wise contrasts of all baseline populations to estimate among population variation of susceptibility.

VIII. REFERENCES

ABSTC. 2003. Updated monitoring Plan for Bt corn. ABSTC unpublished report submitted to the Agency on January 31, 2003.

Head, G.P. 2008. Corn Rootworm Resistance Monitoring Plan for MON 88017 (EPA Reg. No. 524-551) and MON 88017 X MON 810 (EPA Reg. No. 542-552). Monsanto unpublished report to the Agency, MRID 473547-01.

Lang, B. 2007. 2006 Season Monitoring for the Susceptibility of Neonate Western Corn Rootworm Larvae to the Bacillus thuringiensis Cry3Bb1 protein. Monsanto unpublished report to the Agency, MRID 473894-01.

Lang, B. 2008. 2007 Season Monitoring for the Susceptibility of Neonate Western Corn Rootworm Larvae to the Bacillus thuringiensis Cry3Bb1 protein. Monsanto unpublished report to the Agency, MRID 475231-01.

Levine, E. and Oloumi-Sadeghi, H. 1991. Management of diabroticite rootworms in corn. Ann Rev Entomol. 36: 229-255.

Nowatzki, T., S.A. Lefko, R.R. Binning, S.D. Thompson, T.A. Spencer, and B.D. Siegfried. 2008. Validation of a novel resistance monitoring technique for corn rootworm (Coleoptera: Chrysomelidae) and event DAS-59122-7 maize. J. Appl. Entomol. 132:177-188

Pleau, M.J., J.E. Huesing, G.P. Head, and D.J. Fier. 2002. Development of an artificial diet for the western corn rootworm. Ent. Exp. Appl. Vol. 105: 1-11

Siegfried, B., T. Spencer, and T.T. Vaughn. 2005. Baseline susceptibility of western corn rootworm (Coleoptera: Chrysomelidae) to the Cry3Bb1 Bacillus thuringiensis toxin. J. Econ. Entomol. Vol. 98: 1320-1324.

Siegfried, B., T. Spencer, and B. Koehler. 2006. Susceptibility of Neonate Rootworm Larvae to the Cry3Bb1 toxin from Bacillus thuringiensis: 2005 Data Summary. Monsanto unpublished report to the Agency, MRID 469491-01.

Tabashnik, B.E., F. Gould, and Y. Carrière. 2004. Delaying evolution of insect resistance to transgenic crops by decreasing dominance and heritability. J. Evol. Biology 17: 904-912

Other:

BPPD, 2004. Technical review of resistance monitoring plan, IRM compliance assurance program, technology stewardship agreement system, and annual confirmation system submitted by Monsanto for MON 863 Bt corn registration. Memo from T. Milofsky to M. Mendelsohn, June 03, 2004.

13544

R184102

Chemical Name: Bacillus thuringiensis Cry1Ab protein and the genetic material necessary for its production (via elements of p2062) in Event 3243M corn

PC Code: 006505
HED File Code: 41500 BPPD Tox/Chem
Memo Date: 8/24/2009
File ID: 00000000
Accession #: 000-00-0135

HED Records Reference Center
8/25/2010