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

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC
SUBSTANCES

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MEMORANDUM

SUBJECT: Review of Syngenta's Revised Insect Resistance Management Strategy for Event MIR604 Maize (modified Cry3A) (Reg. No: 67979-L, D# 343070, DP# 343070, MRID# 465206-01)

TO: Michael Mendelsohn
Senior Regulatory Policy Specialist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

FROM: Sharlene R. Matten, Ph.D. Biologist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

Tessa S. Milofsky, M.S. Agronomist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

Mika Hunter, B.S. Entomologist 9-6-06
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

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

CONCLUSIONS AND RECOMMENDATIONS

The proposed IRM strategy and data to support it are "acceptable" except that the in-field strip refuge must be at least 4 rows wide (≥6 rows wide preferred) based on recent larval movement data. If resistance is recessive, then the proposed IRM plan using a 20% structured refuge will be adequate to delay resistance for at least 15 years given the assumptions of Syngenta's model. If MIR604 maize is planted in areas with observable rotation-resistance in WCRW, then planting transgenic corn only in rotated maize fields

is a good IRM strategy that will delay the evolution of resistance by at least 15 years regardless of gene expression.

1. It is recommended that the IRM "terms and conditions" of registration be analogous to what has previously been required for other registered non-high dose corn rootworm-protected *Bt* corn products.
2. Specific resistance monitoring and remedial action plan recommendations are described below within the body of the review.
3. Specific cross-resistance studies are recommended. Syngenta should establish strains of CRW that are resistant to mCry3A to investigate the nature, inheritance, and fitness costs of specific mechanisms of resistance to the mCry3A protein expressed in MIR604 maize.
4. It is recommended that the behavioral deterrence (avoidance) mechanism be studied further by Syngenta and appropriate results should be submitted to the Agency when this research is completed.
5. It is recommended that that Syngenta continue its studies on the biological impact of adults surviving on MIR604 maize and submit these results when they are completed.
6. The EPA-approved changes granted on June 16, 2006 to the annual IRM reporting requirements and the Compliance Assurance Program for the lepidopteran-protected *Bt* maize registrations should be incorporated into Syngenta's IRM Compliance Assurance Program for MIR604 Corn Products.

BACKGROUND

Syngenta submitted an amended insect resistance management strategy in support of their FIFRA Section 3 Registration for Event MIR604-derived transgenic maize that expresses modified Cry3A (mCry3A) protein (MRID# 465296-01, McCaffery et al., 2005). Modified Cry3A in MIR604 is expressed at a non-high dose against infestation by the Western corn rootworm (WCRW; *Diabrotica virgifera virgifera* Le Conte) and the Northern corn rootworm (NCRW; *D. longicornis barberi* Smith and Lawrence). A behavioral avoidance mechanism is also present. BPPD reviewed Syngenta's previously submitted IRM strategy and noted several deficiencies in the simulation modeling and evaluation of cross-resistance potential (Matten, 2005). These deficiencies are addressed in the amended IRM strategy submission (McCaffery et al., 2005, MRID# 465296-01), the subject of this review.

SUMMARY OF SYNGENTA'S SUBMISSION

Syngenta's submission is divided into three main parts: 1) Sustainable deployment of Event MIR604 maize through effective insect resistance management (IRM), 2) IRM plan for MIR 604, and 3) Justification for the proposed IRM plan for MIR604. The first two sections of the revised Syngenta's submission were originally submitted by Syngenta (McCaffery and Stein, 2004) and reviewed by BPPD (Matten, 2005). There were significant deficiencies identified in the IRM modeling and evaluation of cross-resistance

potential. Syngenta has addressed these deficiencies in the section entitled, "Justification for the proposed IRM plan for MIR604."

Syngenta's IRM plan for MIR604 maize contains the following elements:

1. 20% structured refuge and its deployment and management;
2. IRM as a component of an integrated pest management program; and
3. product stewardship program.

The product stewardship program consists of the following aspects:

1. grower education plans;
2. grower obligations;
3. grower compliance;
4. annual resistance monitoring;
5. action plan in the event of unexpected levels of CRW damage;
6. remedial action plans; and
7. annual IRM plan review.

Syngenta has provided the following areas of support for its proposed IRM plan for MIR604 maize:

1. dose of mCry3A expressed in MIR604 (root expression assays, laboratory feeding assays using excised roots, field efficacy, and adult emergence studies);
2. simulation models for development of resistance;
3. impact on MIR604 maize on biology of CRW;
4. cross-resistance potential.

BPPD REVIEW

Nature and Derivation of Syngenta's MIR604 CRW-Control Maize

A *cry3A* gene from *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*) (Sekar *et al.*, 1987) was synthesized by Syngenta scientists for optimal expression in maize. The gene was then modified (hereinafter referred to as "mCry3A" or "modified Cry3A") to enhance activity against the *Diabrotica virgifera virgifera* (Western corn rootworm, WCRW) and *Diabrotica longicornis barberi* Smith and Lawrence (Northern corn rootworm, NCRW). The introduction of a particular serine protease recognition site (both chymotrypsin and cathepsin G are serine proteases) into the native Cry3A results in a much more rapid and complete processing of the 67 kDa protein to 55 kDa and thus increased activity against WCRW and NCRW. After binding to the receptor, the mCry3A protein inserts rapidly and irreversibly into the plasma membrane of the cell to form ion permeable pores in a similar fashion as other Cry toxins (Van Rie *et al.*, 1989; Garcia-Alonso and Vlachos,

2003). These ion channels cause a loss in membrane potential and the formation of these lesions leads to death resulting from septicaemia and/or starvation (Schnepf *et al.*, 1998).

Corn Rootworm Species Controlled

Native Cry3A is primarily active against Colorado potato beetle (*Leptinotarsa decemlineata*) and has minimal activity against NCRW, both members of the Chrysomelidae family of beetles. The mCry3A protein has a similar spectrum of activity to the native Cry3A, but with enhanced toxicity to NCRW and WCRW, both major coleopteran pests of maize in the USA. Modified Cry3A has some activity against *Diabrotica balteata* (Banded cucumber beetle). Trials were conducted in 2005 (data not available for this review) to determine the level of control of *Diabrotica virgifera zea* (Mexican corn rootworm; MCRW) by mCry3A expressing maize plants (Event MIR604). Modified Cry3A has no activity against non-coleopteran pests including various lepidopteran pests of maize. MIR604 maize is most effective against larvae, especially the first instar. Syngenta's IRM strategy focuses entirely on rootworms.

Pest Biology

A clear understanding of pest biology and ecology is essential to the development of a sound IRM plan. MIR604 *Bt* corn was developed to control two primary corn rootworm species: western corn rootworm (*Diabrotica virgifera virgifera* LeConte, WCRW) and northern corn rootworm (*D. Barberi* Smith & Lawrence, NCRW). WCRW is the most prevalent target pest in the United States and throughout most of the Corn Belt[1]. NCRW, also found throughout the Corn Belt, is considered the second most prevalent rootworm pest in the United States, and is the primary target pest of the north-central region[2]. Key factors believed to influence CRW adaptation to MIR604 corn include distribution, univoltinism, adult dispersal among fields, adult dispersal within fields, larval dispersal across rows, larval mortality due to density-dependent processes, insecticide use, egg mortality, fecundity, and adult and larval population density. Syngenta has provided a sufficient summary of the biology and ecology of the corn rootworm target pest(s) both in the current IRM strategy submission (McCaffery et al., 2005, MRID# 465296-01) and the original IRM strategy submission (McCaffery and Stein, 2004, MRID# 462656-17). See Matten (2005) for BPPD's review of Syngenta's original IRM strategy submission and pest biology information.

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[1] WCR is the primary rootworm pest in Colorado, Kansas, Nebraska, Ohio, Indiana, Illinois, Iowa, Missouri, and Michigan.

[2] NCR is the primary rootworm pest in Wisconsin, Minnesota, South Dakota and northern Iowa.

IRM Plan for MIR604

Syngenta's IRM plan for MIR604 maize contains three major components: 1) a 20% structured refuge; 2) IRM is part of an integrated pest management program; and 3) product stewardship. Each of these components will be discussed below.

1) 20% structured refuge and its deployment and management

There are two ways a grower can implement the refuge requirement: a non-Bt corn refuge can be planted as a continuous block adjacent to the MIR604 maize fields or as non-transgenic strips planted within transgenic field. Considering the limited movement of CRW larvae, planting refuges close to transgenic fields in large blocks is preferred to narrow strips (Gray 1999, Meinke *et al.* 2001). Syngenta will require growers to plant a minimum structured refuge of non-corn rootworm-control (CRW) maize on at least 20% of their maize acres. Refuge fields must not be planted with other transgenic corn used to control rootworm because neither acts as a refuge for the other. Use of a 20% refuge with MIR604 maize complements other technologies and provides a degree of uniformity for CRW control as well as with products with both CRW and lepidopteran control. This uniformity provides growers with a straightforward and understandable message about refuge requirements and promotes compliance. Justification of the 20% refuge requirement is discussed later in this review.

Syngenta will require growers to plant the structured refuge as blocks, strips, perimeter borders or pivot corners. Encouragement will be given to growers to plant these non-CRW control corn acres within their CRW-control acres. Syngenta has proposed that in-field strips be at least 6 to 12 non-rootworm protected *Bt* corn rows, although this row width size may be larger than necessary based on the current understanding of rootworm biology. BPPD has reviewed larval movement data published by Hibbard et al. (2003). This study indicated that between 0.75% and 6% of larvae moved across corn rows. These results represent a relatively high-end estimate of the number of larvae that cross rows. This means that much narrower in-field strips should be sufficient to provide adequate protection from sub-lethal selection caused by CRW larval movement across rows and maintain low functional recessiveness. Any increase in sublethal selection would be offset by a greater probability that potentially resistant adults emerging from the *Bt* corn rows would be mated by susceptible adults from the refuge row. Single-row strips would likely be too narrow and allow too much larval movement across rows to sufficiently maintain low functional recessiveness. Therefore, seed mixes would not be a good refuge strategy for rootworms. In-field strips of ≥4 rows would provide the advantage of being more compatible with the current in-field strip width requirement for lepidopteran-protected *Bt* corn hybrids (≥4 row strips, with ≥6 row strips preferred). In-field strips of ≥4 rows would also be more practical and flexible for the grower because of the compatibility with split-planter designs. Because mCry3A will be stacked with Cry1Ab (submission pending), a recommendation of ≥4 row strips will provide the grower a more easily understandable and consistent message regarding the width of in-

field strips. Overall, BPPD's recommendation is that Syngenta require growers to plant in-field strip refuges with widths of ≥4 rows (≥6 rows preferred) for rootworm-protected *Bt* corn hybrids. This will simplify refuge deployment and potentially increase grower compliance with refuge requirements. Use of an in-field strip refuge is not intended for fields planted to increase inbred seed since these fields need to be isolated from external corn pollen sources. An in-field or adjacent non-Bt corn refuge would be inconsistent with inbred seed production practices.

Management of the structured refuge must conform to the rotational and management practices to achieve synchronous CRW development in both refuge and MIR604 maize fields. Growers will have the option of treating the refuge with conventional insecticides (seed treatments, soil applications and foliar applications) to control severe damage by CRW and to control secondary pests. These treatments will impact the overall number of beetles that emerge from the refuge fields even though their efficacy is less than 100%. Syngenta's modeling study examines one scenario in which a nominal 50% of refuge insects are removed by insecticide control which impacted the potential development of resistance (see modeling section below). Syngenta indicates that there is no evidence for any cross-resistance, synergy, or antagonism between the mCry3A in MIR604 maize and Cry1Ab and other *Bt* toxins in Lepidoptera-control maize varieties. There are also no known interactions between mCry3A and insecticides.

Syngenta notes that the use of continuous corn acres for refuge fields is always to be encouraged. It is also permissible for growers to plant the refuge acres on first-year corn acres, but only where the CRW-control corn is also planted on first-year corn acres. This distinction is made for areas in which WCRW has adapted to crop rotation and sizable populations of these insects will emerge from first-year corn. There is also variant of NCRW in which there is extended diapause. Both rotation-resistant CRW variants impact the efficacy of crop rotation.

2) IRM is Part of an Integrated Program

Syngenta stressed that the IRM strategy for MIR604 maize is part of an overall package of integrated crop management techniques. These tactics include:
1. Crop rotation. Growers will be encouraged to maintain crop rotation as a vital part of CRW management. The presence of rotation-resistant phenotypes, however, may render such an approach ineffective in some areas.
2. Refuge quality. Growers will be educated on management practices to maximize the effectiveness of their refuges.
3. Insecticide use. When moderate to high CRW population pressures in the refuge, growers will be encouraged to treat the refuge with a soil insecticide or use seed treatments in a manner to maintain overall survival of CRW to function effectively as refuge mating partners.

3) Product Stewardship

Growers are crucial to the success of any IRM plan because they are responsible its implementation on the practical level. Syngenta has developed a product stewardship program that stresses the importance of implementation of the IRM plan for MIR604 maize. Syngenta's product stewardship program consists of the following aspects:

1. grower education plans (e.g., Syngenta guide),
2. grower obligations (grower agreements),
3. grower compliance,
4. annual resistance monitoring,
5. action plan in the event of unexpected levels of CRW damage,
6. remedial action plans, and
7. annual IRM plan review.

Compliance programs are important in that they encourage growers to comply with IRM requirements, while providing mechanisms by which registrants can be held accountable for noncompliant growers. The compliance program presented in this submission mirrors those developed for existing lepidopteran-protected *Bt* maize registrations. The specific compliance assurance program components are: 1) Annual IRM Survey, 2) Mechanism for Handling Tips and Complaints, 3) On-Farm Visits, and 4) Phased Compliance Approach. Syngenta will respond to instances of non-compliance through the Phased Compliance Approach and will also use grower agreements and an annual affirmation scheme to reinforce grower understanding and compliance. Grower education programs give growers a clear understanding of the importance of IRM and its implementation. The IRM program will be communicated to growers by Syngenta through its authorized sales agents using both print and other media including workshops, educational pamphlets/brochures, and an assortment of public relation activities. BPPD finds Syngenta's compliance assurance program to be "acceptable" with the following caveats. The EPA-approved changes (granted on June 16, 2006) to the annual IRM reporting requirements and the Compliance Assurance Program for the lepidopteran-protected *Bt* maize registrations should be incorporated into Syngenta's IRM Compliance Assurance Program for MIR604 Corn Products.

The need for proactive resistance detection and monitoring is critical to the survival of *Bt* technology. Consequently, the Agency mandates that a resistance monitoring plan must be implemented for all registered *Bt* maize products. Resistance can evolve regionally or as a local increase in resistance (*r*-) allele frequency. The resistance monitoring plan designed for MIR604 maize is an adaptation of the ABSTC program developed for lepidopteran-protected *Bt* maize. This program will attempt to detect either local or regional resistance early enough to initiate effective remedial action. As for all other *Bt* maize products, Syngenta's resistance monitoring program will be implemented through a two-pronged approach, including field reports of unexpected damage and population testing and sampling. The initial emphasis of their monitoring plan will be on establishing the baseline susceptibility for WCRW. Growers will be asked to report to

Syngenta any unexpected field damage. Diagnostic bioassays of larvae from eggs laid by field-collected WCRW adults will be used to survey susceptibility of the insects to mCry3A. The monitoring efforts will focus on regions of the Corn Belt where WCRW populations are known to regularly reach high numbers and where the highest MIR604 adoption is expected. Detecting shifts in the frequency of resistance genes (i.e. susceptibility changes) through resistance monitoring can be an aggressive method for detecting the onset of resistance prior to widespread crop failure. As such, the utilization of sensitive and effective resistance monitoring techniques is critical to the success of an IRM plan. BPPD finds that Syngenta's basic resistance monitoring plan and remedial action plan for the mCry3A protein expressed in MIR604 maize are "acceptable," although certain issues need to be addressed. These are discussed below.

1. The monitoring plan only focuses on WCRW. CRW species are challenging to rear in the laboratory. Of the identified target pest species, scientists have had the most success rearing WCRW, and have had little success with NCRW. Consequently, BPPD agrees with Syngenta that initial monitoring through population sampling will focus on WCRW, but should include NCRW populations when available. No information exists on MCRW. These data should be provided to BPPD when they become available.
2. A diagnostic method cannot be developed until the dose-response relationships for WCRW to establish the baseline sensitivity to mCry3A. While BPPD agrees that a sensitive and reliable bioassay should be developed, these efforts are currently under development. Once this work is completed, the registrants need to develop guidelines as to what level of root damage will be expected under various conditions, and what level of rootworm control is normally achieved. Growers will have to report any "unexpected damage." However, without guidelines as to what is "acceptable" rootworm damage then it will not be possible to determine what the "unexpected" rootworm damage is. Once these guidelines are established it will be possible to define what is "suspected resistance" as described under the "Remedial Action Plans' to mitigate the spread of putative resistant populations. Because of the importance of these guidelines, it should be required that the registrant develops an interim rootworm damage guidelines by 2008 and final guidelines by 2010 and submits these to the Agency for review.

It is recommended that MIR604 maize be given the following resistance monitoring requirements:

1. The registrants should monitor for resistance and/or trends in increased tolerance for corn rootworm. Sampling should be focused in those areas in which there is the highest risk of resistance development. The registrants should submit to EPA an appropriate sampling protocol as part of its monitoring plan.
2. The registrants should provide EPA a description of its resistance monitoring plan by January 31, 2007. The description would include: sampling (number of locations and samples per locations), sampling methodology, bioassay methodology,

standardization procedures (including QA/AC provisions), detection technique and sensitivity, and the statistical analysis of the probability of detecting resistance. A final resistance monitoring plan is required by January 31, 2008.

3. The registrants should develop an appropriate discriminating or diagnostic dose assay by January 31, 2008.

4. The registrants should follow-up on grower, extension specialist or consultant reports of unexpected damage or control failures for corn rootworm.

5. The registrants should provide EPA with an annual resistance monitoring report.

The remedial action plan is designed as a tiered approach for mitigating potential WCRW, NCRW, and MCRW resistance development to the Cry34/35Ab1 protein. BPPD agrees with the general framework for the Remedial Action Plan; however, because the "baseline sensitivity" has not been calibrated, this plan cannot be implemented. The submission states that mitigation measures will be initiated when unexpected levels of CRW damage occur. However, levels of "expected" damage cannot be identified until baseline sensitivity is determined (see discussion above "Resistance monitoring"). Consequently, it should be required that baseline sensitivity be established within two years of product commercialization, so that expected levels of crop damage and target pest resistance can be established, and a remedial action plan initiated when needed.

The following program summary describes, in order of events, the steps that should be taken to implement a remedial action plan if resistance to target pests is confirmed (this general process has been implemented for other lepidopteran and CRW Bt corn products).

1. **Definition of Suspected Resistance:** Resistance will be suspected if investigations of unexpected damage reports show that:

a. implicated maize plant roots were expressing the mCry3A protein at the expected level,

b. alternative causes of damage or lodging, such as non-target pest insect species, weather, physical damage, larval movement from alternate hosts, planting errors, and other reasonable causes for the observations, have been ruled out;

c. the level of damage exceeds guidelines for expected damage.

If resistance is "suspected", the registrants will instruct affected growers to use alternate pest control measures such as adulticide treatment, crop rotation the following year, or use of soil or seed insecticides the following year. These measures are intended to reduce the possibility of potentially resistant insects contributing to the following year's pest population.

2. **Confirmation of Resistance:** Resistance will be confirmed if all of the following criteria are met by progeny from the target pest species sampled from the area of "suspected resistance":

a. the proportion of larvae that can feed and survive on mCry3A roots from neonate to adult is significantly higher than the baseline proportion (currently being established];
b. the LC_{50} of the test population exceeds the upper limit of the 95% confidence interval for the LC_{50} of a standard unselected population and/or survival in the diagnostic assay is significantly greater than that of a standard unselected population, as established by the ongoing baseline monitoring program;
c. the ability to survive is heritable;
d. mCry3A plant assays determine that damage caused by surviving insects would exceed economic thresholds;
e. the identified frequency of field resistance could lead to widespread product failure if subsequent collections in the affected field area(s) demonstrated similar bioassay results.

3. **Response to Confirmed Resistance:** When resistance is "confirmed", the following steps will be taken:
a. EPA will receive notification within 30 days of resistance confirmation;
b. affected customers and extension agents will be notified about confirmed resistance;
c. affected customers and extension agents will be encouraged to employ alternative CRW control measures;
d. sale and distribution of mCry3A maize in the affected area will cease immediately;
e. a long-term resistance management action plan will be devised according to the characteristics of the resistance event and local agronomic needs. [The details of such a plan should be approved by EPA and all appropriate stakeholders. This process may take longer than 30 days following confirmation of resistance.]

Support for Syngenta's IRM Plan for MIR604 Maize

Dose

Identifying the level of dose, as related to selection intensity, is crucial when determining size and structure of a refuge needed to delay CRW resistance to MIR604 maize. Syngenta conducted a series of field efficacy trials across the Corn Belt in 2003 and 2004 to assess dose. This information was used in the modeling study detailed later in this review. Additional data were obtained from root expression assays and *in vitro* feeding assays in the laboratory. Based on the review of Syngenta's data, it can be concluded that MIR604 maize does not provide a high dose for CRW control.

Root expression assays

Levels of expression in leaves, roots, and whole plants were determined at two developmental stages for two MIR604 hybrids. Levels of expression were numerically higher in leaves than in roots at both the whorl stage and at anthesis and somewhat higher in roots at the whorl stage than at anthesis, but there were few, if any statistically significant differences at $p<0.05$. Root expression was found to be uniform throughout the root architecture implying that there are no microhabitats. Therefore, larvae feeding on MIR604 roots should be uniformly exposed to the mCry3A toxin. Average values of mCry3A in roots range from 1.8 to 3.0 µg mCry3A/g fresh weight.

WCRW laboratory feeding assays

In the first laboratory feeding study, nine replicate groups of ten larvae were placed in plastic dishes containing 3 cm root sections excised from MIR604 hybrids. Five of nine replicates exhibited only minimal root damage (≤ 1 feeding hole) with an average of 22% mortality; while, three of nine replicates exhibited two to three feeding holes with an average of 13.3% mortality. Only one replicate had more than four or more feeding holes.

In the second laboratory feeding study, nine replicate groups of ten larvae were placed in plastic dishes were given a choice of two 3 cm root sections excised from MIR604 and non-transgenic (control) hybrids. In this second experiment, larvae preferred the non-transgenic hybrid to the MIR604 hybrid. Approximately 75% of non-transgenic hybrid roots were heavily damaged with more than four feeding holes per root section. As in the first experiment, the mortality levels on transgenic root sections were low, only 17%. A third experiment, using a similar choice protocol, produced similar results to the second experiment, but there was considerably more variation.

Following investigations by Syngenta and its co-operators, there is evidence for both a direct mortality mechanism of first instar CRW larvae as well as evidence that MIR604 hybrids have properties that might deter feeding. Thus, in terms of root protection, MIR604 plants exhibit a much higher dose than would otherwise be assumed from the consideration of the mortality data alone. The behavioral deterrence mechanism needs to be studied further by Syngenta.

Field efficacy

In 2003 and 2004 Syngenta conducted field-based studies of MIR604's efficacy against WCRW and NCRW and study results were used to develop models of MIR604. Trials included naturally and artificially infested sites and study endpoints were survival until adult stage, timing of adult emergence, sex ratio, and adult weight. Both artificial and natural infestation trials were conducted. A brief summary of the results is provided below.

Efficacy trials compared root damage among MIR604 and non-MIR604-derived corn hybrids. Experimental plots were artificially infested (400 to 1450 eggs/trial), located in fields with naturally occurring populations of corn rootworm, or both approaches to infestation were combined. When artificially infestation was used, at the V2 to V3 leaf stage rootworm eggs were mechanically placed 3 inches deep in soil, within 2 to 3 inches of the corn stalk base.

Artificial Infestation with WCRW:
The percentage of artificially infested CRW eggs known to survive to the larval stage is low, and even lower for adult survival. Consequently, at each trial location the ratio of viable eggs to larvae was assessed, and comparative estimates of mortality made.

Results indicate that MIR604 provided better control of WCRW than untreated non-transgenic controls. Emergence data from 2003 show that MIR604 controlled an average of 89.9% of CRW, with a low of 74% at Walcott, IA and a high of 97% at Bloomington, IL (Table 1). On an emergence per hectare basis, non-transgenic control plots averaged 740,410 beetles per hectare, while MIR604 plots averaged 72,019 beetles.

Table 1. Summary of artificial infestation trials that evaluate control of WCRW by MIR604 corn. Product efficacy is estimated by the reduction in adult emergence from MIR604 plants relative to untreated, non-transgenic control plants.

Location	2003 Walcott IA	2003 Stanton MN	2003 Bloomington IL	2003 Stanton MN	Artificial infestation trials	Artificial infestation averages
Plants/ha	61,507	61,507	61,507	61,507		61,507
Viable eggs/plant	350	428	405	224		352
Initial density eggs/ha	21.6 M	26.4 M	25.0 M	13.8 M		21.7 M
Emergence in MIR604 total (F+M)	38	48	14	18	118	30
Emergence in negative control total (F+M)	145	314	406	209	1164	291
Area evaluated per entry (ha)	0.00047904	0.00008323	0.00038323	0.00018723	0.0016287	0.00040718
MIR604 total (F+M)/ha	79,325	125,250	36,531	46,969	288,075	72,019
Negative cont. total (F+M)/ha	302,688	819,344	1,059,407	780,203	2,961,642	740,410
% control	73.8%	84.7%	96.0%	94.0%	90.3%	90.3%

Natural Infestation with WCRW:
Field trials using natural infestation were implemented at six locations in 2004. Three of these locations (Clay Center, NE, Ames, IA, and Higginsville, MO) were

lightly infested – characterized by few beetles and minimal plant root damage. The remaining three locations (Bloomington, IL, Urbana, IL, and Mead, NE) had a high level of infestation – characterized by high beetle population densities and a high level of plant root damage.

Percent control was determined by comparing emergence in MIR604 plots against the non-transgenic control. This method assumed that the natural infestation of WCRW was evenly distributed across treatments (an unlikely scenario), although the procedure may provide a rough estimate of the efficacy of MIR604. At the lightly infested locations, MIR604 provided greater control of WCRW than non-transgenic controls, with a mean of 92.2% beetle control compared to the controls (Table 2). At heavily infested locations MIR604 provided an average of 11.1% beetle control, with a low of -13.5% and a high of 44.5%, compared to non-transgenic control treatments (Table 3).

Table 2. Summary of light pressure natural infestation trials that evaluate control of WCRW by MIR604 corn. Product efficacy is estimated by the reduction in adult emergence from MIR604 plants relative to untreated, non-transgenic control plants.

Location	2004 Clay Center NE	2004 Ames IA	2004 Higginsville MO	Light pressure natural infestation totals	Light pressure natural infestation averages
Plants/ha	61,507	61,507	61,507		61,507
Emergence in MIR604 total (F+M)	23	35	16	74	25
Emergence in negative control total (F+M)	263	276	412	951	317
Area evaluated per entry (ha)	0.00038323	0.00038323	0.00038323	0.00111497	0.00038323
MIR604 total (F+M)/ha	60,016	91,328	41,750	193,094	64,365
Negative cont. total (F+M)/ha	686,266	720,188	1,075,063	2,481,517	827,172
% control	91.3%	87.3%	96.1%	92.2%	92.2%

Table 3. Summary of heavy pressure natural infestation trials that evaluate control of WCRW by MIR604 corn. Product efficacy is estimated by the reduction in adult emergence from MIR604 plants relative to untreated, non-transgenic control plants.

Location	2004 Bloomington IL	2004 Urbana IL	2004 Mead NE	Heavy pressure natural infestation totals	Heavy pressure natural infestation averages
Plants/ha	61,507	61,507	61,507		61,507

Location	2004 Bloomington IL	2004 Urbana IL	2004 Mead NE	Heavy pressure natural infestation totals	Heavy pressure natural infestation averages
Emergence in MIR604 total (F+M)	126	386	345	857	286
Emergence in negative control total (F+M)	227	340	397	964	321
Area evaluated per entry (ha)	0.00038323	0.00038323	0.00038323	0.00011497	0.00038323
MIR604 total (F+M)/ha	328,781	1,007,219	900,235	2,236,235	745,412
Negative cont. total (F+M)/ha	592,328	887,188	1,035,922	2,515,438	838,479
% control	44.5%	-13.5%	13.1%		11.1%

Natural Infestation with NCRW:

High natural infestations of NCRW were seen at one location in 2003 and six locations in 2004. Infestations were light at Stanton, MN, Cavour, SD, Bloomington, IL, and Mead, NE, while pressure was heavier in Walcott, IA, Ames, IA, and Higginsville, MO. Percent control was determined by comparing emergence in MIR604 plots against the non-transgenic control. This method assumed that the natural infestation of NCRW was evenly distributed across treatments (an unlikely scenario), although the procedure may provide a rough estimate of the efficacy of MIR604. At the Ames and Higginsville locations NCRW emergence reached one third of total CRW adult emergence. For these locations, control of NCRW ranged from 45 to 95%. Control ranged from 34.7 to 100% in areas with lower NCRW pressure (Table 4).

Table 4. Summary of natural infestation trials that evaluate control of NCRW by MIR604 corn. Product efficacy is estimated by the reduction in adult emergence from MIR604 plants relative to untreated, non-transgenic control plants.

Location	2003 Walcott IA	2004 Stanton MN	2004 Cavour SD	2004 Bloomington IL	2004 Ames IA	2004 Higginsville MO	2004 Mead NE
Plants/ha	61,507	61,507	61,507	61,507	61,507	61,507	127,859
Emergence in MIR604 total (F+M)	28	2	0	5	63	10	32
Emergence in negative control total (F+M)	73	22	53	12	115	218	49
Area evaluated per entry (ha)	0.00047944	0.00038323	0.00038323	0.00038323	0.00038323	0.00038323	0.00038323
MIR604 total (F+M)/ha	58,450	5,219	0	13,047	164,391	26,094	83,500
Negative cont. total (F+M)/ha	152,388	57,406	138,297	31,313	300,078	568,844	127,859
% control	61.6%	90.9%	100.0%	58.3%	45.2%	95.4%	34.7%

Adult emergence

To make realistic judgments regarding the likelihood that a resistant CRW beetle will pass on resistance alleles to the next generation, adult fitness and timing of

emergence must be understood. Both the fitness of the surviving beetles and the timing of their emergence from the crops have a major impact on their ability to mate with other WCRW adults which will have an impact on their ability to contribute genetically to the subsequent generation. Based on all MIR604 replicate treatments, there was a delay in the emergence of adults from the crop when compared to the untreated negative isoline control treatments.

Over all locations, a mean delay of 7.7 days was seen for adult emergence in all MIR604 treatments when compared to non-transgenic controls. No discernable differences were seen between treatments with light or heavy pressure and between male and female beetles.

Artificial infestation with WCRW:
In trials that used artificial infestation, delays of 6 to 10 days compared to non-transgenic controls were commonly seen among male and female beetles (Table 5).

Table 5. Emergence delay times (days) for adult male and female WCRW in emerging from artificially infested MIR604 corn fields in 2003 and 2004.

Location	2003 Walcott IA	2003 Stanton MN	2003 Bloomington IL	2004 Stanton MN	Artificial infestation averages
Emergence parameter	Emergence (days)				
5% male emergence	6.1	4.2	0.1	18.4	7.2
50% male emergence	4.6	9.8	0.9	11.0	6.6
80% male emergence	2.6	14.3	1.6	10.2	7.1
5% female emergence	14.8	3.5	1.3	20.4	10.0
50% female emergence	6.9	8.4	-0.3	9.0	6.0
80% female emergence	5.0	8.1	6.6	8.5	7.0
Mean emergence delay for M+F	6.7	8.0	1.7	12.9	7.3

Natural infestation with WCRW:
Emergence delays of over 10 days were seen among female CRW beetles in locations with light natural infestation (Table 6). Few males were identified in these plots, so emergence time could not be reliably measured. Emergence delays in locations with heavy natural infestation were shorter than delays seen in sites with light emergence (Table 7). However, this finding was heavily influenced by the Urbana IL results, which were atypical and for efficacy purposes, considered an outlier. Removing the Urbana results from the heavily infested data set brings the mean value for emergence to 6.5 days, which is more similar to results seen in the artificial infestation trial.

Overall, statistical analysis showed no significant difference in rates of emergence among sexes, locations or treatments. The most reliable data were collected from three trials in 2004 which recorded over 100 adults in emergence cages. For these sites, a mean delay of 5.8 days was calculated for males and females.

Consequently, the modeling study (described in modeling section) uses a standard delay of 6 days for males and females emerging from MIR604 corn fields.

Table 6. Emergence delay times (days) for adult male and female WCRW in emerging from light pressure naturally infested MIR604 corn fields in 2003 and 2004.

Location	2004 Clay Center NE	2004 Ames IA	2004 Higginsville MO	Light pressure natural infestation averages
Emergence parameter	Emergence (days)			
5% male emergence	N/A*	N/A*	N/A*	N/A*
50% male emergence	N/A*	N/A*	N/A*	N/A*
80% male emergence	N/A*	N/A*	N/A*	N/A*
5% female emergence	8.6	12.3	11.5	10.8
50% female emergence	15.4	12.1	7.0	11.5
80% female emergence	19.4	12.5	5.8	12.6
Mean emergence delay for M+F	14.4	12.3	8.1	11.6

N/A* = < 3 beetles emerged from this entry at this location, and therefore data are excluded from delay calculations

Table 7. Emergence delay times (days) for adult male and female WCRW in emerging from heavy pressure naturally infested MIR604 corn fields in 2003 and 2004.

Location	2004 Bloomington IL	2004 Urbana IL	2004 Mead NE	Heavy pressure natural infestation averages
Emergence parameter	Emergence (days)			
5% male emergence	1.1	6.9	9.5	5.8
50% male emergence	3.4	4.8	5.0	4.4
80% male emergence	11.2	-3.2	5.9	4.6
5% female emergence	2.4	4.5	10.6	5.8
50% female emergence	7.0	-4.9	9.4	3.8
80% female emergence	8.7	-6.5	4.1	2.1
Mean emergence delay for M+F	5.6	0.3	7.4	4.4

Artificial infestation with NCRW:

In 2003 and 2004 data on NCRW emergence was collected from four locations. Although there was some variability among locations, emergence delays ranged from 5.5 to 11 days. As noted with WCRW emergence data, beetle sex was not shown to affect rate of emergence.

Table 8. Emergence delay times (days) for adult male and female NCRW in emerging from naturally infested MIR604 corn fields in 2003 and 2004.

Location	2003 Walcott IA	2004 Ames IA	2004 Higginsville MO	2004 Mead NE	NCRW natural infestation averages
Emergence parameter	Emergence (days)				
5% male emergence	7.5	0.0	N/A*	12.9	6.8
50% male emergence	2.9	6.1	N/A*	9.5	6.2
80% male emergence	1.4	3.9	N/A*	19.6	8.3
5% female emergence	18.4	6.4	13.1	14.4	13.1
50% female emergence	9.7	10.5	5.6	6.3	8.0
80% female emergence	6.3	6.1	-0.5	3.2	3.8
Mean emergence delay for M+F	7.7	5.5	6.1	11.0	7.6

N/A* = 3 beetles emerged from this entry at this location, and therefore data are excluded from delay calculations

Adult body weight

The body weights of adult WCRW beetles were recorded at the artificially infested trial in Stanton, MN. Results show that the mean body weight of adult beetles emerging from MIR604 corn fields was significantly greater than the weight of those emerging from non-transgenic plots.

Table 9. Mean dry weight of adult WCRW emerging from artificial infestation field trial at Stanton, MN in 2003.

Treatment	% Control w.r.t. untreated control	Mean adult weight (g x 10-5)
MIR604-3-I1	87.0	206.5 bc
MIR604-3-I2	90.3	183.4 bc
Untreated, non-transgenic, control	0.0	155.4 c
Non-transgenic + FORCE 3G	85.9	222.8 ab

Simulation Models for Development of Resistance

Simulation modeling was used by Syngenta to predict the evolution of WCRW resistance to the mCry3A toxin expressed in MIR604 maize. Models have been

useful tools in the development of IRM strategies. Previously, Monsanto modified a Ciprio model for cotton bollworm (*Helicoverpa zea*) to predict the risk of CRW developing resistance to MON863 corn (U.S. EPA, 2002). Andow and Alstad (2002), Onstad *et al.* (2001), and Onstad *et al.* (2003) used deterministic models to predict the evolution of WCRW resistance using a variety of management strategies. The Andow and Alstad model predicted that, under certain conditions, a 20% refuge would probably delay resistance for greater than 15 generations when the dose was low, but would be ineffective for resistance management of a high dose (U.S. EPA, 2003). In Onstad *et al.* (2001), the model showed that when resistance was dominant, the resistance allele frequency exceeded 3% within 2-5 years as refuge size ranged from 5 to 30% for all doses of toxin, but the resistance allele frequency never exceeded 3% when resistance was recessive. This model was further modified to examine the impact of landscape on WCRW resistance evolution (Onstad *et al.* 2003). Both of the Onstad models were further expanded to evaluate the risk of resistance by WCRW to both transgenic crops and crop rotation in areas with or without rotation-resistant phenotypes (Crowder *et al.*, 2005). Storer (2003) developed a stochastic, spatially explicit model to simulate adaptation (resistance) of WCRW in much of the US Corn Belt. In this model, the relative rate of adaptation was affected by the refuge size and the manner in which the non-transgenic refuge maize was deployed. Specifically, the adaptation rate was lowest if the non-transgenic refuge maize was planted in the same fields each year (Storer, 2003). This model was further modified to predict the evolution of resistance to Cry34Ab1/Cry35Ab1 and considered the influence of rotation-resistant phenotypes (U.S. EPA, 2005).

Syngenta's Specific Modeling for MIR604 Maize

Methods

Syngenta worked with Dr. David Onstad of the University of Illinois to customize the Crowder *et al.* (2005) model using the value for efficacy (i.e. dose) generated by Syngenta (see section on "Dose" above). The model includes patches of crops without explicit spatial structure. Each patch had a basic spatial unit of a 160 ha farm and was in a homogeneous region consisting of similar farms. The typical model landscape had four crops and MIR604 transgenic maize planted to both continuous and rotated cornfields. The crops include continuous corn, rotated corn, rotated soybean, and extra vegetation. Six scenarios were studied. Scenario I consisted only of 100% continuous maize fields in the landscape with no rotation-resistant WCFW in this area. Scenarios II-V had landscapes with 5% extra vegetation, 10% continuous corn, 42.5% soybean, and 42.5% rotated corn based on the standard set in the work of Crowder *et al.* (2005). In these four scenarios the area was inhabited by rotation-resistant WCRW. Scenario VI was similar to scenario I, but in this case a soil insecticide was used in the refuges, giving 50% survival of refuge insects. In scenarios IV-V, transgenic corn was planted only in rotated maize fields. Crowder *et al.* (2005) demonstrated that planting transgenic maize only in continuous maize fields in these kinds of landscapes was an inferior strategy so this scenario was not evaluated. In areas

with rotation-resistant WCRW (scenarios II-V), there were two kinds of landscapes evaluated: one with an initial resistance allele frequency of 0.0001 of the rotation-resistance allele (Y) in the rootworm population (scenarios II and IV) and the second had an initial Y-allele frequency of 0.1 (scenarios III and V). In both cases, the expression of the rotation resistance allele was dominant. Previously, Crowder *et al.* (2005) and Crowder and Onstad (2005) demonstrated that the simulation of realistic evolution of rotation resistance required either additive or dominant expression.

Three refuge levels were evaluated: 10%, 20%, and 30%. The refuge fields and MIR604 fields were assumed to be planted together in either continuous maize or in rotated maize. The refuge was assumed to be planted in the MIR604 field or adjacent to it. If the refuge was continuous maize, the refuge had to remain in the same place every year; otherwise, the refuge in rotation maize fields could change location from year to year.

The time horizon used was 15 years after introduction of MIR604 transgenic maize. This model had a daily time step during July-September to simulate adult activity.

Resistance to MIR604 transgenic maize and rotation was modeled as dominant, recessive, or additive. Precise additive expression for resistance to transgenic maize was used. Resistance to crop rotation was modeled as dominant (Y allele).

The initial adult density was assumed to be 50,000/ha of maize for all types. The sex ratio of adults was 50:50. The initial allele frequency for resistance to MIR604 transgenic maize was 0.0001. The initial allele frequency for resistance to rotation was 0, 0.0001, or 0.1.

A seed insecticide treatment was not included in most scenarios except for Scenario VI which included soil insecticide treatment in refuge in continuous corn. Larval survival was 50% before density-dependent survival.

The model used a range of MIR604 transgenic maize mCry3A doses derived from the adult emergence data provided by Syngenta (see discussion above) and calculated using two larval survival functions described by Onstad *et al.* (2001, 2003). These calculations are not shown in this review, but can be found in McCaffery et al. (2005, MRID# 465296-01). Homozygous resistant individuals (RR) were assumed to always have 100% survival to MIR604 maize. Heterozygous individuals with R dominant (sR) were assumed to always have a 100% survival to the transgenic crop. The survival of homozygous susceptible individuals (SS) and heterozygote individuals with R recessive (Sr) were assumed to have a survival of 0, 0.001, 0.05, and 0.20 to represent a theoretical high, practical high, medium, or low toxin dose, respectively.

The standard density-dependent survival of larvae per state is 0.21 X exp (-0.058 EGG), where EGG is the density of eggs in millions per ha. The maximum larval survival based on this function was 21%. It was assumed that density-dependent mortality occurred after mortality due to overwintering and toxin exposure. In a second approach, it was assumed that mortality is density-independent based on the field data collected by Hibbard *et al.* (2004); thus, 5% of larvae survived after overwintering and toxin exposure. The Urbana, IL field data collected in 2004 was omitted from the analyses because it was considered an outlier. The data from 2003 and 2004 were combined. Assuming that density-dependent survival occurs, the overall mean proportion of survival by susceptibles to the mCry3A toxin in the nine trials was 0.093. For the five trials with natural infestations, the mean survival was 0.14. For the four trials with artificial infestations, the mean survival was 0.038. For density-dependent survival, a range of 0.038, 0.093, to 0.14 for the survival rate of susceptible neonates encountering MIR604 transgenic maize was simulated.

The mean larval survival based on the assumption of density-independent mortality after surviving toxin was calculated based on proportional differences between emergence of adults in control cages and treated cages. The overall mean survival by susceptibles to the toxin in the nine trials was 0.24. For the five trials with natural infestations, the mean survival was 0.34. For the five trials with natural infestations, the mean survival was 0.34. For the four trials with artificial infestations, the mean survival was 0.125. Larval WCRW survival on MIR604 maize was simulated as 0.125, 0.24, and 0.34.

Delays in the emergence of adults were determined from data supplied by Syngenta (see discussion on adult emergence above). In the modeling done by Crowder *et al.* (2005), there was no significant effect of early mortality and delays of 10 and 14 days. For all cases in 2003-2004 in which there were at least 38 adults emerging in the treatment cages, there observed delays ranged from no delay to a delay of almost 15 days. A mean delay of 5.8 days was calculated for both males and females for three situations in which over 100 adults emerged from the treatment cages (most reliable data). The model used a standard delay of 6 days for both susceptible males and females emerging in MIR604 transgenic maize fields. Susceptible genotypes are SS when R is dominant and SS and rS when R is recessive. For the additive case, simulations were run with only SS having early mortality, and then other simulations were run with both SS and RS having early mortality.

Sensitivity analyses were conducted. In all sensitivity analyses a refuge size of 20% was simulated. In the sensitivity analysis all variables were set to standard conditions (unless otherwise noted) except for the function being tested. The effect of initial population size, effect of initial R allele frequencies (resistance to transgenic corn) and the effect of lower fecundity by susceptible adults in transgenic maize were also included in the sensitivity analyses. The sensitivity

analysis also used a 12-day delay (in addition to the 6-day delay) to determine how the results were affected by a longer delay.

Table 10. Six Landscape Scenarios Modeled.

Landscape Scenarios Modeled
I. No rotation resistance (Y allele frequency = 0); only continuous maize
II. Rotated maize with Y allele frequency = 0.0001 5% extra vegetation, 10% continuous maize, 42.5% soybean, 42.5% rotated maize Transgenic MIR604 maize in both rotated and continuous maize fields
III. Rotated maize with Y allele frequency = 0.1 5% extra vegetation, 10% continuous maize, 42.5% soybean, 42.5% rotated maize Transgenic MIR604 maize in both rotated and continuous maize fields
IV. Rotated maize with Y allele frequency = 0.0001 5% extra vegetation, 10% continuous maize, 42.5% soybean, 42.5% rotated maize Transgenic MIR604 maize only in rotated maize fields
V. Rotated maize with Y allele frequency = 0.1 5% extra vegetation, 10% continuous maize, 42.5% soybean, 42.5% rotated maize Transgenic MIR604 maize only in rotated maize fields
VI. No rotation resistance (Y allele frequency = 0); only continuous maize Annual soil insecticide use in refuge with larval survival of 50%

Results from Syngenta's Specific Modeling for MIR604 Maize

Syngenta's modeling results are found in Appendix I of their submission (McCaffery *et al.*, 2005, MRID# 465296-01).

1. Recessive resistance allele. If resistance to transgenic MIR604 maize is recessive, then WCRW never became resistant within 15 years in all simulations for all scenarios.

2. Dominant resistance allele. For scenarios I-III, and VI, either when larval survival was density-dependent or density-independent, resistance to transgenic MIR604 maize evolved in less than 13 years with a 20% refuge (all three toxin scenarios). For scenarios IV and V, either when larval survival was density-dependent or density-independent, resistance to transgenic MIR604 maize did not evolve within the 15 years of the simulation. Resistance evolution using the 10% refuge was worse for scenarios I-III, and VI than using the 20% refuge. The 30% refuge delayed evolution of resistance several years beyond 15 in Scenario III, IV, V when larval survival was density-dependent and density-independent, but resistance still evolved in less than 15 years in Scenarios I, II, and VI.

3. Additive resistance expression. Two simulations representing two possible effects of additive expression on early mortality were run for each combination of toxin survival (3 combinations) and refuge level (3 levels) for a total of 18 simulations. Like the results for dominant resistance expression, the results for scenarios I-II were much different from those for scenarios IV-V when expression was additive.

Density-Dependent. For Scenarios I and II, with density-dependent survival, resistance evolved in less than 15 years using both the 10% and 20% refuge options for all but one simulation that for additive expression case #1 in which the toxin survival was 0.14 using a 20% refuge. No resistance was predicted if a 30% refuge (with density-dependent survival) was used in any of the six landscape scenarios with additive expression (both case #1 and #2) during the 15 years of the simulation. No resistance evolved during the 15 years of the model simulations using scenarios IV and V (MIR604 maize was planted only in rotated maize fields and rotation-resistance existed).

Density -Independent. Resistance did not evolve during the 15 years of the simulation under scenarios I-V using a 20% and 30% refuge if there was density-independent survival except in additive expression case #2 when toxin survival was 0.125 for larvae in scenarios I-III. In these three simulations, resistance evolved in 13 years. A 10% refuge delayed resistance for at least 10 years in scenarios I-III. Resistance did evolve in less than 15 years using all refuge sizes and all toxin combinations with scenario VI (continuous maize treated with soil insecticide). In this case, the 20% refuge with an annual soil insecticide causing 50% mortality will effectively become a 10% refuge. No resistance evolved during the 15 years of the model simulations using scenarios IV and V.

Conclusions from Syngenta's Specific Modeling for MIR604 Maize

The sensitivity analysis indicated that changes in the initial allele frequency had the greatest effect on results. Results were insensitive to recessive expression in MIR604 maize. Simulations using scenario V were insensitive to changes in initial adult density, R-allele frequency, and emergence delay. For scenario I and dominant expression of resistance to transgenic MIR604 maize, the results were generally not sensitive to increases in initial pest density, lengthening of the early-

mortality period, no reduction in fecundity of survivors in transgenic MIR604 maize fields. For scenario I and additive expression or dominant expression, results were sensitive to changes in initial allele frequency.

If rootworm resistance to the mCry3A toxin as expressed in MIR604 maize is recessive (this is thought to be the case), then the modeling study suggests that an IRM plan with a 20% refuge, as proposed by Syngenta, will be adequate for delaying the evolution of resistance for at least 15 years. If MIR604 maize is planted in areas with observable rotation-resistance in the WCRW population, then the simulations indicate that planting the transgenic maize only in rotated maize fields is a good IRM plan that will delay resistance evolution to the mCry3A toxin expressed in MIR604 maize by at least 15 years regardless of gene expression. This strategy will also counteract the rotation resistance in WCRW (see Crowder *et al.*, 2005).

The most complicated cases are for areas without rotation-resistance or for framers who want to plant continuous corn. The simulations show that resistance to MIR604 maize may occur in 9-12 years with a 20% refuge, a toxin survival of 0.093 (the overall mean) and with dominant expression of resistance. If expression is additive, the evolution of resistance is delayed a bit more than for dominant expression by 1-2 years. If the initial resistance allele frequency is higher than assumed then resistance can occur a few years earlier. If soil insecticides are used on an annual basis in the refuge, then a 20% refuge effectively becomes a 10% refuge when resistance expression is dominant or additive and resistance evolves more quickly.

Impact of MIR604 on Biology of CRW

In order to develop an effective IRM plan and appropriate deployment strategies for MIR604, Syngenta submitted information describing CRW biology, ecology, behavior and toxicology related to MIR604. This information is especially important for this product because some individuals survive after feeding on the event. Not only is it important to understand the relative fitness of adults that emerge after feeding on MIR604, it is also important to understand the behavior of surviving larvae. For the most part Syngenta submitted detailed information to address these issues. However, Syngenta considers their research ongoing and BPPD anticipates additional information when it comes available.

Evidence and observations made by Syngenta show that corn rootworm males normally start to emerge before females and this emergence period generally continues for over a month (Hein at al., 1998; Elliot and Hein, 1991; Meinke, 1995). Experiments conducted by Syngenta in 2003 and 2004 indicate that WCRW adults emerging from MIR604 emerge an average of 6-7 days later than WCRW adults emerging from non-transgenic isoline corn. A similar delay in emergence was observed with NCRW. Syngenta found no evidence that there is a difference in the delay in emergence between males and females (although there

was some variability and the emergence profiles did vary). This last observation is especially important because it implies that normal mating patterns are not likely to be disrupted by any shift in the sex ratios normally found in the field. Given that the emergence period of WCRW is over a month, the observed delays of 6-7 days for WCRW adults emerging from MIR604 plots should not impact their availability to mate with adults from refuge plots. Syngenta also investigated the impact of density-dependent and density-independent mortality with respect to delays in emergence. These factors were evaluated for their impact on the evolution of resistance in Sygenta's model (discussed above).

It is clear from Syngenta's submission that the development of surviving larvae is significantly delayed, which is reflected in the delay of emerging adults. Therefore, it is also necessary to establish that adults emerging from MIR604 are not physiologically compromised in a way that would prevent them from mating with refuge adults. Syngenta is currently working with collaborators to fully understand the relative fitness of adults that emerge from MIR604. Currently little is known about sub-lethal effects of toxicants on specific *Diabrotica* species. WCRW females mate soon after they emerge and need to feed for about two weeks before they can lay eggs (Hein and Tollefson, 1985; Hein et al., 1988). Observations have shown early emerging adults survived longer and were more fecund than later emerging adults, conferring reduced fitness to the later emerging results (Boetel and Fuller, 1997). In most cases, the fitness of insects that are exposed to sub-lethal doses of an insecticide is decreased and the number of offspring is reduced (Haynes, 1988). The reduced relative fitness of insecticide-resistant genotypes is also common among insects (Crow, 1957), which has been documented for strains of insects resistant to synthetic insecticides (e.g. Ferrari and Georghiou, 1981; Roush and Plapp, 1982) and strains resistant to *Bacillus thuringiensis* (Groeters et al., 1994; Alyokhin and Ferro, 1999). Syngenta's continued research regarding the biological impact of MIR604 on CRW adults is important.

It is evident from submitted information that the prevention of damage to MIR604 corn roots is not necessarily accompanied by high levels of larval mortality. Syngenta and collaborators conducted studies to better understand the interactions between CRW larvae and MIR604 roots and the means by which roots are protected. To investigate these interactions, both MIR604 and negative isoline plants were from seed and infested with newly hatched WCRW. At several intervals (1, 2, 3, 7 and 14 days) after infestation estimates of root weight, larva number, larval wet and dry weights and larval feeding activity were recorded. Results showed that the weight of MIR604 roots increased significantly after day 3 when compared with the control roots, showing that MIR604 roots were protected from WCRW damage. With regard to larval numbers, the numbers of WCRW larvae on both negative isoline and MIR604 roots declined after infestation, but on MIR604 this decline occurred more quickly and by day 7 no larvae were alive. This finding contrasts with the negative isoline roots where significant survival was observed. Larval feeding was recorded on each

assessment day and significantly fewer larvae were observed on the MIR604 roots compared to the control. For example, on days 1 and 2 less than 10% of the larvae were present on MIR604 roots and none were feeding on day 3, whereas about 50% of larvae on the control roots were observed feeding at any one time. As expected, this difference in feeding resulted in significant differences in larval weight between the two groups. Both wet and dry weights of larvae feeding on MIR604 roots were significantly lower than those feeding on negative isoline roots. Possibly the most importing information from Syngenta's research were the behavioral findings. It was observed that the larvae feeding on MIR604 roots became sick and either died within the first day or survived until day 2 but did not continue to feed on root tissue. Larvae on the MIR604 roots appeared to have one of two behaviors: 1) feeding without inhibition followed by death a short time later, or 2) movement through the root zone, not feeding on the root tissue, but sampling root hairs and continuing to search for food. Applying these finding to a field setting, it is possible that larvae can survive to the 2nd instar by feeding on root hairs of MIR604 plants, taking in small amounts of root tissue, and supplementing their diet with surrounding grassy weeds and plant roots (Wilson and Hibbard. 2004; Clark and Hibbard, 2004). By surviving to the 2nd instar stage, which is less susceptible to mCry3A than the 1st instar stage, WCRW could re-establish on MIR604. Although a re-establishment of the pest is possible, it is documented that natural loss of WRCW between root penetration and adult emergence is high (Elliot et al., 1989).

Cross-Resistance

It is important to consider the impact of cross-resistance on the evolution of corn rootworm resistance to MIR604 maize. Cross-resistance occur when a pest becomes resistant to one *Bt* toxin, for example, and by virtue of this resistance, it confers resistance to another distinct *Bt* toxin. The degree to which this cross-resistance might occur depends largely on the mechanism of resistance characterizing the original resistance and the degree to which the two (or more) toxins are independently compromised by those mechanisms. For example, Cry1Ac and Cry1F, two *Bt* Cry toxins that target Lepidoptera, have a least one midgut receptor in common and if target site modification is responsible for resistance then a degree of cross-resistance is probably (Granero *et al.*, 1996; Bal'ester *et al.*, 1999).

Syngenta discussed the potential for cross-resistance involving the mCry3A toxin in MIR604 maize in the IRM submission. To date, no receptor for Cry3 toxins has been definitely isolated or characterized. Recently, a cadherin-link protein has been identified in the midgut of WCRW (Siegfried *et al.*, 2005).

Syngenta's event MIR604 CRW-control maize will be deployed into a landscape consisting of conventional corn and other CRW-control varieties. At present there are two commercially available CRW-control varieties that must be included in the evaluation of cross-resistance: Monsanto's YieldGard® Rootworm

(MON863) maize that expresses the Cry3Bb1 toxin and Dow AgroSciences Herculex® RW maize that expresses the Cry34Ab1/Cry35Ab1 toxins. The IRM plan for MIR604 must therefore consider the likelihood and possible consequences of the evolution of resistance in CRW that confers cross-resistance to multiple transgenic varieties.

The mode of action of mCry3A expressed in MIR604 CRW-control maize is similar to that of all known Cry toxins (see discussion earlier in this review). The degree of sequence homology may influence the potential for cross-resistance, but this depends on the specificity of the mechanism of resistance that might arise. For *Bt* Cry toxins, only two modes of resistance have been observed: 1) altered detoxification by protease enzymes in the midgut lumen which cleave the toxin and 2) modification of the target site receptor on the brush border membrane of the midgut epithelium so that binding of the toxin is prevented or hindered (Ferré and Van Rie, 2002). The latter mechanism was observed in diamondback moth (*Plutella xylostella*) that were selected with formulated microbial *Bt* products which resulted in field resistance as described in Tabashnik *et al.* (1997). Other non-target site mechanisms of *Bt* resistance have also been described: reduced or impaired gut proteolytic activity in *Plodia interpunctella* (Indian meal moth) (Oppert *et al.*, 1994, 1997; Herrero *et al.*, 2001) and alternation of toxin processing or some other metabolic step in laboratory-selected strains of *Heliothis virescens* (tobacco budworm) (Jurat-Fuentes *et al.*, 2003). A non-target site mechanism of resistance would be detrimental to IRM strategies based around reduced selection resulting from stacked Cry toxin genes or mosaic plantings of varieties expressing different toxins. The degree to which such non-target site mechanisms of resistance might impact resistance to Cry toxins in CRW is currently unknown.

As described in Ballester *et al.* (1999), modifications of the target site binding protein resulting from selection are likely to confer a very specific spectrum of resistances to closely related Cry proteins that also have some affinity with the binding site in question. Between mCry3A and native Cry3Bb1 there is a 61.7% identity and 69.1% similarity in amino acid sequences. Taking into consideration the size difference between the native and modified Cry3A proteins (mCry3A starts at residue #48), the identity is 74.3% and similarity is 66.5%. This level of amino acid homology is not considered to be particularly high and such differences indicate that there may be a differential recognition of these two proteins in the insect midgut. Galitsky *et al.* (2001) note that there are differences between Cry3A and Cry3Bb1 in certain oligomeric structures in domain II and domain III that affect pore formation in the midgut membrane, regulation of channel function, and specificity towards target pests. These differences may affect the behavior of each of these proteins and reduce the likelihood of target-site cross-resistance, although this is unknown.

The Cry34Ab1 and Cry35Ab1 toxins expressed in the Dow AgroScience's CRW product, Herculex® RW represent a new family of insecticidal crystal proteins

(Ellis *et al.*, 2002). Although the target site for these CRW-active proteins is completely unknown, it can be argued that cross-resistance at the target-site between them and the Cry3 toxins is extremely unlikely give the marked divergence in structure of these proteins from the Cry3 group. Therefore, cross-resistance between MIR604 and Herculex RW is considered to be unlikely and need not be considered further.

By in large, the mechanisms of resistance that might evolve to *Bt* toxins in Coleoptera like the CRW are largely unknown. Based on the review of the literature, the only genuine cases of resistance to *Bt* toxins suggest that target site resistance is the most common and probably responsible for the majority of resistance cases. Whether this would actually be the case for CRW is unknown. However, work by Siegfried *et al.* (2005) suggested that there is similarity of the target sites in Coleoptera and Lepidoptera so that if resistance to Cry3 toxins evolves in CRW it might involve a target site modification. For a brief period when there was a commercially available Cry3A-expressing potato in the US, potential resistance mechanisms were studied in the laboratory. Based on these experiments, Colorado potato beetle was shown to survive for a short period of time on transgenic plants (Rahardja and Whalon, 1997). No further characterization of this resistance was ever undertaken as NatureMark stopped marketing these *Bt* potatoes in 2001.

More recent studies by Rausell *et al.* (2004) compared the toxin-binding capacities of proteolytically-processed Cry3A, Cry3B and Cry3C toxins to midgut brush border membranes of Colorado potato beetle. *In vitro* heterologous competition binding experiments showed that the three proteolytically-activated Cry3 toxins all shared a common binding site, but Cry3Aa and Cry3Ca have an extra binding site that is not shared with the Cry3Ba toxin. This means that there could be some differences in the binding of mCry3A in MIR604 and that of Cry3Bb1 in YieldGard RW. The mCry3A has been modified to promote processing so that the availability of active toxin for interaction with the binding site may be different for different Cry3 toxins in the natural situation in CRW midguts.

Receptors for Cry3 toxins have never been isolated and characterized. To date, cadherin and aminopeptidase-N are most frequently associated with Cry toxin binding in Lepidoptera (e.g., Gahan *et al.*, 2001; Luo *et al.*, 1996), although actin (McNall and Adang, 2003), alkaline phosphatase (McNall and Adang, 2003), and glycolipids (Griffith *et al.*, 2005) have also been identified more recently in binding. Only insect cadherins have been proven to mutate to give resistance to Cry toxins in Lepidoptera (Gahan *et al.*, 2001; Morin *et al.*, 2003). Siegfried *et al.* (2005) used an expressed sequence tag to identify the first Coleopteran cadherin gene in CRW. Cadherin could be a receptor for *Bt* proteins in CRW, but further studies are necessary to confirm this possibility. Genetic and biochemical studies with different insect species have shown that resistance mechanisms based on target-site genes such as cadherin are inherited as recessive or incompletely

recessive traits (Ferré and Van Rie, 2002). On the other hand, non-target site mechanisms, such as altered metabolism or processing, are more likely to be inherited as incompletely dominant traits. One would therefore predict that a recessively-inherited target site mechanism is the most likely to evolve if resistance occurs to mCry3A in CRW. The modeling studies (discussed above) include varying degrees of dominance.

Rausell *et al.* (2004) found that CryBb1 might confer some cross-resistance to Cry3A (mCry3A) through modification of the shared receptor. However, the reverse is not necessarily true. That is, resistance to mCry3A could occur through modification of a unique binding site with which Cry3Bb1 does not interact. Thus, CRW developing resistance to mCry3A expressed in MIR604 maize may not confer complete resistance to Cry3Bb1 maize. While Rausell *et al.* (2004) did demonstrate specific binding of processed toxin they did not demonstrate functional receptor binding. Because there is a lack of real information on the nature of resistance, especially in CRW, it is best to assume complete cross-resistance between Cry3Bb1 and mCry3A. This "worst-case" was assumed in the modeling studies discussed above. It is more likely, however, that if CRW resistance does occur to mCry3A, only partial cross-resistance to Cry3Bb1 is expected.

To study these issues further, it is recommended that Syngenta conduct cross-resistance studies using CRW colonies resistant to mCry3A. Experiments should be conducted to investigate the nature, inheritance, and fitness costs of specific mechanisms of resistance to the mCry3A protein expressed in MIR604 maize.

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Chemical:	Modified Cry3A protein and the genetic material necessary for its production (via elements of pZM26) in Event *MIR604 corn*
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