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

OFFICE OF PREVENTION
PESTICIDES AND TOXIC
SUBSTANCES

FEB 09 2006

MEMORANDUM

SUBJECT	Environmental Fate and Risk Assessment for mCry3A Protein in Syngenta's MIR604 field corn (MRIDs 462656-01, 462656-13, and 462656-14).	
TO	Michael Mendelsohn Senior Regulatory Action Leader Microbial Pesticides Branch Biopesticides and Pollution Prevention Division (7511C)	
FROM	Tessa Milofsky, M.S. Agronomist Microbial Pesticides Branch Biopesticides and Pollution Prevention Division (7511C)	signed 2-9-06
THROUGH	Zigfridas Vaituzis, Ph.D, Microbiologist Microbial Pesticides Branch Biopesticides and Pollution Prevention Division (7511C)	signed 2-9-06

Recommendations
It is recommended that additional studies should be conducted to evaluate plant-produced mCry3A protein degradation, accumulation, and persistence in a variety of soil types, including those high in clay and humic acids, into which all non-harvested corn plant material is incorporated.

Background
Syngenta, Ltd. is requesting a full and unrestricted FIFRA Section 3 registration for commercialization of MIR604 corn rootworm protected field corn, a new end-use product containing the mCry3A protein and the genetic material necessary for its production in corn, which includes the PMI inert marker. This memo includes summaries and DERs for the Environmental Safety Assessment, Environmental Fate Assessment, and Laboratory Soil Degradation studies submitted in support of the MIR604 registration. Reviews of the non-target studies included in this data package are covered in a separate memo.

Study: Environmental Safety Assessment (summary document) of Modified Cry3A Protein and Event MIR604 to Non-Target Organisms
MRID No.: 462656-01
Classification: Supplemental
Study Summary:
Summary information is presented in support of the Section 3 registration of MIR604 rootworm protected field corn, which contains the mCry3A protein. The safety assessment document provides summaries of the non-target laboratory studies that were used to evaluate the potential adverse effects of mCry3A on non-target organisms. According to expected environmental concentration (EEC) calculations performed and justified by Syngenta, non-target test species were exposed to 11.2 to 2,600 times the EEC or daily dietary dose of mCry3A without significant adverse effects.
BPPD Review:
The reviewer generally agrees with summary information presented by the study authors. Justifications/explanations provided for EEC calculations seem reasonable. It is noted, however, that many of the assumptions used to calculate margins of exposure for test species were not submitted to EPA for

review and therefore, could not be verified by the reviewer. It is further noted that the potency of corn-derived protein was found to be twice the potency of bacteria-derived MCRY3A-0102 (MRID 461556-03). Despite the lower relative toxicity of the bacteria-derived protein, the reviewer agrees that, based on study results and EEC calculations presented by the registrant, it is unlikely that exposure to plant-produced mCry3A protein will result in significant adverse effects to non-target organisms.

Study: Environmental Fate Assessment of Modified Cry3A Protein in Event MIR604 Corn
MRID No.: 462656-13
Classification: Supplemental
Study Summary:
MIR604 corn plants have been shown to express mCry3A protein in leaves, kernels, roots, and silks, but the protein was not detected in corn pollen. Corn leaf assays were used to verify that mCry3A expression is stable over multiple generations and a soil degradation study showed that MCRY3A-0102 degrades readily, with a DT_{50} of 7.6 days in silty clay loam soil. Due to corn's lack of invasive characteristics and the low probability that the *mcry3A* gene from Event MIR604 would transfer to a wild relative of corn, it is unlikely that mCry3A will spread beyond cultivated sites and persist in weedy populations. It is also unlikely that genes present in MIR604 corn would be subject to horizontal gene transfer at a frequency that exceeds the rate of transfer in other plants. In the unlikely event that *mcry3A* is stably integrated and expressed in a soil microorganism, no harmful effects are expected. Laboratory-based non-target studies indicate that expected environmental exposure to mCry3A protein will not result in unreasonable adverse effects to beneficial organisms. The submission also notes the low likelihood that aquatic organisms will be exposed to mCry3A, since it is unlikely that the protein will enter watercourses via soil particle movement, pollen dispersal, or seed spillage.
BPPD Review:
The reviewer agrees with the above conclusions.

Study: Laboratory Soil Degradation of Modified Cry3A Protein (MCRY3A-0102)
MRID No.: 462656-14
Classification: Supplemental
Study Summary:
Degradation of MCRY3A-0102 (microbe-produced mCry3A protein) in silty clay loam soil was evaluated in a laboratory study, where treated soil samples were maintained under conditions that mimicked the field environment. MCRY3A-0102 activity in soil was evaluated with a Colorado potato beetle (CPB) (*Leptinotarsa decemlineata*) larval bioassay. The test protein was incorporated into the test soil at a nominal rate of 230 µg MCRY3A-0102/g of dry soil. Soil samples were collected at days 0, 1, 3, 7, 12, and 30 and frozen until needed for CPB diet formulation. CPB larvae were maintained on each soil/diet mixture for 72 hrs, after which mortality was determined. Larval mortality was 48-53% when fed diets containing soil from days 0, 1, 3, and 7; mortality declined to 9% when fed diet containing soil from day 30. Based on these results, a simple first-order kinetic model determined that the DT_{50} for mCry3A in this silty clay loam soil is 7.6 days.
BPPD Review:
These results indicate that microbe-produced MCRY3A-0102 insecticidal protein degrades rapidly in silt loam soil. However, silt loam soil is just one of many soil classes used for corn production in the United States. A more useful study would evaluate protein degradation, accumulation, and persistence in a range of soil types, including those with high clay and humic acid content, due to their known binding affinity for proteins.

It is also noted that this study utilized field soil spiked with microbe-derived insecticidal protein. This approach is useful because dose responses can be easily quantified. However, the degradation and accumulation of Cry proteins found within decaying plant tissue may behave differently than proteins in artificially spiked soil and MCRY3A-0102 was shown to be half as potent as plant-produced protein (MRID 461556-03). Consequently, aside from showing that MCRY3A-0102 does degrade in soil, the studies are incomplete and the long term relevance of these study results is unclear.

To account for the above concerns, it is recommended that additional studies should be conducted to evaluate plant-produced mCry3A protein degradation, accumulation, and persistence in a variety of soil types, including those high in clay and humic acids, into which all non-harvested corn plant material is incorporated. Sampling should be conducted each year for three years in a field sown with continuous MIR604 corn. Soil should be monitored for a minimum of one growing season after harvest and continued until the mCry3A protein can no longer be detected. It is recommended that these studies should be included as a condition of registration.

DATA EVALUATION RECORD

Primary Reviewer: Eric B. Lewis, M.S.
EPA Secondary Reviewer: Tessa Milofsky, M.S.

STUDY TYPE:	Nonguideline
MRID NO:	46265601
DP BARCODE:	DP303605
TEST MATERIAL:	mCry3A Protein in Event MIR604 Corn
STUDY NO:	Not provided
SPONSOR:	Syngenta Seeds, Inc., Research Triangle Park, NC
TESTING FACILITY:	N/A
TITLE OF REPORT:	Environmental Safety Assessment of Modified Cry3A Protein and Event MIR604 Corn to Non-Target Organisms
AUTHOR:	Raybould, A.
STUDY COMPLETED:	April 27, 2004
CONFIDENTIALITY CLAIMS:	None
GOOD LABORATORY PRACTICE:	A signed GLP statement was provided. The study is a compilation and is not subject to GLP standards.
STUDY SUMMARY:	Summary information is presented in support of the Section 3 registration of MIR604 rootworm protected field corn, which contains the mCry3A protein. The safety assessment document provides summaries of the non-target laboratory studies that were used to evaluate the potential adverse effects of mCry3A on non-target organisms. According to expected environmental concentration (EEC) calculations performed and justified by Syngenta, non-target test species were exposed to 11.2 to 2,600 times the EEC or daily dietary dose of mCry3A without significant adverse effects.
CLASSIFICATION:	Supplemental

Introduction

Laboratory studies were conducted using a range of non-target organisms to evaluate the environmental safety of modified Cry3A (mCry3A) protein (Table 1). To ensure an adequate "safety margin," the studies were designed to expose non-target arthropods to at least 10 times the estimated environmental concentration (EEC) of mCry3A, which was calculated to be 50 µg mCry3A/g based on preliminary studies indicating that the mCry3A concentration in leaves of MIR604 hybrid corn was 5 µg/g fresh weight. Other plant tissues contained less than this value, and because non-target arthropods tend not to eat corn leaf tissue, this exposure estimate was considered to be a worst-case exposure scenario. To achieve this safety margin (10 x EEC), most of the test species were exposed using a preparation of microbe-produced mCry3A (test substance MCRY3A-0102, 90.3% w/w mCry3A) in artificial diets, rather than using plant material containing mCry3A (lower dose test substance). The mCry3A in MCRY3A-0102 was shown to be chemically and biologically equivalent to mCry3A produced in Event MIR604 corn.

Further evaluation of corn leaf tissue indicated that mCry3A expression over four time points ranged from 3.8 µg mCry3A/g (anthesis) to 7.8 µg mCry3A/g at seed maturity, with an average over all time periods of 6.6 µg mCry3A/g fresh weight. Based on these findings, the 50 µg mCry3A/g exposure level is less than 10 x the exposure level in leaves. However, Syngenta argues that "mCry3A concentration in corn leaf tissue is not a realistic EEC, because (by definition) non-target organisms do not consume significant quantities of crop plant tissues".

The EEC for earthworms was based on the concentration of mCry3A protein present in senescent corn leaf tissue, while the exposure level used for the bird study was calculated from mCry3A concentrations present in corn grain. The fish study was the only non-target organism study that did not use microbe-produced MCRY3A-0102 as the test substance. In this case, corn grain was incorporated in fish feed.

TABLE 1. Ecotoxicolgy studies using exposure to mCry3A

Test species	Exposure route	Minimum Margin of Exposure	Dosing	Endpoints
Bobwhite quail (*Colinus virginianus*)	Capsule	1400 X DDD[1]	Single oral	14 d mortality, body wt, feed consumption
Rainbow trout (*Oncorhyncus mykiss*)	Feed formulated with 50% MIR604 grain	37.0 X EEC[2]	Daily	28 d mortality and growth
Flower bug (*Orius insidiosus*)	Protein in artificial diet	10.6 X EEC	Daily	Pre-imaginal mortality
Ladybird beetle (*Coccinella septempunctata*)	Aphids dipped in solution of protein	12.3 X EEC	Daily until pupation and after adult emergence	Pre-imaginal mortality and development, adult mortality
Rove beetle (*Aleochara bilineata*)	Protein in artificial diet	15.6 X EEC	Daily for first 35 days of test	Fecundity
Carabid beetle	Protein	11.2 X EEC	Daily until	Pre-imaginal mortality

(*Poecilus cupreus*)	injected into blowfly pupae		pupation	
Honeybee (*Apis mellifera*)	Protein in sugar solution	36 X EEC	Daily for first 5 days of test	Brood development, adult and larval mortality
Earthworm (*Eisenia foetida*)	Protein in artificial soil	46 X EEC	Single	14 d mortality, body wt
Mouse[3]	Protein in methylcellulose solution	2600 X DDD	Single oral	14 d mortality, body wt, organ wt, feed consumption

[1]DDD = Daily dietary dose
[2]EEC = Estimated environmental exposure
[3]Mammalian toxicity study

Results of Toxicity Tests

Bobwhite quail (MRID 46155616)

Five male and five female young adult northern bobwhite quail (*Colinus virginianus*) were administered a single oral dose of 722 mg MCRY3A-0102/kg body wt (nominally equivalent to approximately microbe-produced 652 mg mCry3A protein/kg body wt) and observed for 14 days. There were no treatment-related adverse clinical signs or mortality. Body weight and feed consumption of the test birds were comparable to those of controls. The acute oral nominal LD_{50} was greater than 722 mg MCRY3A-0102/kg body wt (microbe-produced 652 mg mCry3A protein/kg body wt), and the nominal NOEL was equal to 722 mg MCRY3A-0102/kg body wt (microbe-produced 652 mg mCry3A protein/kg body wt).

The margin of exposure for seed-eating birds was calculated using the formula DDD = (FIR/bw) x C, where DDD is daily dietary dose, FIR is food intake rate, bw is body weight, and C is the mCry3A concentration in the food. Based on a FIR/bw ratio of 0.11 to 0.35 for seed-eating birds and an average mCry3A concentration of 1.3 µg mCry3A/g fresh weight in kernels of MIR604 hybrids, the daily dietary dose for seed-eating birds ranges from 0.14 to 0.46 mg mCry3A/kg body weight. The 652 mg MCRY3A-0102/kg dose used in the quail study would therefore provide 1417 to 4657 times the estimated daily dietary dose of mCry3A for seed-eating birds that ate a diet of 100% fresh kernels from MIR604 plants.

Rainbow trout (MRID 46155617)

In a 28-day flow-through acute toxicity study, 40 juvenile rainbow trout (*Onchorhynchus mykiss*) were fed twice daily with a formulated fish feed containing 50% by weight Event MIR604 grain, which contains mCry3A protein. Transient discoloration, sounding, and surfacing were seen in one to three fish in the test group after day 15, and one fish was found dead on day 21. No significant differences were detected in the weight of the control or test fish at 0, 14, or 28 days, and no significant difference in length was seen at 14 or 28 days. The actual concentration of mCry3A in the grain used to prepare the fish feed was subsequently determined to be 0.30 µg/g fresh weight, and the concentration of mCry3A in the formulated feed was 0.09 ± 0.005 µg/g fresh weight (MRID 46265602).

The margin of exposure via the feed prepared in the study was calculated to be 37 times the EEC, based on the assumptions that feed would not likely be formulated using 100% grain from MIR604 hybrids and that the adoption rate of MIR604 hybrids would be no more than about 5% of field corn grown in the U.S.

Insidious Flower Bug (MRID 46265609)

Predatory bug (*Orius insidiosus*) nymphs were provided artificial meat-based diet containing MCRY3A-0102 at a rate nominally equivalent to 50 µg microbe-produced mCry3A/g diet for 21 days. A negative control group received artificial diet mixed with deionized water only, and a positive control group received diet containing teflubenzuron (0.01 mg a.i./g of diet). At the end of the test, there was no statistically significant difference in the percent mortality of the MCRY3A-0102 and negative control groups. Mortality in the positive control group was appropriate. The nominal LC_{50} was >50 µg microbe-produced mCry3A/g diet, and the nominal NOEC was 50 µg mCry3A/g diet. Subsequent analysis of the treated diet recovered 95.6% of the mCry3A, indicating mCry3A was present at the intended concentration (50 µg/g diet), and a CPB larvae assay showed the mCry3A was intact in the diet for at least 4 days (MRID 46265610).

Potential routes of field exposure of *Orius* to mCry3A include ingestion of corn silks and spider mites (*Tetranychus urticae*) that feed on MIR604 hybrids. Assuming a concentration of 1.7 µg mCry3A/g fresh weight of MIR604 corn silks, the exposure for *Orius* in the above study (50 µg/g diet) was calculated to be 29.4 times the EEC. Assuming a concentration of 4.7 µg mCry3A/g fresh weight of spider mites, the exposure was 10.6 times the EEC.

Ladybird beetle (MRID 46265603)

Four-day-old ladybird beetle larvae (*Coccinella septempunctata*) were fed daily with live pea aphids (*Acyrthosiphon pisum*) that had been immersed for 30 seconds in diluted wetting solution containing approximately 50 µg of MCRY3A-0102 protein/ml solution. Negative control larvae received aphids dipped in wetting solution only, and positive control larvae received aphids dipped in teflubenzuron. The larvae were allowed to pupate (approximately 9 to 10 days from study initiation to pupation), and adult beetles that emerged were fed treated aphids for 14 days. There were no statistically significant differences in pre-imaginal or adult survival between the negative control and mCry3A treatments; all larvae in the positive control treatment died in the pre-imaginal stage. There was no significant difference in the mean number of days for pupae to form in the negative control and mCry3A treatments, but the mean number of days to adult emergence was significantly lower in the mCry3A group. A subsequent study (MRID 46265604) determined that the actual mCry3A concentration in the treated aphids was 9 µg MCRY3A-0102/g of aphid (9 ng MCRY3A-0102/aphid).

Potential routes of field exposure of *Coccinella* to mCry3A include aphids and spider mites feeding on MIR604 corn hybrids. Based on the aphids containing 0.026 µg mCry3A/g fresh weight and the mites containing 4.7 µg mCry3A/g fresh weight, the EEC for ladybird beetle larvae consuming a diet of 85% aphids and 15% spider mites would be 0.73 µg mCry3A/g fresh weight of diet. The exposure in the above ladybird beetle toxicity test (9 µg mCry3A/g of aphid) was therefore 12.3 times the EEC.

Rove beetle (MRID 46265607)

Adult rove beetles (*Aleochara bilineata*) were provided beef paste diet containing MCRY3A-0102 at a rate nominally equivalent to 50 µg microbe-produced mCry3A/g of diet for 35 days. Negative control beetles were fed diet mixed with deionized water only, and positive control beetles were fed diet mixed with teflubenzuron (0.01 mg a.i./g of diet). To assess the effects of treatment on fecundity, approximately 500 onion fly (*Delia antiqua*) pupae, which are parasitized by rove beetles, were placed in the beetle habitat on days 14, 21, and 28 of the study. During days 35 to 76, emergence of adult rove beetles from the onion fly pupae was monitored. There was no statistically significant difference in day 35 mortality among any of the rove beetle groups, and no statistically significant difference in the mean number of progeny produced by the negative control and MCRY3A-0102 groups from days 35 to 76. Reproduction in the positive control group was significantly reduced. Subsequent analysis (MRID 46265608) of the treated diet recovered 91.7% of the mCry3A, indicating the test material was present at the intended concentration (50 µg/g diet).

Rove beetles (Staphylinidae) generally feed on other invertebrates, although some species feed on decaying plant material or parasitize other insects. A worst-case EEC was calculated using an MIR604 root concentration of 3.7 µg mCry3A/g fresh weight. If soil invertebrates incorporated the root mCry3A at 1.4 times less than the root concentration, the soil invertebrates would average 2.6 µg mCry3A/g fresh weight. The rove beetle exposure in the above study (50 µg/g diet) was then 19.2 times the EEC. If rove beetles consumed decaying plant material containing 3.2 µg mCry3A/g fresh weight (based on 15-day silage), the margin of exposure in the above study would be 15.6 times the EEC.

Ground-dwelling beetle (MRID 42665605)

Ground-dwelling beetle (*Poecilus cupreus*) larvae were fed daily with blowfly (*Calliphora vomitoria*) pupae that had been injected with MCRY3A-0102 at a rate nominally equivalent to 50 µg microbe-produced mCry3A protein/g fly pupa. A negative control of fly pupae injected with deionized water only, and a positive control of fly pupae injected with teflubenzuron (0.664 ng a.i./g of fly pupa) were also used in the test. When the *P. cupreus* larvae pupated, feeding stopped and adult emergence was monitored. There was no statistically significant difference in the percent pre-imaginal mortality or mean weight of emerged *P. cupreus* adults between the MCRY3A-0102 and negative control groups. Treatment with the positive control produced 100% pre-imaginal mortality. A subsequent study (MRID 46465606) found the mean concentration of microbe-produced mCry3A in the fly pupae used in the test was 12 µg mCry3A/g pupa.

The likely route of field exposure of non-target Carabids to mCry3A would be prey that had ingested tissue of MIR604 hybrids, e.g., black cutworm (*Agrotis ipsilon*) larvae. Assuming the cutworm larvae contained 0.15 µg mCry3A/g fresh weight (based on studies with Cry1Ab), the beetle exposure in the above study (12 µg/g pupa) was 80 times the EEC. In a worst case exposure based on the cutworm larvae containing 1.07 µg mCry3A/g fresh weight (using a spider mite mCry3A concentration 1.4 times lower than that of MIR604 tissue), the exposure in the beetle study would be 11.2 times the EEC.

Honeybee (MRID 46155618)

Honeybees (*Apis mellifera*) were exposed via in-hive feeders to a nominal concentration of 50 µg MCRY3A-0102/g of sucrose solution, 0.375 mg of diflubenzuron insect growth regulator/mL of sucrose solution, or 50% w/v sucrose solution alone for 5 days and monitored for 21 days. There were no statistically significant differences in egg cell mortality, larval cell mortality, or pre- and post-test hive condition between the test and sucrose solution-only groups. The diflubenzuron positive control produced 100% mortality in both egg and larval cells, and significantly reduced post-treatment hive condition. Adult bees were generally not affected by any of the treatments.

Honeybees are not likely to be exposed to mCry3A via MIR604, since corn does not produce nectar and detectable amounts of mCry3A are not found in the pollen. The honeybee was used to represent the order Hymenoptera, which contains wasps that are parasites of corn pests, primarily Lepidoptera. There is currently no validated test to expose parasitic Hymenoptera larvae to high doses of protein, so honeybee larvae were used. Assuming the average concentration of mCry3A in Lepidoptera larvae is 1.4 µg/g fresh weight (based on studies with Cry1Ab), the margin of exposure for honeybee larvae in the above study was 35.7 times the EEC.

Earthworm (MRID 46265611)

Groups of 10 earthworms (*Eisenia fetida*) in artificial soil were exposed to MCRY3A-0102 equivalent to a nominal concentration of 250 µg microbe-produced mCry3A/g moistened soil for 14 days. Controls were exposed to the same soil without MCRY3A-0102. The LC_{50} for a positive control (2-chloroacetamide) group in the same soil was also determined. At test end, the test material group

worms had a mortality rate of 5% and a mean weight loss of 5.8%. Negative control worms had a mortality rate of 0% and a mean weight loss of 11.4%. The LC_{50} for the positive control worms was 18.0 mg/kg dry soil. Subsequent extraction of mCry3A from treated soil samples collected on days 0, 3, 7, and 14 of the study recovered 18.1%, 14.8%, 6.3%, and 7.6% of the nominal concentration, respectively (MRID 46265612). The low recovery rate at day 0 was attributed to low extractability from the soil, because a CPB larval bioassay showed that the test material was active for at least 5 days, and a Western blot assay showed intact mCry3A was present at 14 days, although some degradation did occur.

The most likely route of field exposure of earthworms to mCry3A would be ingestion of senescent plant material incorporated into the soil. Assuming that earthworms ate 100% senescing MIR604 leaves (conservative scenario) containing 5.5 µg mCry3A/g fresh weight, the 250 µg mCry3A/g moistened soil in the above study would be 46 times the EEC.

Other toxicity tests

No ecotoxicity studies were carried out using wild mammals, but an acute oral toxicity study with mice was conducted. Mice (5/sex) received a single oral dose of MCRY3A-0102 representing approximately 2377 mg mCry3A/kg body weight. After 14 days, there were no signs of toxicity, and all endpoints were comparable between the treated and control groups. The LD_{50} for males and females was >2377 mg MCRY3A-0102/kg body weight.

Rodents such as squirrels, mice, voles, and woodchucks, and larger mammals such as raccoons and deer ingest corn seed or ripening ears. The DDD of mCry3A for mammals can be calculated using the formula described above for birds. The FIR/bw ratios for the harvest mouse (*Micromys minutus*) and the wood mouse (*Apodemus sylvaticus*) consuming cereal seeds have been estimated to be 0.33 and 0.28, respectively. Based on MIR604 hybrid kernels averaging 1.3 µg mCry3A/g fresh weight, and assuming 100% of the diet is kernels, the DDD for rodents was estimated to be 0.43 mg mCry3A/kg body weight. The dose used in the acute oral toxicity study with mice discussed above would be over 5500 times the DDD.

Animals grazing on corn ears may consume both leaf material and kernels. Assuming that the ears contain 10.1 µg mCry3A/g fresh weight (a worst-case exposure, since MIR604 hybrid leaves average 10.1 µg mCry3A/g fresh weight, while the kernels, which would compose most of the ear, average only 1.6 µg mCry3A/g fresh weight) and the FIR/bw ratio for deer is 0.09, the DDD for large mammals eating a 100% diet of MIR604 corn ears is 0.91 mg mCry3A/kg body weight. The dose used in the acute oral toxicity study with mice discussed above would be 2600 times the large mammal DDD.

Potential for exposure of non-target Coleoptera

The registrant states that mCry3A activity is expected to be limited to beetles, particularly some members of the Chrysomelidae (leaf beetles, flea beetles, rootworms), Curculionidae (weevils and snout beetles), and Tenebrionidae (darkling beetles) families. The Chrysomelidae and Curculionidae are herbivores and could potentially be exposed to MIR604 pollen. However, since mCry3A has not been detected in MIR604 pollen, exposure of these beetles is unlikely. Tenebrionidae larvae and adults are plant material scavengers, and some mealworm species are pests of stored grain. Darkling beetles are not recorded as a pest of corn, but may be exposed to mCry3A by feeding on senescent MIR604 tissue in cornfields. The LD_{50} of a native Cry3A to yellow mealworm (*Tenebrio molitor*), a representative Tenebrionid, has been found to be 11.4 µg Cry3A/g larvae. Assuming that the activity of mCry3A to *T. molitor* is roughly equal to that of native Cry3A, and the mCry3A concentration in senescing MIR604 hybrid leaves is 5.5 µg/g fresh weight, a larva would need to ingest about 2.1 g of senescing leaf tissue to receive the median lethal dose. This represents about 3500 times the body weight of a first instar larva and about 15 times the body weight of a final instar. Since Tenebrionidae

are omnivorous and mCry3A rapidly degrades in soil, darkling beetles are unlikely to be exposed to sufficient mCry3A to be significantly impacted.

Endangered species considerations

The registrant states that no harmful effects of mCry3A, native Cry3A, or other Cry3 proteins have been shown in taxa outside Coleoptera. The activity of mCry3A is expected to be limited to certain species of the Chrysomelidae, Curculionidae, and Tenebrionidae families, and there are currently no endangered or threatened species in these families. Additionally, there are no known endangered or threatened beetle species in habitats where corn is grown. Therefore, no endangered or threatened beetles are expected to be harmed by mCry3A pollen expressed in MIR604 hybrids.

The lack of detectable expression of mCry3A in MIR604 pollen, the lack of weediness in corn, the absence of sexually compatible wild relatives of corn in the U.S., and the low probability of significant mCry3A dispersal in soil indicates that endangered or threatened species would only be exposed to mCry3A by eating MIR604 plants or organisms that had fed on MIR604 tissue. The Agency has reviewed the likelihood of endangered bird and bat species exposure to Cry3Bb1, a coleopteran-active protein expressed in Event MON863, concluding that endangered birds and bats rarely forage in agricultural fields and that insectivorous species tend to take flying insects rather than larvae (the life stage most likely to contain Cry protein). Similarly, cultivation of MIR604 is unlikely to have any harmful effects on endangered or threatened species.

DATA EVALUATION RECORD

Primary Reviewer: Eric B. Lewis, M.S.
EPA Secondary Reviewer: Tessa Milofsky, M.S.

STUDY TYPE:	Nonguideline
MRID NO:	46265613
DP BARCODE:	DP303605
TEST MATERIAL:	mCry3A Protein in Event MIR604 Corn
SPONSOR:	Syngenta Seeds, Inc., Research Triangle Park, NC
TESTING FACILITY:	N/A
TITLE OF REPORT:	Environmental Fate Assessment of Modified Cry3A Protein in Event MIR604 Corn
AUTHOR:	Raybould, A.
STUDY COMPLETED:	April 27, 2004
CONFIDENTIALITY CLAIMS:	None
GOOD LABORATORY PRACTICE:	A signed GLP statement was provided. The study is a compilation and is not subject to GLP standards.
STUDY SUMMARY:	MIR604 corn plants have been shown to express mCry3A protein in leaves, kernels, roots, and silks, but the protein was not detected in corn pollen. Corn leaf assays were used to verify that mCry3A expression is stable over multiple generations and a soil degradation study showed that mCry3A degrades readily, with a DT_{50} of 7.6 days in silty clay loam soil. Due to corn's lack of invasive characteristics and the low probability that the *mCry3A* gene from Event MIR604 would transfer to a wild relative of corn, it is unlikely that mCry3A will spread beyond cultivated sites and persist in weedy populations. It is also unlikely that genes present in MIR604 corn would be subject to horizontal gene transfer at a frequency that exceeds the rate of transfer in other plants. In the unlikely event that *mcry3A* is stably integrated and expressed in a soil microorganism, no harmful effects are expected. Laboratory-based non-target studies indicate that expected environmental exposure to mCry3A protein will not result in unreasonable adverse effects to beneficial organisms. The submission also notes the low likelihood that aquatic organisms will be exposed to mCry3A, since it is unlikely that the protein will enter watercourses via soil particle movement, pollen dispersal, or seed spillage.

Introduction

The *cry3A* gene from *Bacillus thuringiensis* subsp. *tenebrionsis* was synthetically recreated to optimize for expression in corn, and changed so that the encoded modified Cry3A (mCry3A) protein has enhanced activity against the western corn rootworm (*Diabrotica virgifera virgifera*) and the northern corn rootworm (*D. longicornis barberi*). Corn plants transformed with mCry3A, such as Event MIR604, display resistance to these pests.

mCry3A Levels in MIR604 Hybrids

In MIR604 corn plants sampled at four growth stages (whorl stage, 6 weeks after planting; anthesis, 10-11 weeks after planting; seed maturity, 18-20 weeks after planting; and senescence, about 24 weeks after planting), mCry3A was found in the leaves, kernels, roots, and silks at each growth stage, but not in pollen (limit of detection = 0.07 µg/g fresh wt). The expression profile appears to be stable over multiple generations. In four successive backcrosses under greenhouse conditions, leaf tissue sampled at anthesis showed that the concentration of mCry3A in each generation was similar, with no trend toward increased or decreased expression.

Degradation in Soil

Evidence indicates that Cry proteins undergo enzymatic degradation by soil proteases. Laboratory soil degradation studies on lepidopteran-active proteins (Cry1Ab, Cry1Ac, Cry1F) indicate they are rapidly degraded, with a DT_{50} (time for the Cry protein concentration or bioactivity to fall to half its initial value) of 2 to 22 days for Cry1A proteins and a DT_{50} of just over 3 days for Cry1F. The DT_{50} for Cry3Bb1 protein, which is active against certain Coleoptera, is between less than 1 day and up to 9 days. In a laboratory degradation test using mCry3A incorporated into a silty clay loam soil (MRID 46265614), a Colorado potato beetle bioassay determined the DT_{50} of MCRY3A-0102 protein was 7.6 days.

Spread and Persistence of the mCry3A gene

The probability of the *mcry3A* gene from MIR604 transferring to wild relatives of corn is very low. Corn will hybridize with a group of taxa collectively known as teosinte, which are native to Central America. Teosinte species have co-existed with cultivated corn for several thousand years, but have remained genetically distinct from cultivated varieties despite occasional introgression. While teosinte species are grown in the U.S. in botanical gardens, fertilization of these plants by MIR604 pollen is unlikely.

Species of the genus *Tripsacum*, three of which occur in the U.S., are considered to be close relatives of corn. However, hybrids between *Tripsacum* and corn are difficult to obtain and are often sterile. *T. dactyloides* is widespread, but does not hybridize readily with corn, and the probability of backcross or F_2 progeny of *Tripsacum* x *Zea* hybrids being produced in the field is negligible. In the environmental assessment section of the BRAD for Bt-plant incorporated protectants (2001), the Agency concluded that the chance of natural introgression of genes from corn to *Tripsacum* was extremely remote.

In its reassessment of the environmental safety of *Bt* plant-incorporated protectants, the Agency reviewed information concerning the theoretical risks of horizontal gene transfer (HGT), and found no evidence for HGT under field conditions. Non-agency reviews found very few examples of HGT, and these cases involved artificially high sequence homology between the transgene and the potential recipient. Data comparing full genomic sequences of various prokaryotes and eukaryotes have identified putative HGT events, although the data are subject to interpretation. There is no reason to suppose that corn derived from Event MIR604 is likely to transfer genes by HGT at a higher rate than any other plant.

The likelihood of exposure to mCry3A via microorganisms expressing the *mcry3A* gene from MIR604 is minimal. Should a *cry* gene be transferred from a transgenic plant to a microorganism, there should be no significant hazards. *B. thuringiensis* is common in soil, and *cry* genes have been available for HGT to other species for long periods. No harmful effects appear to have resulted from the prolonged exposure. Soil microorganisms have not previously been exposed to the *mcry3A* gene contained in MIR604. The DNA sequence of *mcry3A* was substantially altered from native *cry3A* to optimize codon use for expression in plants, so the *mcry3A* gene likely has lower homology to potential recombination sites in soil microorganisms than the native *cry3A* gene does. Should *mcry3A* be integrated into a plasmid or chromosome of a bacterium, mCry3A protein is unlikely to be produced because the maize metallothionen promoter linked to *mcry3A* in MIR604 is unlikely to function in bacteria and codon use in *mcry3A* is optimized for expression in corn, not bacteria. Laboratory tests of the effects of mCry3A protein on a variety of non-target organisms have revealed no adverse effects. In the unlikely event that *mcry3A* is stably integrated and expressed in a soil microorganism, no harmful effects are expected.

Weediness

Corn has lost the ability to survive outside cultivation, and is unlikely to form self-sustaining populations as a weed since a) it is easily controlled with herbicides, b) seed dispersal is limited, c) the seeds lack dormancy and are exposed to winter conditions, d) it requires disturbed ground to germinate, and e) it is not competitive with perennial vegetation. The lack of invasiveness of corn in non-agricultural habitats makes it unlikely that mCry3A will spread from cultivated sites and persist as weedy populations of MIR604.

Environmental Exposure and Fate

Ecologically significant exposure to mCry3A outside cultivation is unlikely to occur through contact with pollen, since mCry3A protein has not been detected in MIR604 hybrid pollen. Due to its rapid degradation in soil, mCry3A is not likely to spread from cultivation to surface or ground water. Exposure to mCry3A during cultivation would be limited to contact with MIR604 tissues, and possibly short-term exposure to exuded proteins in soil. After harvest, mCry3A may remain in MIR604 plant tissue until the tissue degrades. The mCry3A is expected to degrade rapidly and not move offsite.

Aquatic organisms are not likely to be exposed to mCry3A since it is unlikely that mCry3A will enter watercourses via soil particle movement, pollen dispersal, or seed spillage. Other than humans or animals that eat corn grain, forage, or silage, the only organisms likely to be exposed to mCry3A from cultivation of MIR604 are pests and non-target organisms that feed on corn tissue, predators and parisitoids of these animals, and soil organisms. Exposure of non-target organisms feeding on the above-ground parts of corn will be limited to the period of cultivation.

Soil organisms may be exposed to mCry3A during cultivation and afterward by material incorporated into the soil following harvest. Due to the rapid degradation of mCry3A in soil, the post-harvest exposure of soil organisms to significant amounts of mCry3A would be limited to a few weeks. A second exposure of non-target organisms could arise if MIR604 volunteers occur. This exposure would be much lower.

DATA EVALUATION RECORD

Primary Reviewer: Eric B. Lewis, M.S.
EPA Secondary Reviewer: Tessa Milofsky, M.S.

STUDY TYPE:	Nonguideline
MRID NO:	46265614
DP BARCODE:	DP303605
TEST MATERIAL:	MCRY3A-0102
STUDY NO:	SSB-015004
SPONSOR:	Syngenta Seeds, Inc., Research Triangle Park, NC
TESTING FACILITY:	Syngenta Biotechnology, Inc., Regulatory Science Laboratory, Research Triangle Park, NC 27709-2257
TITLE OF REPORT:	Laboratory Soil Degradation of Modified Cry3A Protein (MCRY3A-0102)
AUTHORS:	Kramer, C. and R. Joseph
STUDY COMPLETED:	April 27, 2004
CONFIDENTIALITY CLAIMS:	None
GOOD LABORATORY PRACTICE:	A signed GLP statement was provided. The study was GLP compliant.
STUDY SUMMARY:	Degradation of MCRY3A-0102 (microbe produced mCry3A protein) in silty clay loam soil was evaluated in a laboratory study, where treated soil samples were maintained under conditions that mimicked the field environment. MCRY3A-0102 activity in soil was evaluated with a Colorado potato beetle (CPB) (*Leptinotarsa decemlineata*) larval bioassay. The test protein was incorporated into the test soil at a nominal rate of 230 µg microbe-produced mCry3A/g of dry soil. Soil samples were collected at days 0, 1, 3, 7, 12, and 30 and frozen until needed for CPB diet formulation. CPB larvae were maintained on each soil/diet mixture for 72 hrs, after which mortality was determined. Larval mortality was 48-53% when fed diets containing soil from days 0, 1, 3, and 7; mortality declined to 9% when fed diet containing soil from day 30. Based on these results, a simple first-order kinetic model determined that the DT_{50} for mCry3A in this silty clay loam soil is 7.6 days.
CLASSIFICATION:	Supplemental

Test Material

The test substance, MCRY3A-0102, *E. coli*-produced mCry3A protein, Batch Number not provided, was supplied by the sponsor with a reported purity of 90.3% w/w. No expiration date was reported. The test substance was stored at -20°C prior to use.

Test Methods

The test soil was a silty clay loam collected from a corn-growing region of Iowa. Characterization of the soil is provided in Appendix A of MRID 462656-14. The soil was passed through a 2 mm sieve and acclimated at 25 ± 1°C at a constant moisture level for 10 days prior to the test. One day prior to the test, 50 g of equivalent dry weight soil (~59 g) at 18.25% moisture level was added to 250-mL glass incubation flasks.

At test start, MCRY3A-0102 was prepared in deionized water at a concentration of 2.3 mg microbe-produced mCry3A/mL and applied in a volume of 5 mL to each of 24 flasks of soil for a dose corresponding to 230 µg microbe-produced mCry3A/g of soil on a dry weight basis. One control flask and 6 biomass determination flasks were prepared using soil dosed with 5 mL of deionized water only. The flasks were capped, shaken, and weighed to determine moisture content. Flasks were maintained at 25 ± 1°C and moisture levels were adjusted throughout the test to 75 ± 12% of field moisture capacity at 1/3 bar. Samples were collected from the dosed flasks after 0, 1, 3, 7, 12, and 30 days of incubation and frozen prior to their use in a degradation bioassay using Colorado potato beetle (CPB) (*Leptinotarsa decemlineata*) larvae. Microbial activity of soil in the biomass determination flasks was measured on days 0 and 42 using a Micro-Oxymax analyzer (procedure described in Appendix A of MRID 462656-14).

For the bioassay, soil collected at each incubation time point was incorporated into a stock CPB diet at a concentration of 10% w/w, and the resulting suspension was poured into Petri dishes. A negative control dosed with sterile water only was prepared in the same manner, and a positive control containing no soil was prepared by adding MCRY3A-0102 test solution to CPB diet to give a concentration of 23 µg microbe-produced mCry3A/g of diet (equivalent to that in the day 0 soil samples). Ten freshly-hatched CPB larvae (from eggs obtained from New Jersey Dept of Agriculture, Trenton) were added to each dish of diet, covered, and maintained under ambient laboratory conditions. The test material and negative control treatments were replicated 12 times (120 larvae total each) and the positive control plates were replicated 4 times (40 larvae total). Larval mortality was assessed at 72 hours.

Results Summary

Results of the larval bioassay are given in Table 1. Mean CPB larvae mortality in MCRY3A-0102- treated soil was 48-54% during the first week, then declined rapidly to 9% on day 30. The loss rate of mCry3A activity was estimated by plotting the mean percent mortality against days of incubation and fitting simple first order kinetics to the data using ModelManager (Cherwell Scientific Publishing, Oxford, UK). The DT_{50} value (time for 50% of initial bioactivity to dissipate) for degradation of MCRY3A-0102 in this soil was estimated to be 7.6 days. Biomass determinations of soil at study start and end showed that microbial activity was maintained during the study.

TABLE 1. MCRY3A-0102 activity in treated soil as measured by Colorado potato beetle mortality (%)							
Mean percent mortality							
Negative control	Positive control	MCRY3A-0102					
		Day 0	Day 1	Day 3	Day 7	Day 12	Day 30
18	53	53	54	48	51	11	9

Data from p. 15, MRID 46265614

Study Authors' Conclusions

The study authors concluded that mCry3A was rapidly degraded in soil, with a first-order DT_{50} of 7.6 days.