



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

OFFICE OF PREVENTION,  
PESTICIDES AND TOXIC  
SUBSTANCES

FEB 11 2009

**MEMORANDUM**

**SUBJECT:** Addendum to the Environmental Risk Assessment for Plant-incorporated Protectant (PIP) Vip3Aa20 protein as expressed in Event MIR162 maize (*Zea mays*) to review its associated combined PIP products: Bt11 x MIR162 (expressing *Bacillus thuringiensis* Cry1Ab and Vip3Aa20 proteins, respectively) [EPA Reg. No. 67979-RG] and Bt11 x MIR162 x MIR604 (expressing *Bt* Cry1Ab, Vip3Aa20, and modified Cry3A, respectively) [EPA Reg. No. 67979-RE]; [MRID No: 471372-07, -08, -10, 471374-03 and 471530-05, Decision No. 379488 and 379490; DP Barcode: 345910 and 345913]; submitted by Syngenta Seeds, Inc.- Field Corps- NAFTA.

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**THROUGH:** Zigfridas Vaituzis, Ph.D. Senior Scientist  
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**TO:** Jeannine Kausch, Regulatory Action Leader  
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**ACTION REQUEST:** To review the environmental fate and effects data in support for Sec. 3 Registration for Bt11 x MIR162 and Bt11 x MIR604 x MIR162 combined PIP corn products.

**CONCLUSION:**

At present, the Agency has not identified any significant adverse effects of the Vip3Aa variants, Cry1Ab, and mCry3A proteins on the abundance of non-target organisms (NTOs) in any field population, whether expressed individually or as combined PIP products: Bt11 x MIR162 and Bt11 x MIR162 x MIR604. The potential for synergistic effects has been evaluated and the data that were reviewed for the individual parental events (Bt11, MIR162, and MIR604) can be bridged to support the Sec. 3 Registration of the associated combined PIP products: Bt11 x MIR162 and Bt11 x MIR162 x MIR604.

It is unlikely that direct or indirect harmful effects to NTOs, including federally-listed threatened or endangered species, would result from the insecticidal proteins Vip3Aa in Event MIR162 corn or in combination as with Event Bt11 and/or Event MIR604 corn, expressing Cry1Ab and mCry3A proteins, respectively, as a result of the proposed Sec. 3 registration. The Agency anticipates that for full commercial cultivation, no hazard will result to the environment.

#### **I. Environmental Risk Assessment for Bt11 x MIR162 and Bt11 x MIR162 x MIR604 Corn Hybrids**

##### **SUMMARY**

Syngenta Seeds, Inc. – Field Corps- NAFTA submitted an application and is seeking Sec. 3 Registration for two combined PIP products- Bt11 x MIR162 [EPA Reg. No. 67979-RG] and Bt11 x MIR162 x MIR604 [EPA Reg. No. 67979-RE], as expressed in transgenic corn [*Zea mays*]. These combined PIP products were created by crossing events: Bt11, MIR162, and MIR604 corn (expressing *Bt* Cry1Ab, Vip3Aa20, and mCry3A insecticidal transgenic proteins, respectively), via traditional breeding methods.

Vip3Aa and Cry1Ab proteins are intended to control several lepidopteran pests of corn including: *Agropis ipsilon* (black cutworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigui* (beet armyworm), *Heliothis virescens* (tobacco budworm), and *Helicoverpa zea* (corn earworm). Modified Cry3A protein will provide additional control for coleopteran pests of corn including: *Diabrotica virgifera virgifera* (western corn rootworm) and *Diabrotica longicornis barberi* (northern corn rootworm). The Bt11 x MIR162 corn hybrid expresses both the Cry1Ab and Vip3Aa20 proteins, while the Bt11 x MIR162 x MIR604 expresses Cry1Ab, Vip3Aa20, and mCry3A.

It was previously established by the Agency that the relative potency of plant-produced Cry1Ab, Vip3Aa20, and mCry3A proteins is similar to their corresponding microbial-produced proteins, indicating that plant-produced protein was similar in toxicity to the microbial-produced protein (Barsoum, 2008; Edelstein, 2008; and Waggoner and Kough, 2008). Each event also had comparable protein expression levels to the Bt11 x MIR162 and Bt11 x MIR162 x MIR604 corn hybrids (MRID No. 470176-07; Waggoner and Kough, 2008). Since no systematic increase was observed in expression of Cry1Ab, Vip3Aa20, and mCry3A proteins in the combined PIP products compared to the individual events, the margins of exposure that were previously determined for the insecticidal proteins in the individual events can be cited for the risk assessments of these proteins in the stacked hybrid.

For environmental risk assessment purposes, development of new non-target species data would not be required if there is no indication of synergistic effects or increased levels of proteins expressed in the combined PIP products. If these two points are confirmed, then the reviewed non-target data and the environmental risk assessments for the combined and single PIP lines are applicable to the Bt11 x MIR162 and Bt11 x MIR162 x MIR604 corn. Previous studies to determine the potential for synergistic effects were conducted (via sensitive insect

diet-incorporation bioassays) on subsets of the following pesticidal mixtures: Cry1Ab and Vip3Aa proteins and Cry1Ab and mCry3A proteins. In addition, new data on the effects of combined Cry1Ab, Vip3Aa, and mCry3A proteins were also developed to confirm this hypothesis. The results of the interaction study show that there is no change in the level of activity among susceptible insects when the three traits are combined. Therefore, the reviewed non-target data and the environmental risk assessments for the single PIP lines are applicable to the Bt11 x MIR162 and Bt11 x MIR162 x MIR604 corn hybrids. As a result the environmental risk assessment of Cry1Ab, Vip3Aa, and mCry3A proteins concludes that there will be no unreasonable adverse effects to the environment, including endangered species, by either Bt11 x MIR162 and Bt11 x MIR162 x MIR604 corn.

## **BACKGROUND**

Vip3A is a novel class of recently discovered insecticidal proteins that occur naturally in *Bacillus thuringiensis* (*Bt*), a gram-positive soil bacterium (Estruch, *et al.* 1996). The vegetative insecticidal proteins are produced during vegetative bacterial growth and are secreted as soluble proteins into the extracellular environment. Syngenta Seeds, Inc. developed Event MIR162, a corn line that expresses *Bt* insect control protein, known as Vip3Aa.

Although Vip3Aa protein shares no homology with Cry1Ab or other known Cry proteins, extensive testing by Syngenta has established that Vip3Aa demonstrates similar toxicity against larvae of certain lepidopteran species, including key pests of corn. While the modes of action differ between Vip and Cry proteins, the general symptoms displayed by sensitive lepidopteran larvae following ingestion of Vip proteins resembles that caused by Cry1Ab proteins (*i.e.*, cessation of feeding, loss of gut peristalsis, overall paralysis of the insect, and death) (Yu, *et al.*, 1997). Since the effects of Vip and Cry proteins are considered similar, the studies submitted on non-target organisms for Event MIR162 were conducted and evaluated according to the same environmental risk assessment criteria of previously reviewed PIP products containing Cry protein. This approach was previously used for the review and registration of Event COT102 cotton (US EPA, 2007).

Syngenta submitted additional data on the potential synergistic interaction between Vip3Aa, Cry1Ab, and mCry3A proteins and these data are summarized in this report to demonstrate the lack of synergism among the proteins and support the Sec. 3 registration of Bt11 x MIR162 and Bt11 x MIR162 x MIR604 combined PIP products.

## **ENVIRONMENTAL ASSESSMENT**

This document is an addendum to the Agency's environmental risk assessment for Vip3Aa20 protein expressed in Event MIR162 (Waggoner and Vaituzis, 2008b) reviewing MIR162's associated combined PIP products: Bt11 x MIR162 and Bt11 x MIR162 x MIR604 maize hybrids. On November 26, 2008, the Vip3Aa20 protein produced in Event MIR162 corn (EPA Reg. No. 67979-14) was granted a Sec. 3 registration for use as a PIP (Waggoner and

Vaituzis, 2008b). The MIR162 environmental risk findings are summarized in Section A below.

The second protein expressed in the Bt11 x MIR162 combined PIP product is Cry1Ab protein, providing protection against the European corn borer and other lepidopteran pests. The Cry1Ab protein produced in Event Bt11 corn (EPA Reg. No. 524-528) was reassessed in 2001 (US EPA, 2001) and the Bt11 environmental risk findings are summarized in Section B below.

The third protein expressed in the Bt11 x MIR162 x MIR604 combined PIP product is mCry3A, which provides resistance to western corn rootworm and northern corn rootworm. The mCry3A protein produced in Event MIR604 corn (EPA Reg. No. 524-528) was granted a Sec. 3 registration in addition to its associated stacked product Bt11 x MIR604 (US EPA, 2007). The MIR604 environmental risk findings are summarized in Section C below.

#### **A. Event MIR162 (lepidopteran active) Environmental Risk Assessment**

Potential adverse effects to non-target organisms by Vip3Aa protein has been reviewed (Waggoner and Vaituzis, 2008a and b). The following is a summary of the Vip3Aa environmental risk assessment.

Event MIR162 corn specifically expresses Vip3Aa20<sup>1</sup>, a variant of the naturally occurring Vip3Aa1 protein isolated from *Bacillus thuringiensis* strain AB88, differing from the Vip3Aa1 protein by one amino acid. Another Vip3Aa protein variant is also expressed in the recently registered Event COT102 cotton as Vip3Aa19 and in Syngenta's experimental Event Pacha corn. The Agency previously determined that "all proteins designated as Vip3Aa are more than 95% identical," and "there is sufficient information to support the safety of all Vip3Aa proteins" (Edelstein, 2008). All the previously submitted data developed for Vip3Aa protein can be cited in support of the registration of Event MIR162 corn.

Prior to registration of the *Bt* Vip3Aa20, EPA conducted ecological risk assessments on plants, wild mammals, birds, fish, aquatic invertebrates, estuarine and marine animals, earthworms, terrestrial non-target insects (including honey bee adults and larvae, parasitic wasps, green lacewings, several lady beetle species, rove beetles, minute pirate bugs, springtails (*Collembola* toxicity/reproduction)), and field evaluations of the effects on several Vip3Aa protein variants. The Vip3Aa exposure to non-target invertebrates, soil degradation/persistence studies and an endangered species impact assessment were reviewed and found acceptable (US EPA, 2008; Waggoner and Vaituzis, 2008a and b). In addition, gene flow and weediness assessments via pollen and Cry protein DNA uptake by plants and soil microorganisms were also performed. EPA concluded that there is sufficient information to believe that there is no risk from the registered uses of Vip3Aa cotton or corn to non-target wildlife, aquatic, and soil organisms.

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<sup>1</sup> Prior to receiving the Crickmore designation of Vip3Aa19, the protein produced in Events COT102 and Pacha were referred to as VIP3A, Vip3A or Vip3Aa.

At present, the Agency is aware of no identified significant adverse effects of Vip3Aa protein on the abundance of non-target beneficial organisms in any population in the field environment, whether they are pest parasites, pest predators, or pollinators. Further, the EPA believes that cultivation of Event MIR162 corn may have fewer adverse impacts on non-target organisms than use of chemical pesticides for corn production, because under normal circumstances, MIR162 corn requires substantially fewer applications of chemical pesticides, compared to production of non-*Bt* corn. Fewer chemical insecticide applications generally result in increased populations of beneficial organisms that control secondary pests, such as aphids and leafhoppers. In addition, no adverse effect on Federally-listed endangered and threatened species is expected from the proposed lepidopteran-resistant corn registration. Furthermore, the EPA has determined that there is no significant risk of gene capture and expression of Vip3Aa protein by wild or weedy relatives of corn in the U.S., its possessions, or territories. Available data do not indicate that Vip proteins have any measurable adverse effect on microbial populations in the soil, nor has horizontal transfer of genes from transgenic plants to soil microbes been demonstrated.

In conclusion, the risk assessment found no hazard to the environment from cultivation of Event MIR162 corn expressing Vip3Aa protein.

#### **B. Event Bt11 (lepidopteran active) Environmental Risk Assessment**

Potential adverse effects to non-target organisms by Cry1Ab protein has been reviewed (US EPA, 2001). The following is a summary of the Cry1Ab environmental risk assessment.

Prior to registration of the first *Bt* plant-incorporated protectants in 1995, EPA conducted ecological risk assessments for all *Bt* Cry proteins expressed in potato, corn, and cotton. EPA evaluated studies of potential effects on a wide variety of non-target organisms that might be exposed to the *Bt* protein (US EPA, 2001). This included Cry1Ab protein as expressed in Syngenta's Event Bt11 corn. EPA performed risk assessments on plants, wild mammals, birds, fish, aquatic invertebrates, estuarine and marine animals, earthworms, terrestrial non-target insects (including honey bee adults and larvae, parasitic wasps, green lacewings, several lady beetle species, springtails (*Collembola* toxicity/reproduction), monarch butterflies), field evaluations of the effects of Cry1Ab exposure on non-target invertebrates, soil degradation/persistence studies, and an endangered species impact assessment (US EPA, 2001). In addition, gene flow and weediness assessments via pollen and Cry protein DNA uptake by plants and soil microorganisms were also performed. EPA concluded that there is sufficient information to believe that there is no risk from the uses of Cry1Ab corn to non-target wildlife, aquatic, and soil organisms.

At present, the Agency is aware of no identified significant adverse effects of Cry protein on the abundance of non-target organisms in any population in the aquatic or terrestrial field environment, whether they are animals, plants, 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 Cry1Ab corn than non-*Bt* corn. Multi-year invertebrate abundance studies do not show a shift in biodiversity in Cry1Ab corn fields,

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 invertebrate populations.

The movement of Cry1Ab transgenes from the host plant into weeds and other crops was also considered. The Agency determined that there is no significant risk of gene capture and expression of Cry1Ab protein by wild or weedy relatives of corn in the U.S., its possessions or territories. The fate of Cry1Ab protein in soils and indirect effects on soil biota have also been evaluated (US EPA, 2001). The 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 (Milofsky and Vaituzis, 2006). 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 microbes has not been demonstrated (Sanvido, *et al.* 2007). Published studies of *Bt* Cry protein in soil show no effect on bacteria, actinomyces, fungi, protozoa, algae, nematodes, springtails or earthworms (Saxena and Stotzky, 2001). In addition, new plants grown in *Bt* Cry protein-containing soil do not take up the *Bt* protein.

In conclusion, the risk assessment found no hazard to the environment from cultivation of Event Bt11 corn expressing Cry1Ab protein.

### **C. MIR604 (coleopteran active) Environmental Risk Assessment**

Potential adverse effects to non-target organisms by modified Cry3A (mCry3A) protein has been reviewed (US EPA, 2007). The following is a summary of the mCry3A environmental risk assessment.

For registration of mCry3A as expressed in Event MIR604 corn, EPA reviewed studies conducted on representative non-target species and performed risk assessments on plants, wild mammals, birds, fish, aquatic invertebrates, estuarine and marine animals, earthworms, terrestrial non-target insects (including honey bee adults and larvae, rove beetles, minute pirate bugs, carabid beetles, lady beetles), and soil degradation/persistence studies (US EPA, 2007). In addition, gene flow and weediness assessments via pollen and Cry protein DNA uptake by plants and soil microorganisms were also performed. EPA concluded that there is sufficient information to believe that there is no risk from the uses of mCry3A corn to non-target wildlife, aquatic, and soil organisms.

An endangered species impact assessment of possible effects on Hungerford's crawling water beetle was performed for this registration. Hungerford's crawling water beetle species is currently known to occur in only six streams - five in mostly northern Michigan and one in Ontario, Canada. These are not major corn growing areas. The beetles are found in the cool riffles of clean, slightly alkaline streams. All streams where this beetle has been found have moderate to fast water flow, good stream aeration, inorganic substrate, with an open to

partially open canopy just below beaver dams or similar human-made structures. Adults prefer gravel and cobble riffles while larvae occupy areas with slower current and dense growth of microalgae, especially Chara. Since the Hungerford's crawling water beetle larvae are reported to feed on filamentous algae (and possibly periphytic diatoms), no dietary exposure to anti-coleopteran Cry protein in corn tissue is expected. Therefore, the No Effect (NE), direct or indirect, from cultivation of anti-coleopteran Cry protein containing corn to Hungerford's crawling water was confirmed.

At present, the Agency is aware of no identified significant adverse effects of Cry protein on the abundance of non-target organisms in any population in the aquatic or terrestrial field environment, whether they are animals, plants, 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 mCry3A corn than non-Bt corn. Two year invertebrate abundance studies do not show a shift in biodiversity in Cry3Bb1 corn fields, 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 invertebrate populations.

Furthermore, the EPA has determined that there is no significant risk of gene capture and expression of mCry3A protein by wild or weedy relatives of corn in the U.S., its possessions, or territories. The fate of mCry3A protein in soils and indirect effects on soil biota have also been evaluated (US EPA, 2007). Available data do not indicate that Cry proteins have any measurable adverse effect on microbial populations in the soil, nor has horizontal transfer of genes from transgenic plants to soil microbes been demonstrated.

In conclusion, the risk assessment found no hazard to the environment from cultivation of Event MIR604 corn expressing mCry3A protein.

#### **D. Synergism studies**

The purpose of these studies was to characterize the potential for interaction between the lepidopteran-active proteins Vip3Aa, Cry1Ab, and coleopteran-active mCry3A proteins. In order to bridge the ecological effects and environmental fate data of the individual parental events to the combined PIP products, the effects of the pesticidal mixture of the combined PIP product must be tested on a susceptible pest species, via diet-incorporation bioassays. Interactions between the test materials can be assessed by comparing the larval mortality observed for the mixed proteins with the predicted responses based on the bioassay of each protein individually. If there is no greater mortality than expected over the range of concentrations in a sensitive pest species, it is likely that there will be no synergism of the mixture against non-target organisms.

### **i. Potential Interactions in between Cry1Ab and mCry3A proteins**

#### **MRID No. 467956-04**

A study was conducted to assess the combined effects of Cry1Ab and mCry3A insecticidal proteins on two sensitive insect species: European corn borer (ECB, *Ostrinia nubilalis*) and Colorado potato beetle (CPB, *Leptinotarsa decemlineata*). A series of dilutions were conducted in which 1<sup>st</sup> instar ECB and CPB larvae were exposed to a high and a low concentration of the first protein (Cry1Ab or mCry3A), represented by the LC<sub>70</sub> and LC<sub>30</sub>, respectively, in combination with a high concentration of the second protein (mCry3A or Cry1Ab), represented by the LC<sub>90</sub> to the corresponding sensitive species. Neither the ECB nor the CPB data revealed evidence of synergistic or antagonistic interactions between Cry1Ab and mCry3A, after analysis of the proportional mortality of sensitive bioassay species at the intended endpoint of the experiments. These results indicated that the effect of a mixture of mCry3A and Cry1Ab on non-target Lepidoptera and Coleoptera species can be predicted from the effects of the individual proteins alone (Hunter and Vaituzis, 2007; US EPA, 2007).

### **ii. Potential Interactions in between Vip3Aa and Cry1Ab proteins**

#### **MRID No. 470176-21**

Four laboratory feeding bioassays were conducted to assess any synergistic or antagonistic interactions between Vip3A and full-length Cry1Ab proteins in the lepidopteran pest, tobacco budworm (TBW, *Heliothis virescens*). Five dilution series of the test materials were prepared in buffer for each test: one series each of Vip3Aa and FLCry1Ab alone, and three series of the two proteins mixed together in different ratios (up to 1600 µg/mL Vip3Aa and 100 µg/mL FLCry1Ab together). There was no evidence of either a synergistic or antagonistic interaction between Vip3A and FLCry1Ab in *H. virescens*, indicating that the effect of a mixture of Vip3A and FLCry1Ab on non-target Lepidoptera can be predicted from the effects of the individual proteins alone.

#### **MRID No. 470176-22**

Three laboratory feeding bioassays were conducted to assess any synergistic or antagonistic interactions between Vip3Aa19 and full-length Cry1Ab proteins in the lepidopteran pest, cotton bollworm (CBW, *Helicoverpa zea*). Five dilution series of the test materials were prepared in buffer for each test: one series each of Vip3Aa and FLCry1Ab alone, and three series of the two proteins mixed in different ratios (up to 25,600 ng/cm<sup>2</sup> Vip3Aa and 12,800 ng/cm<sup>2</sup> FLCry1Ab together). No evidence of either a synergistic or antagonistic interaction between Vip3A and FLCry1Ab in *H. zea*, indicating that the effect of a mixture of Vip3A and FLCry1Ab on non-target Lepidoptera can be predicted from the effects of the individual proteins alone.

**Conclusions/Recommendations:** The results of the interaction studies from sub-sets of the combined proteins (Cry1Ab and mCry3A against ECB and CPB; and Vip3Aa and FLCry1Ab against TBW and CBW) indicate that there is no change in the level of activity among susceptible insects. Collectively these data provide evidence that Vip3Aa, Cry1Ab, and mCry3A proteins do not interact in an antagonistic or synergistic manner. These data were previously reviewed and found acceptable by the Agency (Hunter and Vaituzis, 2007; Waggoner and Vaituzis, 2008a).

## **E. Effects of Combined PIP products on Non-target Organisms**

The potential for interaction among the Cry1Ab, Vip3Aa20, and mCry3A proteins was tested using three species of non-target organisms: the rove beetle (*Aleochara bilineata*) and the pink-spotted ladybeetle (*Coleomegilla maculata*), which are related to the target pest of mCry3A in MIR604 maize, and the monarch butterfly (*Danaus plexippus*), which is sensitive to Cry1Ab.

### **i. Rove beetle (MRID No. 471530-05)**

Adult rove beetles were exposed to a mixture of 15 µg Cry1Ab + 50 µg Vip3Aa20 + 25 µg mCry3A per gram of a meat-based diet for 35 days. The microbially-produced protein concentrations were chosen to represent at least the highest concentrations in the tissues of maize plants derived from the relevant events, or in breeding stacks containing the transgenes introduced in these events. Reproduction of beetles fed the diet containing the test materials was compared with that of control beetles fed untreated diet or diet containing the buffer used to dissolve Vip3Aa20 and mCry3A in the test material diet. There was no statistically significant difference in reproduction of the test material group compared to the control groups. Previous studies also found no effect of Vip3Aa20 and mCry3A on the rove beetle (US EPA, 2007 and 2008), and Cry1Ab is not known to be toxic to Coleoptera at the concentrations found in Bt11 maize.

**Conclusions/Recommendations:** No adverse effects were seen on the reproduction of rove beetles after exposure to the combined effects of Vip3Aa20, Cry1Ab, and mCry3A proteins in a treated meat-based diet. The NOEC was greater than 50 µg/g Vip3Aa20 + 15 µg/g Cry1Ab + 25 µg/g mCry3A diet for the reproduction of *Aleochara bilineata* and the LC<sub>50</sub> was greater than 50 µg/g Vip3Aa20 + 15 µg/g Cry1Ab + 25 µg/g mCry3A, when exposed orally via a treated meat-based diet.

### **ii. Ladybird beetle (MRID No. 471372-08)**

Ladybird beetle (*Coleomegilla maculata*) larvae were exposed to 11.23 µg Cry1Ab + 50 µg Vip3Aa20 + 24 µg mCry3A per gram of a moth egg/bee pollen diet. The protein concentrations were chosen to represent at least the highest concentrations that would be present in tissues of maize hybrids derived from the relevant events, or breeding-stack hybrids derived from these events. There were no statistically significant differences in days to pupation or adulthood, pupal mortality, or percent larval or adult mortality for larvae fed the test material diet compared to larvae fed untreated control diet. Previous studies have shown no effects of Cry1Ab, Vip3Aa19, and mCry3A on ladybird beetles (US EPA, 2007 and 2008).

**Conclusions/Recommendations:** No adverse effects were seen in *C. maculata* after exposure to the combined effects of Vip3Aa, Cry1Ab, and mCry3A proteins in a moth egg/bee pollen diet. The NOEC was greater than 50 µg Vip3Aa20 + 11.23 µg Cry1Ab + 24 µg mCry3A per g of diet on the development and survival of *Coleomegilla maculata* and the LC<sub>50</sub> was greater than 50 µg Vip3Aa20 + 11.23 µg Cry1Ab + 24 µg mCry3A per g of diet, when exposed orally via a treated bee pollen and moth egg-based diet.

### iii. Monarch butterfly (MRID No. 471372-10)

First-instar monarch butterfly larvae were exposed to non-transgenic maize pollen, Bt11 pollen, or Bt11 x MIR162 x MIR604 pollen at a density of 680 grains/cm<sup>2</sup> on leaves of the food plant milkweed (*Asclepias curassavica*). The larvae were fed pollen-treated leaves for four days, and then fed untreated leaves. In two separate runs of the test, the control mortality validity criterion of 20% was exceeded on day 6, and day 7, respectively. As a result, the data from day 5 and day 6 were analyzed for differences in mortality between the test material groups and control groups fed untreated leaves only. There was no significant difference in mortality of the test material pollen group compared to that of the control group in either run of the experiment.

#### Conclusions/Recommendations:

No statistically significant differences were noted in mortality from the combined effects of Bt11 x MIR162 x MIR604 corn when compared to the assay control group on monarch butterfly larvae. In addition, no adverse effects were test treatment related and were attributed to experimental shortcomings of high control mortality. The NOEC was greater than 0.425 mg Bt11 x MIR162 x MIR604 maize pollen / cm<sup>2</sup> of milkweed leaf diet on *Danaus plexippus* larvae and the LC<sub>50</sub> was greater than 0.425 mg Bt11 x MIR162 x MIR604 maize pollen / cm<sup>2</sup> diet, when exposed orally via a liquid suspended test pollen and milkweed leaf diet.

## F. Field Studies

### i. Efficacy Studies

#### MRID No. 476049-01

The efficacy of Bt11, MIR162, MIR604, Bt11 x MIR162, and Bt11 x MIR162 x MIR604 maize was compared against several pests (including black cutworm, fall armyworm, European corn borer, and western corn rootworm). The results were provided to the Agency as supplemental data to demonstrate the lack of interaction (i.e., no synergism or antagonism) among the insecticidal proteins produced in Bt11, MIR162, and MIR604 maize (MRID No. 476049-01, Waggoner and Kough, 2009). The efficacy of Bt11 x MIR162 and Bt11 x MIR162 x MIR604 maize was consistent with an additive effect of the individual efficacies of Bt11, MIR162, and MIR604 maize alone in all the field studies (described in Huber *et al.*, 2007; White *et al.*, 2007a, b, c, and d). Therefore, the efficacy studies also support the lack of synergistic effects by showing no interaction between the Cry1Ab, Vip3Aa20, and mCry3A proteins produced in the combined PIP products events Bt11 x MIR162, and Bt11 x MIR162 x MIR604 maize.

### ii. Non-target Organism Field Studies

Another Vip3Aa protein variant is also expressed in the recently registered Event COT102 cotton as Vip3Aa19 and in Syngenta's experimental Event Pacha corn. Event Pacha expresses Vip3Aa19 protein in corn, which is over 99.8% identical to Vip3Aa20 protein (Barsoum, 2008). In a three-year field study of Bt11 x Pacha maize, no significant differences in the composition of non-target organism communities were seen between Bt11 x Pacha and a non-transgenic near-isogenic maize that was not treated with insecticide (Dively, 2005).

### **G. Overall Synergism Conclusion:**

Collectively, these studies along with the single-species, NTO toxicity testing and field studies reviewed for the parental events Bt11, MIR162 and MIR604, indicate its associated combined PIP products, Bt11 x MIR162 and Bt11 x MIR162 x MIR604 maize, will not result in any unexpected interaction related to an antagonistic or synergistic action to target and non-target insects. Therefore, it is extremely unlikely that the Vip3Aa, Cry1Ab, and mCry3A proteins contained in a single plant will impart any hazard to non-target organisms exposed to these hybrids in the environment. Based on the information presented, the compilation of ecotoxicity studies on non-target organisms, evaluation for synergism between the test proteins, efficacy and field data support the bridging of the environmental risk assessment from the original parental events to the combined PIP Bt11 x MIR162 and Bt11 x MIR162 x MIR604 corn products.

### **H. Supplemental data needed to confirm Bt11 x MIR162 and Bt11 x MIR162 x MIR604 Non-Target Hazard Assessment**

The Agency has sufficient information to believe that there is no risk from the proposed uses of Bt11 x MIR162 and Bt11 x MIR162 x MIR604 corn to non-target wildlife, aquatic, and soil organisms. In previous Section 3 registrations of PIPs, the Agency required registrants to conduct post-registration long term invertebrate population/community studies and protein accumulation in soils studies. However, the issue of long range effects of cultivation of these Cry proteins on the invertebrate community structure in corn and cotton fields has since been adequately addressed by the meta-analysis of field studies performed during 10 years (Marvier, *et al.* 2007; Sanvido, *et al.* 2007). No unexpected adverse effects on invertebrate community structure were reported (Dively, 2005). The Agency is in agreement with these conclusions. Likewise, no unexpected accumulation of Cry proteins in agricultural soils was seen in published studies (Icoz and Stotzky, 2007; Sanvido, *et al.* 2007) and in numerous studies submitted directly to the EPA for the currently registered *Bt* PIP products containing Cry or Vip proteins (Milofsky, 2006; Waggoner and Vaituzis, 2008a and b).

However, additional aquatic invertebrate data are required for the Event MIR162 corn product as a condition of registration, in light of the published laboratory studies showing reduced growth in shredding caddisflies exposed to anti-lepidopteran Cry1A protein corn litter (Rosi-Marshall, *et al.* 2007). Therefore, a condition of registration for the MIR162 stacked products is based on the registrant's data submission to satisfy the conditions of registration for the parental event- MIR162 corn product and that these reviews must be found acceptable by the Agency.

### **CONCLUSION**

The environmental risk assessment indicates for the Bt11 x MIR162 and Bt11 x MIR162 x MIR604 corn hybrids, based on prior assessments conducted on Vip3Aa, Cry1Ab, mCry3A proteins individually, that no unreasonable harm will result to the environment or any federally-listed threatened or endangered species from commercial cultivation Bt11 x

MIR162 and Bt11 x MIR162 x MIR604 corn hybrids. The Agency has determined that Bt11 x MIR162 and Bt11 x MIR162 x MIR604 corn hybrids will have No Effect (NE) on endangered and/or threatened species listed by the US Fish and Wildlife Service (USFWS) and the National Marine Fisheries Services (NMFS), including mammals, birds, terrestrial and aquatic plants, and invertebrate species. Therefore, no consultation with the USFWS is required under the Endangered Species Act.

The Agency believes that cultivation of Bt11 x MIR162 and Bt11 x MIR162 x MIR604 corn hybrids may result in fewer adverse impacts to non-target organisms than result from the use of chemical pesticides. Under normal circumstances, *Bt* 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. Therefore, the overall result of cultivation of Bt11 x MIR162 and Bt11 x MIR162 x MIR604 corn hybrids, expressing Vip3Aa, Cry1Ab, and mCry3A proteins, is that the number of chemical insecticide applications for non-target pest control will be reduced for management of multiple pest problems.

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**DATA EVALUATION RECORD**

**CRY1Ab PROTEIN  
VIP3Aa20 PROTEIN  
mCRY3A PROTEIN  
(Bt11 x MIR162 x MIR604 Maize)**

**STUDY TYPE: Non-target Insect Testing, Tier I (OPPTS 885.4340)**

**MRID 471530-05**

Prepared for  
Biopesticides and Pollution Prevention Division  
Office of Pesticide Programs  
U.S. Environmental Protection Agency  
One Potomac Yard  
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Prepared by  
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Task Order No. 07-070

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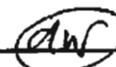
Date: \_\_\_\_\_

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**DATA EVALUATION RECORD****Primary Reviewer:** Eric B. Lewis, M.S., Oak Ridge National Laboratory**EPA Reviewer:** Annabel Waggoner, Environmental Protection Specialist, BPPD

**STUDY TYPE:** Non-target Insect Testing, Tier I (885.4340)

**MRID NO:** 471530-05

**DP BARCODE:** 345913

**DECISION NO:** 379490

**TEST MATERIAL:** Microbial-produced proteins: TRYCRY1AB-0105 (containing Cry1Ab), MIR162VIP3A-0106 (containing Vip3Aa20), and MCRY3A-0102 (containing modified Cry3A)

**STUDY NO:** T002321-06

**SPONSOR:** Syngenta Seeds, Inc., P.O Box 12257  
3054 E. Cornwallis Road  
Research Triangle Park, NC 27709

**TESTING FACILITY:** Syngenta Jealotts' Hill International Research Centre,  
Bracknell, Berkshire, RG42 6EY, UK

**TITLE OF REPORT:** Vip3Aa20 + Cry1Ab + mCry3A: A Laboratory Study to Determine Effects of Vip3Aa20 + Cry1Ab + mCry3A Proteins, in Combination, on the Rove Beetle *Aleochara bilineata* (Coleoptera: Staphylinidae)

**AUTHOR:** Stacey, D.A., and R.J. Blake

**STUDY COMPLETED:** May 8, 2007

**CONFIDENTIALITY CLAIMS:** None

**GOOD LABORATORY PRACTICE:** A signed and dated GLP statement was provided. The study was conducted in compliance with UK and OECD GLP Regulations.

**STUDY SUMMARY:** In a laboratory bioassay, adult rove beetles (*Aleochara bilineata*) were exposed to a prepared meat diet containing 50 µg/g Vip3Aa20 + 15 µg/g Cry1Ab + 25 µg/g mCry3A microbially-produced proteins to assess the combined effect of Bt11 x MIR162 x MIR604 maize on reproduction. A negative control diet, a buffer control diet, and a reference control diet were also included in the test. To assess reproduction, onion fly (*Delia antiqua*) pupae were provided for parasitization by the beetles during the test. Second-generation beetles emerging from the parasitized pupae were counted until emergence ceased on

test day 84. There was no statistically significant difference in the number of emerged beetles in the test material group compared to the pooled control groups. The response of the reference control group was appropriate. No adverse effects were noted on the reproductive effects of Vip3Aa20, Cry1Ab, and mCry3A proteins on *A. bilineata*. Furthermore, the NOEC was greater than 50 µg/g Vip3Aa20 + 15 µg/g Cry1Ab + 25 µg/g mCry3A diet for the reproduction of *Aleochara bilineata* and the LC<sub>50</sub> was greater than 50 µg/g Vip3Aa20 + 15 µg/g Cry1Ab + 25 µg/g mCry3A, when exposed orally via a treated meat-based diet.

**CLASSIFICATION: ACCEPTABLE**

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## **I. STUDY DESIGN:**

### **Test Material**

MIR162VIP3A-0106, a microbially-expressed Vip3Aa20 protein, a white powder with a reported purity of 84% (w/w).

TRYCRY1AB-0105, a microbially-expressed Cry1Ab (trypsinised) protein, a cloudy solution with a reported purity of 0.0127% w/v (127 µg/mL).

MCRY3A-0102, a microbially-expressed modified Cry3A (mCry3A) protein, a white powder with a reported purity of 90.3% w/w.

All test materials were received from the study sponsor, and were stored in a freezer at -20°C with a desiccant.

The reference material was Dimilin Flo (SC) (diflubenzuron), Batch No. AM4C31F009, with a nominal concentration of 480 g/L and a re-analysis date of May, 2007. Prior to the test, the reference material was stored at < 30°C and away from direct sunlight.

### **Test Methods**

The study was conducted to assess the combined effect of the test materials on reproduction of the rove beetle (*Aleochara bilineata*), via a diet incorporation assay. The test organisms were obtained as parasitized pupae of the onion fly (*Delia antiqua*) from de Groene Vlieg, Nieuwe Tonge, Netherlands. After emergence the adult beetles were maintained individually in cool, dark conditions and fed *Chironomus* larvae. Prior to test start, beetles <7 days old were collected for mating pairs.

The test consisted of four groups: an untreated control (diet only), a buffer control (buffer-treated diet), the test material group, and a reference control group (Table 1). Each group contained four replicates of 20 beetles each (10 males, 10 females). The treatments were administered in a diet of cooked minced beef, blended into a paste. Due to poor solubility in water, the test material was

dissolved in two buffer solutions prior to mixing with the diet. Buffer 1 consisted of 10 mM Tris-HCl buffer (pH 10.5) and 0.4 mM EDTA, while buffer 2 consisted of 50 mM Tris-HCl buffer (pH 9.5) and 2mM EDTA. Nominally, 7.14 mg of MIR162VIP3A-0106 was made up to 10 g with buffer 1 solution and mixed until dissolved, after which 10 g of buffer 2 was added to lower the pH. Then 3.32 mg of MCRY3A-0102 and 14.16 mL of TRYCRY1AB-0105 were added and mixed until dissolved. This solution was then made up to 120 g with artificial diet and mixed thoroughly. The finished diet for the test material group nominally contained 50 µg/g Vip3Aa20 + 15 µg/g Cry1Ab + 25 µg/g mCry3A, which approximates the highest concentrations of these proteins in fresh tissue of events MIR162, Bt11, and MIR604 maize, respectively. The reference standard was prepared by bringing 0.5 mL of Dimilin Flo up to 100 mL with deionized water and bringing 1 mL of the resulting solution up to 20 g with deionized water. The solution was then made up to 120 g with artificial diet for an application rate of 0.02 mg a.i./g diet. Samples of the test material and control diets were collected and stored frozen for analysis of integrity and stability.

<b>Treatment</b>	<b>Amount of test or reference material used</b>	<b>Amount of buffer* or water used</b>	<b>Amount of diet used</b>
Untreated control	NA	20.03 g of deionized water	100.20 g
Negative control (Buffer only)	NA	10.02 g of buffer 1 + 10.01 g of buffer 2	100.02 g
Vip3Aa20 + Cry1Ab + mCry3A	00.0074 g MIR162VIP3A-0106 0.034 g MCRY3A-0102 14.16 mL TRYCRY1AB-0105	10.01 g buffer 1 + 10.03 g buffer 2	99.97 g
Reference control (Diflubenzuron)	1 mL of a solution of 0.05 mL Dimilin Flo in 100 mL deionized water	20 mL deionized water	100.62 g

Data from p. 25, MRID 47153005

\*Buffer 1 = 10 mM Tris-HCl buffer (pH 10.5), 0.4 mM EDTA; buffer 2 = 50 mM Tris-HCl buffer (pH 9.5), 2 mM EDTA

All diets were prepared at test start and aliquots of the treated diets were frozen at -20°C until needed. Prior to daily use, the treated diets were defrosted and then used immediately. Each test day approximately 0.2 g of the appropriate diet was placed on a 15-mm diameter filter paper disk and transferred into the corresponding test chamber, and any old diet was removed.

For the first 7 days of exposure, the beetles were maintained in 11-cm diameter glass pots lined with filter paper. The lids had a mesh-filled hole in the center to allow ventilation. A few drops of deionized water were placed on the filter paper as a water source. On test day 7, the beetles were transferred to one-liter flat-bottomed glass dishes (14-cm diameter, 7-cm deep) with ventilated lids for an additional 28 days. Each dish contained 750 g of sand moistened with deionized water, and the moisture content was maintained during the test. The test was conducted in a controlled environment chamber set to maintain a temperature of 20 ± 2°C and a relative humidity of 60-90%. The photoperiod was 16 hours light/8 hours darkness.

To assess reproduction of the beetles, 500 *D. antiqua* pupae were placed in the sand of the test chambers on test days 14, 21, and 28. Each introduction was at a different location in the sand. On test day 35, the adult beetles were removed from the sand by sieving and the number of live and

dead beetles was recorded. The sand with the parasitized *D. antiqua* pupae was returned to the test chambers and allowed to dry for 7 days, after which the pupae were sieved out and placed in emergence chambers. The emergence chambers were two 14-cm diameter plastic Petri dishes on top of each other. There was a hole in the bottom of the top dish and in the lid of the bottom dish with a 2-mm mesh in between, which allowed any emerged beetles to fall from the top dish into the bottom dish. Emerged beetles were removed and counted every one to four days until emergence in the negative control group fell below two beetles per replicate per day on day 84.

The number of newly-emerged adults in the untreated and buffer control groups was compared using a two-tailed Wilcoxin test ( $p \leq 0.05$ ). Since the control treatments did not differ significantly, the data for both groups were pooled. The test material group was compared to the pooled controls using Dunnett's test ( $p \leq 0.05$ ). Shapiro-Wilk's test for normality and Levene's test for homogeneity of variance were used to determine that a transformation of data was not required. Statistical analyses of the data were done using the one way ANOVA module in StEve, v 1.1.

## II. RESULTS:

The number of first-generation beetles recovered after 35 days of exposure is given in Table 2. Mean mortality in the negative control, buffer control, test material, and reference control groups was 31.25%, 32.50%, 20.00%, and 15.00%, respectively.

Treatment	Replicate	Found alive	Found dead <sup>1</sup>	Not found <sup>2</sup> (N=20)	% Mortality (N=20)	Mean % mortality
Negative control	1	13	2	5	35	31.25
	2	12	3	5	40	
	3	15	1	4	25	
	4	15	0	5	25	
Buffer control	1	15	0	5	25	32.50
	2	9	0	11	55	
	3	11	4	5	45	
	4	19	0	1	5	
Vip3Aa20 + Cry1Ab + mCry3A	1	13	1	6	35	20.00
	2	20	0	0	0	
	3	18	1	1	10	
	4	13	0	7	35	
Reference control	1	19	0	1	5	15.00
	2	19	0	1	5	
	3	17	0	3	15	
	4	13	2	5	35	

Data from p. 21, MRID 47153005

<sup>1</sup>Includes beetles found dead on surface prior to day 35

<sup>2</sup>Assumed dead

The number of second-generation adults emerged from the pupae is summarized in Table 3. There was no statistically significant difference in the number of beetles that emerged from the test material group compared to the pooled control groups. Response of the reference control group was appropriate, indicating the exposure system was valid.

Table 3. Emergence of second generation adult rove beetles		
Treatment	Mean no. of emerged beetles $\pm$ std dev	Mean reproduction as percent of pooled controls
Negative control	647.75 $\pm$ 57.89	105.54
Buffer control	579.75 $\pm$ 218.95	94.46
Pooled controls	613.75 $\pm$ 235.53	—
Vip3Aa20 + Cry1Ab + mCry3A	470.50 $\pm$ 202.19	76.66
Reference control	249.75 $\pm$ 88.99	40.69

Data from p. 22, MRID 47153005

Confirmation that intact Cry1Ab was present in prepared test material diet was provided in a separate study (Appendix 2 of MRID 47153005). ELISA showed that extraction of Cry1Ab from the treated diet recovered a mean of 16.5  $\mu$ g Cry1Ab/g of diet, 110% of the nominal amount applied. Western blot analysis of the extracted protein showed a dominant band of immunoreactive material corresponding to the expected molecular weight of Cry1Ab (ca. 66,000 Da). No Cry1Ab was detected in the negative control diet.

Bioactivity of the prepared diet was confirmed in a sensitive insect bioassay using European corn borer larvae (*Ostrinia nubilalis*) (Appendix 2 of MRID 47153005). Samples of the test material diet were incorporated into standard lepidopteran diet at 10% or 20% w/w of the standard diet (ca. 1.5 and 3  $\mu$ g Cry1Ab/mL of diet, respectively) and fed to the larvae for six days. Mortality of larvae fed the 10 and 20% diets was 100% and 96%, respectively, compared to 0% for larvae exposed to standard lepidopteran diet containing 10% or 20% w/w of the negative control diet.

### III. CONCLUSION:

The study authors concluded that the treated meat-based diet containing microbially-expressed Vip3Aa20, Cry1Ab, and mCry3A proteins had no adverse effects on the reproduction of the test organism.

### IV. EPA REVIEWER'S COMMENTS:

The average emergence of second generation rove beetles for the pooled controls was 613.8, which was significantly different in comparison to the number of emerged rove beetles (249.8) for the toxic standard, thus indicating a 59.3% reduction in reproduction. In addition, the study met the validity criteria that the mean number of emerged beetles in the controls was  $\geq$  400 each, and that the reference standard produced a  $\geq$  50% reduction in reproduction compared to the pooled controls. More importantly, there was no statistically significant difference in the number of beetles that emerged from the test material group compared to the pooled control groups. Therefore, the  $LC_{50}$  was greater than 50  $\mu$ g/g Vip3Aa20 + 15  $\mu$ g/g Cry1Ab + 25  $\mu$ g/g mCry3A, and the NOEC was greater than 50  $\mu$ g/g Vip3Aa20 + 15  $\mu$ g/g Cry1Ab + 25  $\mu$ g/g mCry3A diet for the reproduction of *Aleochara bilineata*, when exposed orally via a treated meat-based diet.

### V. CLASSIFICATION: Acceptable

### VI. REFERENCES:

Barret, et al. (1994) "Guidance Document on Regulatory Testing and Risk Assessment Procedures for Plant Protection Products with Non-Target Arthropods." From the ESCORT Workshop

(European Standard Characteristics of Non-Target Arthropod Regulatory Testing).  
Wageningen, Holland, March 28-30, 1994.

Grimm, *et al.* (2000) "A test for evaluating the chronic effects of plant protection products on the rove beetle *Aleochara bilineata* (Coleoptera: Staphylinidae) under laboratory and extended laboratory conditions," In: Candolfi, *et al.* (2000). Guidelines to evaluate side-effects of plant protection products to non-target arthropods. IOBC, BART and EPPO Joint Initiative, pp. 1-13. ISBN 92-9067-129-7.

**DATA EVALUATION RECORD**

**CRY1Ab PROTEIN  
VIP3Aa20 PROTEIN  
MCRY3A PROTEIN  
(Bt11 x MIR162 x MIR604 Maize)**

**STUDY TYPE: Environmental Fate and Potency (Non-guideline)**

**MRID 471372-07**

Prepared for  
Biopesticides and Pollution Prevention Division  
Office of Pesticide Programs  
U.S. Environmental Protection Agency  
One Potomac Yard  
2777 South Crystal Drive  
Arlington, VA 22202

Prepared by  
Toxicology and Hazard Assessment Group  
Environmental Sciences Division  
Oak Ridge National Laboratory  
Oak Ridge, TN 37830  
Task Order No. 07-070

Primary Reviewer:  
Eric B. Lewis, M.S.

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Quality Assurance:  
Lee Ann Wilson, M.A.

Signature: \_\_\_\_\_  
Date: \_\_\_\_\_

**Disclaimer**

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

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**DATA EVALUATION RECORD****Primary Reviewer:** Eric B. Lewis, M.S., Oak Ridge National Laboratory**EPA Reviewer:** Annabel Waggoner, Environmental Protection Specialist, BPPD

**STUDY TYPE:** Environmental Fate and Potency (Non-guideline)

**MRID NO:** 471372-07

**DP BARCODE:** 345913

**DECISION NO:** 379490

**TEST MATERIAL:** Bt11 x MIR 162 x MIR 604 Corn (a.i., Cry1Ab, Vip3Aa20, and mCry3A proteins)

**STUDY NO:** SSB-525-07

**SPONSOR:** Syngenta Seeds, Inc., P.O Box 12257  
3054 E. Cornwallis Road  
Research Triangle Park, NC 27709

**TESTING FACILITY:** Not applicable

**TITLE OF REPORT:** The Environmental Fate and Potency of the Insecticidal Proteins Cry1Ab, Vip3Aa20, and mCry3A in Bt11 x MIR162 x MIR604 Stacked Maize Hybrids

**AUTHOR:** Raybould, A.

**STUDY COMPLETED:** May 10, 2007

**CONFIDENTIALITY CLAIMS:** None

**GOOD LABORATORY PRACTICE:** A signed and dated GLP statement was provided. The study is a compilation of information and data from diverse sources, and is not subject to GLP standards.

**STUDY SUMMARY:** Event Bt11-derived, Event MIR162-derived, and Event MIR604-derived maize express the insecticidal proteins Cry1Ab, Vip3Aa20, and modified Cry3A (mCry3A), respectively, to control certain lepidopteran and coleopteran pests. The combined PIP product- Bt11 x MIR162 x MIR604 was created via traditional breeding methods to express the Cry1Ab, Vip3Aa20, and mCry3A proteins in combination. A field trial found no systematic increase in expression of Cry1Ab, Vip3Aa20, and mCry3A in the stacked hybrid compared to the individual events, indicating that margins of exposure previously determined for the insecticidal proteins in the individual events can be used in risk assessments for these proteins in the stacked hybrid. Previous studies to determine the potential for synergistic effects were conducted (via sensitive insect diet-incorporation bioassays) on subsets of the following pesticidal mixtures: Cry1Ab and Vip3Aa proteins and Cry1Ab and mCry3A proteins. In addition, no enhanced toxicity was demonstrated on non-target organisms among these proteins, indicating that results from studies of the individual proteins can be used to predict the effects of the stacked hybrid. Therefore, based

on the results of risk assessments for the individual events, no unreasonable adverse effects are expected on non-target organisms by exposure to Cry1Ab, Vip3Aa20, and mCry3A resulting from cultivation of Bt11 x MIR162 x MIR604 maize.

**CLASSIFICATION: ACCEPTABLE**

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## **I. BACKGROUND:**

Bt11 x MIR162 x MIR604 stacked hybrid maize expresses Cry1Ab, Vip3Aa20, and mCry3A proteins to provide control of lepidopteran pests, western corn rootworm (*Diabrotica virgifera virgifera*) and other closely-related species of *Diabrotica*. The stacked hybrid also includes Event GA21, which confers tolerance to glyphosate herbicides, but GA21 does not express any pesticidal proteins. Individually, events Bt11, MIR162, and MIR604 maize pose negligible environmental risk due to the absence of toxicity of Cry1Ab, Vip3Aa20, and mCry3A to non-target organisms at concentrations exceeding those expected in the field. The environmental risk assessment for Bt11 x MIR162 x MIR604 maize is expected to provide the same conclusions in the environmental risk assessment if the following conditions are met: 1) the insecticidal proteins are not expressed at levels high enough to reduce the margins of exposure (MOEs) found in ecological studies of the individual proteins and invalidate the predicted lack of harm to non-target organisms in the field, and 2) there are no interactions among the proteins that increase their individual toxicities.

Environmental fate data indicate that exposure of non-target organisms to Cry1Ab and Vip3Aa20 will be confined largely to maize fields during and immediately after cultivation of Bt11 x MIR162 x MIR604 maize (US EPA, 2001; Raybould, 2007a; Raybould *et al.*, 2007). None of the environmental fate parameters (protein DT<sub>50</sub>s in soil, gene flow to wild relatives, weediness potential of maize) are likely to be affected by combining Bt11, MIR162, and MIR604 in a breeding stack.

## **II. SUMMARY OF RESULTS:**

### **A. Tests for Differences in Expression**

The expected environmental concentrations (EECs) of the proteins to non-target organisms, based on environmental fate and expression data, have been calculated for Bt11, MIR162, and MIR604 (US EPA, 2001; Raybould, 2007a; Raybould *et al.*, 2007; US EPA 2007). The MOE is the multiple of the EEC to which organisms were exposed in studies to assess the hazard of the proteins. The EECs, and hence the MOEs, for Bt11, MIR162, and MIR604 are applicable to Bt11 x MIR162 x MIR604 provided that the expression of Cry1Ab, Vip3Aa20, and mCry3A is not changed due to their being combined in a breeding stack.

In an Illinois trial Bt11, MIR162, MIR604, and Bt11 x MIR162 x MIR604 maize were grown using standard agricultural practices, and tissues were collected and analyzed using enzyme-linked immunosorbent assay (ELISA) to estimate the dry-weight concentrations of Cry1Ab, Vip3Aa20, and mCry3A. Two small but statistically significant differences were found between Bt11 x MIR162 x MIR604 maize and the relevant single gene events: a 12% reduction in the Cry1Ab concentration in the kernels of the stacked hybrid compared with Bt11, and a 10% reduction in the mCry3A concentration of whole plants of the stacked hybrid compared to MIR604. There were no statistically significant differences in the Vip3Aa20 concentration of the stacked hybrid and

MIR162. These results indicate that the MOEs determined in previous studies for Cry1Ab, Vip3Aa20, and mCry3A in the single maize events are applicable to the stacked hybrid.

## **B. Tests for Synergism**

The expression data show that non-target organisms are unlikely to be exposed to harmful concentrations of Cry1Ab, Vip3Aa20, and mCry3A via Bt11 x MIR162 x MIR604 maize, assuming that there is no synergism among Cry1Ab Vip3Aa20, and mCry3A. Four types of studies were conducted to test for synergism among the proteins - sensitive insect bioassays, non-target organism bioassays, efficacy studies, and non-target organism field studies.

### ***1. Sensitive Insect Bioassays***

The potential for interaction between test materials can be assessed by comparing the larval mortality observed for the mixed proteins with the predicted responses based on the bioassay of each protein individually. The test substances for the sensitive insect bioassays were microbial preparations of FLCry1Ab and Vip3Aa19, which were previously determined by the Agency as suitable test surrogates for Cry1Ab and Vip3Aa20 proteins (Edelstein, 2008; Barsoum, 2008).

The results of three laboratory feeding bioassays on the lepidopteran pest, cotton bollworm (CBW, *Helicoverpa zea*) showed no evidence of synergistic or antagonistic interactions between the Vip3Aa19 and FLCry1Ab proteins [MRID No. 470176-22 (Raybould, 2006b)]. Therefore, the effect of a mixture of Vip3Aa19 and FLCry1Ab proteins on non-target Lepidoptera can be predicted from the effects of the individual proteins alone (Waggoner, 2008).

The results of four laboratory feeding bioassays on the tobacco budworm (TBW, *Heliothis virescens*) showed no evidence of synergistic or antagonistic interactions between the Vip3Aa19 and FLCry1Ab proteins [MRID No. 470176-21 (Raybould, 2007b)]. Therefore, the effect of a mixture of Vip3Aa19 and FLCry1Ab proteins on non-target Lepidoptera can be predicted from the effects of the individual proteins alone (Waggoner, 2008).

### ***2. Non-target Organism Bioassays***

Protein interaction Cry1Ab, Vip3Aa20, and mCry3A was tested using three species of non-target organisms (Raybould, 2006a): the rove beetle (*Aleochara bilineata*) and the pink-spotted ladybeetle (*Coleomegilla maculata*), which are related to the target pest of mCry3A in MIR604 maize, and the monarch butterfly (*Danaus plexippus*), which is sensitive to Cry1Ab.

Adult rove beetles were exposed to a mixture of 15 µg Cry1Ab + 50 µg Vip3Aa20 + 25 µg mCry3A per gram of a meat-based diet for 35 days (Stacey and Blake, 2007a). The microbially-produced protein concentrations were chosen to represent at least the highest concentrations in the tissues of maize plants derived from the relevant events, or in breeding stacks containing the transgenes introduced in these events. Reproduction of beetles fed the diet containing the test materials was compared with that of control beetles fed untreated diet or diet containing the buffer used to dissolve Vip3Aa20 and mCry3A in the test material diet. There was no statistically significant difference in reproduction of the test material group compared to the control groups. Previous studies also found no effect of Vip3Aa20 and mCry3A on the rove beetle (Stacey and Blake, 2007a; Vinall, 2003), and Cry1Ab is not known to be toxic to Coleoptera at the concentrations found in Bt11 maize.

Ladybird beetle larvae were exposed to 11.23 µg Cry1Ab + 50 µg Vip3Aa20 + 24 µg mCry3A per gram of a moth egg/bee pollen diet (Patanaude, 2007a). Again, the protein concentrations were chosen to represent at least the highest concentrations that would be present in tissues of maize hybrids derived from the relevant events, or breeding-stack hybrids derived from these events. There were no statistically significant differences in days to pupation or adulthood, pupal mortality, or percent larval or adult mortality for larvae fed the test material diet compared to larvae fed untreated control diet. Adults in the test material diet group weighed about 11% more than those in the control group, which was a statistically significant difference. Previous studies have shown no effects of Cry1Ab, Vip3Aa19, and mCry3A on ladybird beetles (Teixeira, 2002; Waterman, 2003).

First-instar monarch butterfly larvae were exposed to non-transgenic maize pollen, Bt11 pollen, or Bt11 x MIR162 x MIR604 pollen at a density of 680 grains/cm<sup>2</sup> on leaves of the food plant milkweed (*Asclepias curassavica*) (Patanaude, 2007b). The larvae were fed pollen-treated leaves for four days, and then fed untreated leaves. In two separate runs of the test, the control mortality validity criterion of 20% was exceeded on day 6, and day 7, respectively. As a result, the data from day 5 and day 6 were analyzed for differences in mortality between the test material groups and control groups fed untreated leaves only. There was no significant difference in mortality of the test material pollen group compared to that of the control group in either run of the experiment.

Additionally, most non-target organisms are not sensitive to Cry1Ab, Vip3Aa20, or mCry3A at EECs that would result from cultivation of Bt11 x MIR162 x MIR604 maize (US EPA, 2001; Raybould, 2007a; Raybould *et al.*, 2007; US EPA, 2007), and non-toxins rarely interact to produce a toxic mixture (FIFRA SAP, 2004).

### **3. Non-target Organism Field Studies**

Another Vip3Aa protein variant is also expressed in the recently registered Event COT102 cotton as Vip3Aa19 and in Syngenta's experimental Event Pacha corn. Event Pacha expresses Vip3Aa19 protein in corn, which is over 99.8% identical to Vip3Aa20 protein (Barsoum, 2008). In a three-year field study of Bt11 x Pacha maize, no significant differences in the composition of non-target organism communities were seen between Bt11 x Pacha and a non-transgenic near-isogenic maize that was not treated with insecticide (Dively, 2005).

### **4. Efficacy**

In separate trials, the efficacy of Bt11, MIR162, MIR604, and Bt11 x MIR162 x MIR604 maize was tested against artificial infestations of corn earworm (*Helicoverpa zea*), European corn borer (*Ostrinia nubilalis*), fall armyworm (*Spodoptera frugiperda*), and western corn rootworm (*Diabrotica virgifera virgifera*) (White *et al.*, 2007a, 2007b, 2007c, and 2007d). In all the studies, the efficacy of Bt11 x MIR162 x MIR604 was not different from the additive efficacies of Bt11, MIR162, and MIR604 maize alone.

### **C. Implications for MOEs**

Since no interaction between Cry1Ab, Vip3Aa20 and mCry3A was found, the MOEs determined individually for Cry1Ab, Vip3Aa variants, and mCry3A are suitable for predicting their effects in Bt11 x MIR162 x MIR604. For non-lepidopteran non-target organisms, the MOEs give confidence that the combined effect of Cry1Ab, Vip3Aa20, and mCry3A will be zero at EECs that will result from cultivation of Bt11 x MIR162 x MIR604 maize (US EPA, 2001; Raybould, 2007; Raybould *et al.*, 2007, US EPA, 2007). While mCry3A is toxic to certain species of Chrysomelidae, Curculionidae, and Tenobronidae (all Coleopterans), non-target organisms in these groups are

unlikely to be exposed to significant concentrations of mCry3A (MOE >0), and those likely to be exposed to mCry3A are pests.

#### **D. Endangered Species Assessment**

The toxicity of Cry1Ab and Vip3Aa20 at the concentrations found in Bt11 and MIR162 maize, respectively, will be limited to certain lepidopterans (US EPA, 2001; Raybould, 2007a), and the toxicity of mCry3A at its concentration in MIR604 maize will be limited to certain species of Chrysomelidae, Curculionidae, and Tenebrionidae (Raybould, *et al.*, 2007; US EPA, 2007). Since synergism of Cry1Ab, Vip3Aa20, and mCry3A has not been found in trials, the toxicity of their mixture in Bt11 x MIR162 x MIR604 maize is also expected to be limited to the lepidopterans and coleopterans affected by the individual events.

The only endangered lepidopteran with potential exposure to insecticidal proteins in maize is the Karner blue butterfly (*Lycaeides melissa samuelis*) (US EPA, 2001). The potential route of exposure is consumption of maize pollen that has settled on the leaves of its food plant, wild lupine (*Lupinus perennis*). McKee *et al.* (2001) concluded that the Karner blue would not be impacted by Bt11 maize pollen due to low exposure. This conclusion was supported by Peterson *et al.* (2006), who determined that the exposure is minimal because most lupine populations are separated from maize fields by at least 500 meters, and because maize anthesis usually occurs after the Karner blue feeding period is over.

The only endangered coleopteran with potential to be exposed to the insecticidal proteins in MIR604 maize is the American burying beetle (*Nicrophorus americanus*). This species feeds exclusively on carrion, and any burying beetles that occur in or near maize files would therefore be minimally exposed to mCry3A (US EPA, 2007).

The insecticidal proteins in Bt11 x MIR162 x MIR604 maize have shown no toxicity to taxa outside Lepidoptera and Coleoptera. The dietary exposure of endangered or threatened lepidopterans or coleopterans would be negligible, indicating that Bt11 x MIR162 x MIR604 maize is unlikely to have harmful effects on those species in the United States.

Although it is not endangered or threatened, the monarch butterfly is susceptible to Cry1Ab, and there has been concern that it may be harmed by consuming pollen from transgenic insect-resistant maize. However, due to the distribution of the monarch's food plant (milkweed), its migration pattern, and the timing of maize anthesis, very few monarchs are exposed to harmful concentrations of Cry1Ab (Sears, *et al.*, 2001). Feeding studies detected no significant difference between Bt11 and Bt11 x MIR162 x MIR604 pollen on growth and survival of monarch larvae (Patanaude, 2007b); therefore, the conclusion that Bt11 maize pollen containing Cry1Ab poses low risk to monarchs (Sears *et al.*, 2001; US EPA, 2001) can be extended to Bt11 x MIR162 x MIR604.

## **II. CONCLUSION:**

The study author concluded that protein expression and synergism studies indicate that the effects of Bt11 x MIR162 x MIR604 maize can be predicted from the risk assessments of the insecticidal proteins expressed in maize derived from the individual component events. Therefore, non-target organisms, including endangered and threatened species and monarch butterflies, are unlikely to

be harmed by exposure to Cry1Ab, Vip3Aa20, and mCry3A resulting from cultivation of Bt11 x MIR162 x MIR604 maize.

### III. EPA REVIEWER'S COMMENTS:

Based on the information presented, the compilation of ecotoxicity studies on non-target organisms, evaluation for synergism between the test proteins, efficacy and field data support the bridging of the environmental risk assessment from the original parental events to the combined PIP Bt11 x MIR162 x MIR604 corn product.

### IV. CLASSIFICATION: ACCEPTABLE

### V. REFERENCES:

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White, J. M. Meehan, and M. Meghji. 2007c. Insecticidal Efficacy of a Bt11 x MIR162 x GA21 Maize Hybrid Against Fall Armyworm in the Field. Syngenta Seeds Biotechnology Report # SSB-516-07. Unpublished.

White, J. M. Meehan, and M. Meghji. 2007d. Insecticidal Efficacy of a Bt11 x MIR162 x MIR604 x GA21 Maize Hybrid Against Western Corn Rootworm in the Field. Syngenta Seeds Biotechnology Report # SSB-515-07. Unpublished.

**DATA EVALUATION RECORD**

**CRY1Ab PROTEIN  
VIP3Aa20 PROTEIN  
mCRY3A PROTEIN  
(Bt11 x MIR162 x MIR604 Maize)**

**STUDY TYPE: Non-target Insect Testing, Tier I (OPPTS 885.4340)**

**MRID No. 471372-08**

Prepared for  
Biopesticides and Pollution Prevention Division  
Office of Pesticide Programs  
U.S. Environmental Protection Agency  
One Potomac Yard  
2777 South Crystal Drive  
Arlington, VA 22202

Prepared by  
Toxicology and Hazard Assessment Group  
Environmental Sciences Division  
Oak Ridge National Laboratory  
Oak Ridge, TN 37830  
Task Order No. 07-070

Primary Reviewer:

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Lee Ann Wilson, M.A.

Signature: \_\_\_\_\_

Date: \_\_\_\_\_

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**DATA EVALUATION RECORD**

**Primary Reviewer:** Eric B. Lewis, M.S., Oak Ridge National Laboratory

**EPA Reviewer:** Annabel Waggoner, Environmental Protection Specialist, BPPD



**STUDY TYPE:** Non-target Insect Testing, Tier I (885.4340)

**MRID NO:** 471372-08

**DP BARCODE:** 345913

**DECISION NO:** 379490

**TEST MATERIAL:** Microbial-produced proteins: TRYCRY1AB-0105 (containing Cry1Ab), MIR162VIP3A-0106 (containing Vip3Aa20), and MCRY3A-0102 (containing modified Cry3A)

**STUDY NO:** 1781.6671

**SPONSOR:** Syngenta Seeds, Inc., P.O Box 1225  
3054 E. Cornwallis Road  
Research Triangle Park, NC 27709

**TESTING FACILITY:** Springborn Smithers Laboratories,  
790 Main Street  
Wareham, MA 02571-1037

**TITLE OF REPORT:** Cry1Ab, Vip3Aa20, and mCry3A: Laboratory Study to Determine the Combined Effects of Cry1Ab, Vip3Aa20 and mCry3A on the Predatory Beetle *Coleomegilla maculate*

**AUTHOR:** Patnaude, M.R.

**STUDY COMPLETED:** May 15, 2007

**CONFIDENTIALITY CLAIMS:** None

**GOOD LABORATORY PRACTICE:** A signed and dated GLP statement was provided. The study was conducted in compliance with 40 CFR Part 160 with the following exceptions: routine water screening analyses were conducted at GeoLabs, Inc., Braintree, MA, using standard US EPA procedures, and the positive control substance was not characterized under GLP standards.

**STUDY SUMMARY:** In a laboratory bioassay, predatory beetle (*Coleomegilla maculata*) larvae were exposed to a diet containing bee pollen and *Ephestia kuehniella* eggs contained 50 µg Vip3Aa20 + 11.23 µg Cry1Ab + 24 µg mCry3A per g of

diet for 21 days. The test also included an assay control diet and a potassium arsenate positive control diet. There were no detrimental effects on larval, pupal, or adult survival; days to pupation; or days to adulthood for beetles exposed to the test material diet. The response of the positive control group was appropriate. The stability and bioactivity of the test materials in the prepared diets were confirmed in separate bioassays. The NOEC was greater than 50 µg Vip3Aa20 + 11.23 µg Cry1Ab + 24 µg mCry3A per g of diet on the development and survival of *Coleomegilla maculata* and the LC<sub>50</sub> was greater than 50 µg Vip3Aa20 + 11.23 µg Cry1Ab + 24 µg mCry3A per g of diet, when exposed orally via a treated bee pollen and moth egg-based diet.

**CLASSIFICATION:           ACCEPTABLE**

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## **I. STUDY DESIGN:**

### **Test Material**

The test material was microbially-expressed TRYCRY1AB-0105, supplied by the study sponsor with a reported purity of 0.0127% w/v Cry1Ab protein. Upon receipt at the test facility, the test material was stored refrigerated (2 to 8°C) in the original container.

The test material was microbially-expressed MIR162VIP3A-0106, supplied by the study sponsor with a reported purity of 84% w/w Vip3Aa20 protein. Upon receipt at the test facility, the test material was stored frozen (-25 to -10°C) in the original container.

The test material was microbially-expressed MCRY3A-0102, supplied by the study sponsor with a reported purity of 90.3% w/w mCry3A protein. Upon receipt at the test facility, the test material was stored frozen (-25 to -10°C) in the original container.

The positive control substance was potassium arsenate, Lot No. 013K0602, received from Sigma Chemical Co., St. Louis, MO with a reported purity of 62.8%. The positive control was stored at room temperature and recertification of its insecticidal activity was confirmed.

### **Test Methods**

The study was conducted to assess the combined effect from dietary exposure to the test materials on the predatory beetle (*Coleomegilla maculata*). A toxic reference standard was used to demonstrate the suitability of the test system for detection of toxic effects. The test organisms (SSL Lot No. 06A125) were obtained as eggs from the US Dept. of Agriculture, Byron, GA. Upon receipt at the testing facility, the eggs were incubated in an environmental chamber set to maintain a temperature of 25 ± 2°C and a relative humidity of 40 to 80%. The photoperiod was 16 hours light/8 hours darkness.

The base diet was a 50:50 w/w mixture of bee pollen (General Nutrition Corp., Wareham, MA) and *Ephestia kuehniella* (Mill moth) eggs (Beneficial Insectaries, Redding, CA). Preparation of the test substance diets is provided in Table 1. The test substances were added to the bee pollen and eggs, and the resulting diet was mixed with deionized water (0.85:1, water:dry diet) and vortexed.

Table 1. Test diet preparation for Dietary Exposure to MIR162VIP3A-0106 (Vip3A <sub>20</sub> ) + TRYCRY1AB-0105 (Cry1Ab) + MCRY3A-0102 (mCry3A) proteins						
Deionized water (mL)	TRYCRY1AB-0105 (mL)	MIR162VIP3A-0106 (g)	MCRY3A-0102 (g)	Bee pollen (g)	<i>Ephestia</i> eggs (g)	Final diet concentration (µg a.i./g)
95	NA	NA	NA	56	56	Assay control
76.7	18.3	0.0122	0.0056	56	56	50 + 11.25 + 25

Data from p. 13, MRID 47137208

The positive control diet was prepared by adding 0.0084 g of potassium arsenate to a 50 mL flask and bringing it to volume with deionized water. Forty-eight mL of the stock solution was combined with 10 g each of bee pollen and *Ephestia* eggs for a final dietary concentration of 250 µg of potassium arsenate/g of diet. The treatment and control diets were encapsulated in Parafilm® to form approximately 50 µL pellets. The diets were prepared prior to the test and stored frozen (-20°C) until needed. The pellets were scored with a razor blade and acclimated to room temperature prior to use. Approximately 100-g samples of each diet were taken at test start and stored at -20°C prior to shipment to the sponsor for analyses for concentration and intactness.

The test chambers were 1.5-cm Petri dishes with plastic covers. At test start, moistened dental wicks and the appropriate food pellet were added to each test chamber, followed by a single, second instar (3- to 5- days old) *C. maculata* larva. Each treatment consisted of 40 replicates. The test chambers were randomly placed on trays in an incubator designed to maintain a temperature of 25 ± 2°C and a relative humidity of 40 to 80%. Approximately 50 µg of the appropriate diet was provided to each insect every other day. Unconsumed food was removed when the fresh diet was added. The moistened dental wicks were replaced as needed.

The beetles were observed for mortality, development (pupation and emergence), feeding behavior, and abnormal behavior until the test terminated on day 21. At test end, all emerged adults were frozen and weighed. Any insects that had not emerged or had died were counted.

Samples of each diet were collected at test start and shipped frozen to the study sponsor for analysis of concentration and intactness of the test materials. Additional samples were collected at test start for use in sensitive insect bioassays to confirm the presence and bioactivity of the test materials at test start and after frozen storage.

At test end, survival data were analyzed using Fisher's Exact Test. Days to pupation, days to adulthood, and adult weight, were analyzed by ANOVA, and significant differences were determined using a two-sample t-test. If control mortality exceeded 5%, the observed mortality was corrected using Abbott's formula.

## II. RESULTS:

Results are summarized in Table 2. The weight of *C. maculata* adults exposed to the test material diet was slightly higher than that of the assay control adults. The development time (days to

pupation and days to adulthood) was also slightly shorter in the test material group than the control group. However, these differences were not considered biologically relevant because the test material represented 7.5X to 10X the concentration found in the issues of Bt11 x MIR162 x MIR604 corn and did not display toxic effects. Response to the positive control was appropriate.

Parameter	Assay control	Test diet (50 µg Vip3Aa20 + 11 µg Cry1Ab + 24 µg mCry3A/g of diet)	Positive control (250 µg potassium arsenate/g diet)
Mean no. of days to pupate	12.1 ± 0.93	10.0 ± 1.7 <sup>a</sup>	NA
Living pupae	33	39	NA
Total pupae	35	39	NA
Percent larval pupation	87.5	97.5	0
Dead larvae	5	1	40
Total larvae	40	40	40
Percent larval mortality	12.5	2.5	100
Percent adult emergence	94.3	100	NA
Abbott's corrected pre-imaginal mortality	0.0	-0.2	NA
Mean no. of days to adulthood	14.5 ± 1.3	13.0 ± 1.1 <sup>a</sup>	NA
Dead adults	1	0	NA
Total adults	33	39	NA
Percent adult mortality	3.0	0.0	NA
Mean adult weight (g)	0.0141 ± 0.0033	0.0157 ± 0.0036 <sup>a</sup>	NA

Data from p. 20, MRID 47137208

<sup>a</sup> Significantly different when compared to assay control

Quantitative analysis of the treated diet using ELISA found mean concentrations of 12.1 µg Cry1Ab, 6.5 µg Vip3Aa20, and 16.3 µg mCry3A per g of diet, corresponding to recoveries of 107.3%, 12.9%, and 68.1%, respectively, of the intended dietary concentrations (Appendix I of MRID 47137208). The apparently low concentration of Vip3Aa20 extracted was believed to be due to its low solubility in aqueous solutions. None of the test material proteins was found in the control diet. Western blot analysis of the extracted proteins showed the presence of immunoreactive bands corresponding to the expected Cry1Ab, Vip3Aa20, and mCry3A molecular weights (ca. 66 Da, 89 Da, and 67.7 Da, respectively). The Western blots also indicated that the test materials were substantially stable in the initial and frozen test diet.

The insecticidal activity of the test material diet was confirmed as equivalent at test start and after frozen storage in sensitive insect bioassays using European corn borer (ECB, *Ostrinia nubilalis*, highly sensitive to Cry1Ab), fall armyworm (FAW, *Spodoptera frugiperda*, highly sensitive to Vip3Aa20), and Colorado potato beetle (CPB, *Leptinotarsa decemlineata*, highly sensitive to mCry3A) larvae.

### III. CONCLUSION:

The study author concluded that there were no detrimental effects on larval, pupal, or adult survival, days to pupation, or days to adulthood in *C. maculata* exposed to the combined test diet containing 50 µg Vip3Aa20 + 11.23 µg Cry1Ab + 24 µg mCry3A per g of diet of bee pollen and moth eggs.

#### **IV. EPA REVIEWER'S COMMENTS:**

The study met the criteria established for validity, which were control mortality of  $\leq 20\%$  and positive control mortality of  $> 20\%$ . The NOEC was greater than 50 µg Vip3Aa20 + 11.23 µg Cry1Ab + 24 µg mCry3A/g of diet on the development and survival of *Coleomegilla maculata* and the LC<sub>50</sub> was greater than 50 µg Vip3Aa20 + 11.23 µg Cry1Ab + 24 µg mCry3A/g of diet, when exposed orally via a treated bee pollen and moth egg-based diet.

#### **V. CLASSIFICATION: ACCEPTABLE**

**DATA EVALUATION RECORD**

**CRY1Ab PROTEIN  
VIP3Aa20 PROTEIN  
MCRY3A PROTEIN  
(Bt11 x MIR162 x MIR604 Maize)**

**STUDY TYPE: Non-target Insect Testing, Tier I (OPPTS 885.4340)**

**MRID No. 471372-10**

Prepared for  
Biopesticides and Pollution Prevention Division  
Office of Pesticide Programs  
U.S. Environmental Protection Agency  
One Potomac Yard  
2777 South Crystal Drive  
Arlington, VA 22202

Prepared by  
Toxicology and Hazard Assessment Group  
Environmental Sciences Division  
Oak Ridge National Laboratory  
Oak Ridge, TN 37830  
Task Order No. 07-070

Primary Reviewer:

Eric B. Lewis, M.S.

Signature: \_\_\_\_\_

Date: \_\_\_\_\_

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Anthony O. Armstrong, M.S.

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

Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Quality Assurance:

Lee Ann Wilson, M.A.

Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Disclaimer

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

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**DATA EVALUATION RECORD**

**Primary Reviewer:** Eric B. Lewis, M.S., Oak Ridge National Laboratory

**EPA Reviewer:** Annabel Waggoner, Environmental Protection Specialist, BPPD

*AW*

**STUDY TYPE:** Non-target Insect Testing, Tier I (885.4340)

**MRID NO:** 471372-10

**DP BARCODE:** 345913

**DECISION NO:** 379490

**TEST MATERIAL:** Bt11 x MIR162 x MIR604 maize pollen (expressing *Bt* Cry1Ab, Vip3Aa20, and mCry3A proteins)

**STUDY NO:** 1781.6669

**SPONSOR:** Syngenta Seeds, Inc., P.O Box 12257  
3054 E. Cornwallis Road  
Research Triangle Park, NC 27709

**TESTING FACILITY:** Springborn Smithers Laboratories,  
790 Main Street  
Wareham ,MA 02571-1037

**TITLE OF REPORT:** Bt11 x MIR162 x MIR604 x GA21. Evaluation of Potential Effects of Bt11 x MIR162 x MIR604 x GA21 Maize Pollen on Monarch Butterfly (*Danaus plexippus*) via Dietary Exposure Assays

**AUTHOR:** Patnaude, M.

**STUDY COMPLETED:** May 15, 2007

**CONFIDENTIALITY CLAIMS:** None

**GOOD LABORATORY PRACTICE:** A signed and dated GLP statement was provided. The study was conducted in compliance with 40CFR Part 160 with one exception: routine water screen analyses were conducted using US EPA procedures and are considered facility records under Springborn Smithers Laboratories SOP 7.92.

**STUDY SUMMARY:** A laboratory dietary exposure bioassay was conducted to assess the effects of Bt11 x MIR162 x MRI604 maize pollen diet, which contains the transgenic insecticidal proteins Cry1Ab, Vip3Aa20, and modified Cry3A (mCry3A), on monarch butterfly (*Danaus plexippus*) larvae. The test also included larval exposure to transgenic Bt11 maize pollen, control (non-transgenic) maize pollen, and deionized water (a non-pollen assay control). First instar larvae were provided a diet of milkweed leaves

containing the appropriate pollen at a target density of 680 grains/cm<sup>2</sup> of leaf for a maximum of four days, followed by untreated leaves for an additional five days. Control mortality exceeded the 20% validity criterion on day 6 and was attributed to confinement stress. Statistical analyses on the larval mortality through day 5 showed no significant increase in mortality in the test material and assay control groups. In a repeat of the bioassay, control mortality exceeded 20% on day 7. Statistical analyses on the larval mortality through day 6 also showed no significant increase in mortality between the test material and assay control groups. The NOEC was greater than 0.425 mg Bt11 x MIR162 x MIR604 maize pollen / cm<sup>2</sup> of milkweed leaf diet on *Danaus plexippus* larvae and the LC<sub>50</sub> was greater than 0.425 mg Bt11 x MIR162 x MIR604 maize pollen / cm<sup>2</sup> diet, when exposed orally via a liquid suspended test pollen and milkweed leaf diet.

**CLASSIFICATION:           ACCEPTABLE**

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**I. STUDY DESIGN:**

**A. Test Material**

Bt11 x MRI 162 x MIR604 maize pollen, Batch No. GA21 SBI 8/06, supplied by the study sponsor on September 14, 2006. A second sample of the test material, Reference No. 05MG055768, was received from the study sponsor on December 8, 2006.

The reference material was Bt11 maize pollen, Reference No. 05MG055998, supplied by the study sponsor on September 14, 2006. A second sample of the test material, Reference No. 05MG055998, was received from the study sponsor on December 8, 2006.

The control substance was non-transgenic maize pollen, Batch No. not provided, supplied by the study sponsor on September 14, 2006. A second sample of the control substance, Reference No. 05MG065560, was received from the study sponsor on December 8, 2006.

After receipt at the testing facility, all samples of the test, reference, and control substances were stored in a freezer (-70 to -90°C), each in its original container.

**B. Test Methods**

The study was conducted to assess the effect of Bt11 x MIR162 x MRI604 pollen on larval mortality and biomass of the monarch butterfly (*Danaus plexippus*) via dietary exposure. Bt11 x MIR162 x MRI604 maize plants produce the transgenic insecticidal proteins Cry1Ab, Vip3Aa20, and modified Cry3A (mCry3A). The test organisms were obtained as eggs from Shady Oak Butterfly Farm, Brooker, FL. Upon receipt at the test facility, the eggs were incubated in an environmental chamber set to maintain a temperature of 27 ± 2°C and a relative humidity of about

50%. The photoperiod was 16 hrs light:8 hrs darkness. Late first instars (36- to 48-hrs old) were used the study.

The base diet for the monarch larvae was milkweed plants (*Asclepias curassavica*) grown from commercial seed in 360-Metro mix and maintained in a greenhouse prior to and during the study. The plants were irrigated twice/week with a commercial water-soluble fertilizer, and otherwise watered with tap water. When the plants reached maturity after about eight weeks, leaves were harvested from approximately the same height on the plants on test days 0, 2, and 3, and immediately placed between moist paper towels to reduce desiccation. They were then cut in half and treated with the test material.

The test included four treatments: Bt11 x MIR162 x MIR604 maize pollen, Bt11 maize pollen, control (non-transgenic) maize pollen, and deionized water (a non-pollen assay control). Each treatment consisted of 32 replicates. A single pollen density of 680 grains/cm<sup>2</sup> of milkweed leaf, which was the highest pollen density that could be achieved using the spray apparatus, was used in the test. Dosing solutions of 43.02 mg/mL were prepared on test days 0, 2, and 3 by diluting approximately 1.2906 g of the appropriate pollen to a total volume of 30 mL with deionized water. A magnetic stir plate and bar were used to keep the pollen in a homogenous suspension during application to the leaves.

The dosing solutions were applied to milkweed leaves within six hours after leaf collection. The solutions were applied using a sprayer and application chamber constructed at the testing facility. The sprayer (Spray Systems Company, Wheaton, IL) used an atomizing spray nozzle calibrated to deliver 20 psi to a revolving belt (277 ft/min) that transported the leaves past the nozzle. The system was calibrated to deliver 0.00988 mL/cm<sup>2</sup> of treatment solution (0.4250 mg pollen/cm<sup>2</sup>). The assay control of deionized water was sprayed first. After application, the leaves were allowed to dry prior to presentation to the test organisms. The leaf pollen density was confirmed on test days 0 and 3 by counting the pollen grains on one leaf from each of the control and test pollen groups.

The test arenas were 1.5 oz vessels in 16-vessel trays with ventilated plastic covers. Each test arena contained a thin layer of agar on the bottom. On day 0, two halves of one milkweed leaf from the appropriate treatment were placed on the bottom of each test arena and a single larva was placed on the leaf surface. The test vessels were maintained in an incubator at a temperature of 27 ±2°C and a relative humidity of about 50%. The photoperiod was 16 hrs light:8 hrs darkness. Unconsumed food was removed and replaced every other day. The treated leaves were supplied for four days, after which all larvae were given untreated leaves for an additional five days. On test day 4, the larvae were transferred to 16-oz vessels to avoid confinement stress.

The larvae were observed daily for mortality and at test termination all survivors were weighed. Mortality was analyzed using Fisher's Exact Test, and weight was analyzed using William's Test and Bonferroni's Test.

## **II. RESULTS:**

The pollen grain counts confirmed the pollen density on the milkweed leaves. On day 0, the density ranged from 590 to 664 grains/cm<sup>2</sup> for the pollen treatments (87 to 98% of target), and on day 3 pollen density ranged from 666 to 695 grains/cm<sup>2</sup> (98 to 102% of target).

Results are summarized in Table 1. Control mortality exceeded the validity criterion of 20% on test day 6. Analysis of the day 5 data found no statistically significant difference in mortality of the test material group compared to the assay control group. Day 9 mean larval biomass of the test material group was slightly reduced compared to the assay and non-transgenic control groups. The unexpectedly high control group mortality was believed to be due to confinement stress. Therefore, a second test was initiated using the same protocol.

Table 1. Mortality and biomass of monarch butterfly larvae exposed to Bt11 x MIR162 x MIR604 x GA21 maize pollen – first study						
Treatment	Mean larval mortality (%)					Day 9 mean larval biomass (g)
	Day 5	Day 6	Day 7	Day 8	Day 9	
Non-pollen assay control	16	22	31	59	59	1.15
Non-transgenic maize pollen control	19	25	38	56	63	1.07
BT11 x MIR162 x MIR604 x GA21 maize pollen	28	38	47	56	63	1.02
Bt11 maize pollen	16	31	31	38	53	0.97

Data from p. 22, MRID 47137210

Statistical analysis was performed only for data through day 5, after which control mortality exceeded 20%

Results of the second test are summarized in Table 2. Control mortality exceeded the validity criterion of 20% on test day 7. Analysis of the day 6 data found no statistically significant difference in mortality of the test material group compared to the assay control group. Day 8 mean larval biomass of the test material group was slightly increased compared to the assay control group and slightly decreased compared to the non-transgenic control groups. The unexpectedly high control group mortality in the second test was attributed to reduced ventilation.

Table 2. Mortality and biomass of monarch butterfly larvae exposed to Bt11 x MIR162 x MIR604 x GA21 maize pollen – second study				
Treatment	Mean larval mortality (%)			Day 8 mean larval biomass (g)
	Day 6	Day 7	Day 8	
Non-pollen assay control	16	25	38	0.69
Non-transgenic maize pollen control	19	47	53	0.87
BT11 x MIR162 x MIR604 x GA21 maize pollen	31	47	63	0.80
Bt11 maize pollen	19	22	34	0.88

Data from p. 23, MRID 47137210

Statistical analysis was performed only for data through day 6, after which control mortality exceeded 20%

Confirmation that Vip3Aa20 was present in the test material was provided in a separate study (Appendix 3 of MRID 47137210). ELISA showed that the concentration of Vip3Aa20 in the test pollen was  $59.1 \pm 4.1 \mu\text{g/g}$  pollen. No Vip3Aa20 protein was detected in the non-transgenic maize control pollen.

The insecticidal activity of the Vip3Aa test material was confirmed in a sensitive insect feeding bioassay using fall armyworm larvae (*Spodoptera frugiperda*) (Appendix 3 of MRID 47137210). An extract of the test pollen was overlaid on general insect diet at a concentration of approximately  $100 \text{ ng Vip3Aa20/cm}^2$  of diet surface (corresponding to  $247 \mu\text{g total protein/cm}^2$  of diet surface) and made available to the larvae for 192 hrs. Mortality of larvae fed the test pollen extract was

66.7%. No mortality was seen in larvae given diet overlaid with non-transgenic control pollen extract at a concentration of 270 µg total protein/cm<sup>2</sup> of diet surface.

### **III. CONCLUSION:**

The study author concluded that exposure of monarch larvae to the test material via its application to milkweed leaves at a concentration of 680 grains/cm<sup>2</sup> of leaf produced no statistically significant increase in mortality compared with similar exposure to Bt11 pollen.

### **IV. REVIEWER'S COMMENTS:**

There were no statistically significant differences in mortality from the combined effects of Bt11 x MIR162 x MIR604 corn when compared to the assay control group on monarch butterfly larvae. In addition, no adverse effects were test treatment related and were attributed to experimental shortcomings of high control mortality. The NOEC was greater than 0.425 mg Bt11 x MIR162 x MIR604 maize pollen / cm<sup>2</sup> of milkweed leaf diet on *Danaus plexippus* larvae and the LC<sub>50</sub> was greater than 0.425 mg Bt11 x MIR162 x MIR604 maize pollen / cm<sup>2</sup> diet, when exposed orally via a liquid suspended test pollen and milkweed leaf diet.

### **V. CLASSIFICATION: ACCEPTABLE**