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Non-target organism risk assessment of MIR604 maize expressing mCry3A for control of corn rootworm

A. Raybould[1], D. Stacey[1], D. Vlachos[2], G. Graser[2], X. Li[2] and R. Joseph[2]
[1]Syngenta, Jealott's Hill International Research Centre, Bracknell, Berkshire, UK; [2]Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA

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Abstract: Event MIR604 maize expresses a modified Cry3A protein (mCry3A), for control of corn rootworm. As part of the environmental safety assessment of MIR604 maize, risks to non-target organisms of mCry3A were assessed. The potential exposure of non-target organisms to mCry3A following cultivation of MIR604 maize was determined, and the hypothesis that such exposure is not harmful was tested. The hypothesis was tested rigorously by making worst-case or highly conservative assumptions about exposure, along with laboratory testing for hazards using species taxonomically related to the target pest and species expected to have high exposure to mCry3A, or both. Further rigour was introduced by study designs incorporating long exposures and measurements of sensitive endpoints. No adverse effects were observed in any study, and in most cases exposure to mCry3A in the study was higher than the worst-case expected exposure. In all cases, exposure in the study was higher than realistic, but still conservative, estimates of exposure. These results indicate minimal risk of MIR604 maize to non-target organisms.

Key words: environmental safety, exposure, hazard, hypothesis testing, transgenic plants

1 Introduction

Corn rootworms are serious pests of maize in the USA, where they cause estimated annual losses of 1 billion dollars in crop damage and control costs (Ostlie 2001; Payne et al. 2003). Corn rootworms are larvae of certain species of chrysomelid beetle of the genus *Diabrotica*, including *Diabrotica virgifera virgifera* LeConte [western corn rootworm (WCRW)] and *Diabrotica barberi* Smith and Lawrence [northern corn rootworm (NCRW)] [European and Mediterranean Plant Protection Organization (EPPO) 2004]. *D. v. virgifera* is an alien invasive species in Western and Central Europe. It was introduced from the USA at least three times in the 1990s and early 2000s (Miller et al. 2005), and populations reached economic thresholds soon after the first introduction (Kuhlmann and van der Burgt 1998).

Corn rootworms feed on the root systems of maize seedlings. Moderate pruning of roots by corn rootworms can lead to damage during water shortages because of the inability of the maize plant to adequately translocate water and minerals from the roots to the rest of the plant. Maize plants suffering from moderate to severe root pruning are also susceptible to lodging during rain and wind storms (Levine and Oloumi-Sadeghi 1991). Maize ears on lodged plants may be underdeveloped or not be harvestable because of inaccessibility to harvest equipment.

Control of corn rootworms requires insecticides or crop rotation, and both methods can occasionally fail to prevent yield loss because of adaptation of the pest (Rice 2003). Transgenic maize expressing insecticidal proteins toxic to corn rootworm offer an additional means of control (e.g. Vaughn et al. 2005).

Syngenta has developed MIR604 maize,[1] which expresses a modified Cry3A protein (mCry3A) for corn rootworm control. Native Cry3A, a δ-endotoxin produced by *Bacillus thuringiensis* subsp. *tenebrionis*, has little or no activity against WCRW or NCRW because of the limited processing of protein in the guts of these insects; mCry3A contains introduced cathepsin G-recognition sites, which allow activation of the protein by gut proteases in WCRW and NCRW. mCry3A has greatly increased toxicity to these insects, and expression of mCry3A in maize provides protection against WRCW and NCRW (US Patent no. 7,030,295). This paper is an assessment of the risk to non-target organisms of MIR604 maize.

2 Problem formulation and hypothesis testing

Assessment of the safety of transgenic plants expressing insecticidal proteins relies on the principle of

[1]MIR604 maize will be sold as Agrisure^{TM} RW hybrid maize.

comparative risk assessment: the transgenic plants are compared with non-transgenic, near-isogenic counterparts that are considered to have no unacceptable effects on non-target organisms, and any differences are evaluated (e.g. Kuiper et al. 2002). If plant characterization data show that the only ecotoxicologically relevant difference in the transgenic plant is expression of the intended insecticidal protein, which is the case for MIR604 maize [Food Standards Australia New Zealand (FSANZ) 2006], the risk assessment should seek to predict the likelihood of harmful effects of the protein to non-target organisms. Such predictions are made from tests of risk hypotheses.

The exposure of non-target organisms to an insecticidal protein is estimated from plant expression data, the diets of non-target organisms, the rate of degradation of the protein in soil, and any other relevant environmental fate data. The worst-case concentration of the insecticidal protein to which a particular non-target organism may be exposed is called the expected environmental concentration (EEC). Some species will not be exposed to the protein, and the safety of the transgenic plant to these non-target organisms can be demonstrated without toxicity testing; in effect, the risk hypothesis under test is that the EEC is not greater than zero. Many non-target organisms will be potentially exposed to the protein (EEC >0), and for these species a conservative risk hypothesis is that the no observable effect concentration (NOEC) is greater than or equal to the EEC. Data on the toxicity of the insecticidal protein are required to test this hypothesis.

It is not possible to test all species for which the EEC of the insecticidal protein is greater than zero. Therefore suitable representative indicator species are tested, which act as surrogates for species not tested (e.g. Garcia-Alonso et al. 2006). Confidence in the risk assessment is strengthened by increasing the rigour with which the risk hypothesis is tested, and therefore the best representative indicators are those species most likely to reveal toxicity of the protein at concentrations close to the EEC (Raybould 2006). These species could be taxa closely related to the target pest, and hence likely to have lower NOECs than most of the species for which they are surrogates, or species that have high exposures, and hence likely to have higher EECs than most of the species for which they are surrogates.

The choice of representative indicator species is not the only consideration when testing for hazards of insecticidal proteins; the developmental stage of the representative indicator, the endpoints of the study and the length of exposure can also have strong influences on the ability to rigorously test risk hypotheses. In general, young animals are more sensitive than older animals (e.g. James et al. 1999; Betz et al. 2000; Cappello et al. 2006); endpoints that require passage through a developmental stage, such as reproduction and adult emergence, are more sensitive than survival (e.g. Benoit et al. 1996; Maboeta et al. 1999; Kuhn et al. 2001); and long exposures are more likely to detect effects than short exposures (e.g. Dively et al. 2004; Vojtech et al. 2005). However, when devising tests for detecting effects of insecticidal proteins it should be remembered that endpoints are used to predict effects on organisms in the field; an endpoint may be highly sensitive and have high power to detect an effect, but if it cannot be interpreted biologically then it has no value for risk assessment and will needlessly trigger further evaluation. Power to detect an effect is important, but there must be a hypothesis to link the effect to changes in the assessment endpoint (Raybould 2006), which in this case is the abundance and diversity of non-target organisms potentially exposed to mCry3A via MIR604 maize.

However well-chosen, there is always the possibility that that the representative indicator species are less sensitive or have lower exposure than some of the species for which they are surrogates. Therefore, to increase confidence in the risk assessment, representative indicator species should be tested at concentrations of the insecticidal protein in excess of the EEC. If under these conditions the insecticidal protein has no adverse effects on representative indicator species that have potentially high sensitivity and exposure, there is high confidence of minimal risk of the transgenic plant to all non-target organisms.

Tests using purified proteins are more rigorous tests of risk hypotheses than studies using plants expressing those proteins, because test species can be exposed to high concentrations of the protein under controlled conditions (Romeis et al. 2006; Garcia-Alonso et al. 2006; Raybould 2006). When using a protein from a source other than the transgenic plant, it is necessary to show that it is equivalent to the protein expressed in the plant. The so-called 'bridging studies' test for differences in parameters such as molecular weight, glycosylation, cross-reactivity to antibodies and bioactivity against sensitive species.

Laboratory studies using plant material will be subject to similar constraints as studies using proteins in terms of the number of species that can be assessed: it will always be necessary to extrapolate results from representative indicator species to species that were not tested. However, it is difficult to attain concentrations of the protein greatly in excess of the EEC using plant material, and thus the ability to extrapolate from indicator species to untested species is reduced. Field studies can assess the effects of exposure on many species, but they suffer from lack of control of environmental variables, reducing the power to detect effects. In addition, correlations between confounding environmental variables and the presence or absence of the insecticidal protein may make it difficult to interpret any effects that are detected (Raybould 2006).

From the discussion above, a list of criteria for suitable representative indicator species for hazard assessments of insecticidal proteins can be formulated:

- Close taxonomic relatedness to the target pest.
- High expected exposure to the protein in the field.
- Can be reared in the laboratory with high probability of reaching the endpoint of the study in negative control treatments.
- Young developmental stages can be exposed orally to high concentrations of the protein.

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• Sensitive, but ecologically relevant endpoints can be measured.
• Long exposure to the protein before the endpoint of the study is reached.

In addition, a species may be tested if it is of high protection value, or meets a regulatory data requirement, regardless of any other criteria. Combinations of the above criteria were used to select species for testing the effects of mCry3A, and specifically to test the hypothesis that the NOEC is greater than or equal to the EEC for non-target organisms exposed to mCry3A via the cultivation of MIR604 maize.

3 Estimated environmental concentrations

Estimates of EECs for various groups of non-target organisms are made from measurements of the concentration of mCry3A in the tissues of MIR604 maize. In each case, a worse-case and a more realistic estimate are made: the worst-case EEC represents exposure via a diet of 100% of the relevant plant tissue; the more realistic EECs are refinements of that exposure to represent dilution of the protein through prey, in soil or by other means. Worst-case estimates may be suitable for conservative risk assessments, such as those that seek to protect individuals of endangered species, whereas the more realistic EECs are suitable for protecting populations or ecological functions. Realistic EECs assume that all individuals are present in or adjacent to fields in which MIR604 maize is cultivated, and therefore are conservative values for estimating risks to local or regional populations of non-target organisms, which are the usual assessment endpoints for groups such as non-target arthropods [e.g. European and Mediterranean Plant Protection Organization (EPPO) 2003].

Two MIR604 maize hybrids were grown in field trials at Bloomington, Illinois. The concentration of mCry3A in various plant tissues was estimated by enzyme-linked immunosorbent assay (ELISA) at several developmental stages, and corrected for extraction efficiency to give values for calculation of EECs.

3.1 Above-ground non-target arthropods

Non-target arthropods are non-pest species; pest species that are not intended to be controlled by mCry3A are not classed as non-target arthropods. The highest mean concentration of mCry3A in the above-ground parts of MIR604 maize at any developmental stage was 10.14 μg/g fresh weight leaves at seed maturity. This can be regarded as the worst-case EEC for above-ground non-target arthropods.

By definition, non-target arthropods rarely, if ever, eat leaves of maize. The more likely route of exposure to transgenic proteins is consumption of prey that have fed on maize (e.g. Harwood et al. 2005), or consumption of pollen if prey is scarce (Coll and Guershon 2002).

The concentration of mCry3A in the prey of non-target arthropods will vary depending on the prey species, its developmental stage, and the concentration of mCry3A in plant parts on which they are feeding. Several studies have examined the concentration of Cry proteins in herbivores relative to the concentration of Cry proteins in the plants on which they are feeding; most tested the concentration of Cry1Ab in herbivores feeding on Bt maize (Head et al. 2001; Raps et al. 2001; Dutton et al. 2002; Obrist et al. 2005, 2006a,b), and recently studies have been published of herbivores feeding on cotton and oilseed rape expressing Cry1Ac (Howald et al. 2003; Torres et al. 2006).

In general, the results show that herbivores contain lower concentrations of Bt toxin than the plants on which they are feeding. Phloem-feeding insects, such as aphids, contain only trace amounts of Cry1Ab when feeding on Bt maize (Head et al. 2001; Raps et al. 2001; Dutton et al. 2002; Obrist et al. 2006a). Lepidopteran larvae contain between 0.1 and 0.25 times the concentration of Cry1Ab in Bt maize on which they feed (Raps et al. 2001; Dutton et al. 2002; Obrist et al. 2006b), and similar results were obtained by Torres et al. (2006) with *Spodoptera exigua* feeding on cotton expressing Cry1Ac. Thrips (*Frankliniella tenuicornis*) contain up to 0.35 times the concentration of Cry1Ab in Bt maize, although this concentration is transitory; adults contain about half this amount and pupae less than 1/40th the concentration in larvae (Obrist et al. 2005). The herbivores with the highest concentrations of Cry protein are spider mites (*Tetranychus urticae*); they have been found to contain between approximately 0.7 and 3.0 times the concentration of Cry1Ab in Bt maize (Dutton et al. 2002; Obrist et al. 2006a,b). All of the pests discussed in this paragraph are found in maize, and therefore non-target arthropods may be exposed to mCry3A through consumption of these species.

A precise realistic EEC is difficult to set given the variety of food that non-target arthropods are likely to consume. Setting the EEC at 0.2 times the overall mean leaf concentration at the highest expressing developmental stage seems reasonably conservative as many lepidopteran larvae contain less than this amount, and aphids, lepidopteran eggs and MIR604 pollen contain considerably less. Spider mites may contain higher concentrations of mCry3A than MIR604 leaves, and serious outbreaks of spider mites can occur in maize, particularly under drought (Holtzer et al. 1988); however, most predators in maize fields are generalist feeders that do not depend on a single pest species for food (Steffey et al. 1999), and, some may require a mixed diet to complete development (Toft and Wise 1999). Therefore most non-target arthropods are highly unlikely to consume a diet comprising of solely spider mites containing high concentrations of toxin. Possible exceptions are the specialist spider mite predators *Stethorus* spp. (Coccinellidae). *Stethorus punctillum* is found in maize (e.g. Obrist et al. 2006a); however, this species preferentially eats spider mite eggs (Roy et al. 2002), which are likely to contain low concentrations of toxin compared with adult mites. Hence, 0.2 times the leaf concentration seems a reasonable balance between realism and

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conservatism. The realistic EEC for above-ground non-target arthropods is therefore $10.14 \div 5 = 2.03$ μg mCry3A/g diet.

3.2 Soil-dwellers

The highest mean concentration of mCry3A in MIR604 roots was 4.55 μg/g fresh weight at the whorl stage. This can be regarded as the worst-case EEC for soil-dwelling non-target arthropods.

A realistic EEC can be calculated as the concentration of mCry3A in soil following incorporation of maize plants into soil post-harvest. The average planting density of maize is 65 500 plants per hectare and the average fresh weight of a maize plant is about 750 g. MIR604 plants at senescence contained on average 6.87 μg mCry3A/g fresh weight, and therefore 1 ha of MIR604 maize will contain $65\,500 \times 750 \times 6.87 = 3.37 \times 10^8$ μg mCry3A.

If the maize is ploughed into the top 15 cm of soil, mCry3A will be incorporated into $100 \times 100 \times 0.15 = 1500$ m^3 of soil per hectare. The average density of soil is 1 500 000 g/m^3, and therefore the maize will be incorporated into $1500 \times 1\,500\,000 = 2.25 \times 10^9$ g soil per hectare. Dividing the amount of mCry3A per hectare by the mass of soil per hectare gives the realistic EEC for soil organisms:

$$3.37 \times 10^8 \mu\text{g mCry3A} \div 2.25 \times 10^9 \text{g soil} = 0.15 \mu\text{g mCry3A/g soil.}$$

3.3 Pollinators

Honeybees regularly forage for maize pollen (Severson and Perry 1981) and therefore could be exposed to mCry3A via MIR604 pollen. Honeybees can successfully rear young on a diet of 100% maize pollen; however, it is unlikely that maize pollen regularly comprises more than 50% of their diet (Babendreier et al. 2004). The concentration of mCry3A in MIR604 pollen was below level of quantification (LOQ), which was estimated to be 0.21 μg/g pollen.[2] Assuming a diet of 100% MIR604 pollen containing mCry3A at the LOQ, the worst-case EEC for pollinators is 0.21 μg mCry3A/g pollen. A more realistic, but still conservative, EEC for honeybees and other pollinators is a diet of 50% MIR604 pollen, giving a realistic EEC of $0.5 \times 0.21 = 0.11$ μg mCry3A/g pollen.

3.4 Aquatic organisms

The main route of exposure of aquatic invertebrates to mCry3A is through MIR604 pollen deposited in water bodies adjacent to maize fields. Aquatic invertebrates could feed on pollen, and therefore the theoretical maximum exposure of these animals to mCry3A is the concentration in pollen (≤0.21 μg/g).

[2]Initial measurements indicated that the concentration of mCry3A in MIR604 pollen was below the level of detection (LOD). Later ELISAs using a new antibody detected mCry3A in MIR604 pollen at below the level of quantification. Risk assessments based on mCry3A < LOD in MIR604 pollen are still considered valid.

Aquatic invertebrates are opportunistic feeders (Anderson and Sedell 1979) and pollen usually comprises a small proportion of particulate matter in water bodies (Richerson et al. 1970; Stabel 1986); therefore maize pollen is likely to form a small proportion of the diet of aquatic invertebrates. A realistic EEC should therefore be set at least an order of magnitude below the concentration of mCry3A in pollen (i.e. ≤0.021 μg mCry3A). As the mean LC_{50} to WCRW is 0.20 μg mCry3A/ml diet [United States Environmental Protection Agency (US EPA) 2007], the exposure of aquatic invertebrates to mCry3A can be considered negligible, and hence minimal risk to aquatic invertebrates can be concluded without hazard testing of these organisms (US EPA 2007).

The exposure arguments outlined about also apply to freshwater fish. There is, however, another route of exposure via incorporation of MIR604 grain into fish feed. Although the risks to farmed fish may be considered to be part of a risk assessment for food and feed use, historically in the USA they have been considered as part of environmental risk assessment.

As part of a study to test the hazards of mCry3A to fish (see section 4, below), fish feed was prepared from MIR604 grain, using methods to maximize the amount of mCry3A in the feed: the feed was prepared with the maximum amount of maize grain (50% wt/wt) that would provide a nutritionally balanced feed; about 25% maize by weight is typical in fish feed [National Research Council (NRC) 1983]; and 'cold' pelleting was used to minimize degradation of mCry3A compared with normal preparation methods in which feed is heated during pelleting. The feed was analysed by ELISA and found to contain 0.09 μg mCry3A/g. This can be considered the worst-case exposure of freshwater fish to mCry3A via fish feed prepared from MIR604 grain.

Realistic exposures of farmed fish to mCry3A are likely to be much lower than 0.09 μg mCry3A/g. We have no estimate of the degree to which cold pelleting reduces the degradation of mCry3A, but a worst-case assumption is that it does not reduce degradation compared with normal pelleting. However, the feed used in the hazard study contains about twice the usual proportion of corn grain in fish feed (NRC 1983). Moreover, fish feed is very unlikely to be prepared from 100% grain of MIR604 hybrids. Corn rootworms infest about 18% of US maize cultivation at economic levels (Rice 2003). Assuming all of this maize cultivation is planted to MIR604 maize with a 20% non-transgenic refuge for insect resistance management, a highly conservative realistic mean EEC for farmed fish can be set as $0.09 \times 0.18 \times 0.8 = 0.013$ μg mCry3A/g.

3.5 Wild birds

Birds feed rarely on maize leaves; however birds such as crows (*Corvus brachyrhynchos*), grackles (*Quiscalus quiscula*) and sandhill cranes (*Grus canadensis*) uproot sprouting maize to feed on the germinating seeds (e.g. Steffey et al. 1999; Blackwell et al. 2001; Sterner et al. 2003). Red-winged blackbirds (*Agelaius phoeniceus*) and common grackles destroy over 360 000 metric tons

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per annum of ripening maize in the USA and Canada. Blackbirds typically slit open husks with their bills and puncture kernels in the milk stage (Steffey et al. 1999). Blackbirds are also common in maize stubble where they forage for spilled kernels and weed seeds (Linz et al. 2003). Therefore, the concentration of mCry3A in kernels was used to estimate the exposure of wild birds to mCry3A via cultivation of MIR604 maize.

Exposure to birds may be expressed most suitably as a daily dietary dose (DDD), which is given by a simple formula (Crocker et al. 2002):

$$\mathrm{DDD} = \frac{\mathrm{FIR}}{\mathrm{bw}} \times C$$

where FIR is the food intake rate, bw the body weight and C the concentration of mCry3A in food.

The mean concentration of mCry3A in MIR604 kernels was 1.54 μg/g fresh weight. FIR/bw ratios for cereal seed-eating birds consuming fresh food were estimated by Crocker et al. (2002). Among the seven species represented, values range from 0.11 for the pheasant (*Phasianus colchicus*) to 0.35 for the tree sparrow (*Passer montanus*). Higher FIR/bw ratios give higher DDDs, and therefore the worst-case DDD for birds feeding on MIR604 kernels can be estimated as $0.35 \times 1.54 = 0.54$ μg mCry3A/g body weight ($\equiv$ mg/kg bw).

Wild birds are highly unlikely to consume a diet of 100% maize kernels. More realistic exposures can be estimated from the proportion of maize seeds in the diet of birds feeding in maize-growing areas. Studies by McNichol et al. (1979) and Homan et al. (1994) of the diets of red-winged blackbirds and common grackles, respectively, showed that maize kernels comprise up to 50% of their diet. Therefore a realistic DDD for wild birds is $0.5 \times 0.54 = 0.27$ μg mCry3A/g body weight ($\equiv$ mg/kg bw).

3.6 Wild mammals

The main route of exposure of wild mammals to mCry3A in MIR604 maize is via consumption of kernels. Rodents, such as thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*), deer mice (*Peromysus maniculatus*), house mice (*Mus domesticus*), and prairie and meadow voles (*Microtus* spp.) feed on germinating maize seeds. Frequently these species remove so many seeds that the field needs to be replanted. Woodchucks (*Marmota monax*) also feed on sprouting corn seed, but because they feed along the edges of fields, they usually cause less serious damage than other rodents (Steffey et al. 1999).

The mean concentration of mCry3Ain MIR604 kernels was 1.54 μg/g fresh weight. Crocker et al. (2002) estimated the ratio of food intake rate and body weight (FIR/bw) for several rodent species. The values for the harvest mouse (*Micromys minutus*) and the wood mouse (*Apodemus sylvaticus*) consuming cereal seeds are 0.33 and 0.28, respectively. Higher FIR/bw ratios give higher DDDs, and therefore the worst-case DDD for rodents feeding on MIR604 kernels can be estimated as $0.33 \times 1.54 = 0.51$ μg mCry3A/g body weight ($\equiv$ mg/kg bw).

Wild mammals are highly unlikely to consume a diet of 100% maize kernels. More realistic exposures can be estimated from the proportion of maize kernels in the diet of rodents feeding in maize-growing areas. The proportion of maize kernels in wild rodent diets varies greatly according to species (Houtcooper 1978; Ellis et al. 1998), but can be up to 73%. A realistic, but still conservative, DDD can therefore be calculated based on a diet of 73% maize kernels, giving a realistic DDD for wild mammals of $0.73 \times 0.33 \times 1.54 = 0.37$ μg mCry3A/g body weight ($\equiv$ mg/kg bw).

4 Hazard testing

4.1 Selection of test species

The hazard of mCry3A to non-target organisms was assessed in a series of laboratory studies (table 1). Species for testing were selected according to criteria discussed in section 2, above.

Three beetle species were chosen for close taxonomic relatedness to the target pests, and as representatives of potentially exposed functional groups and groups that are important for biological control. Pest screening data indicate that the activity of native Cry3A is likely to be limited to certain species in the beetle families Chrysomelidae, Curculionidae and Tenebrionidae (e.g. MacIntosh et al. 1990; Johnson et al. 1993; Wu and Dean 1996); similar pest-screening data indicate a comparable range of activity of mCry3A, apart from the intended enhanced activity against certain *Diabrotica* species. There are no non-target species in these families that are potentially exposed to mCry3A via MIR604 maize (although

Table1. *Species used to assess the toxicity of mCry3A to non-target organisms*

Test species	Common name	Order: family	NTO group
Coccinella septempunctata	Seven-spot ladybird	Coleoptera: Coccinellidae	Above-ground non-target arthropod; related to target pest
Orius insidiosus	Insidious flower bug	Hemiptera: Anthocoridae	Above-ground non-target arthropod
Poecilus cupreus	Ground beetle	Coleoptera: Carabidae	Soil-dweller; related to target pest
Aleochara bilineata	Rove beetle	Coleoptera: Staphylinidae	Soil-dweller; related to target pest
Eisenia foetida	Earthworm	Haplotaxida: Lumbricidae	Soil-dweller
Apis mellifera	Honeybee	Hymenoptera: Apidae	Pollinator
Colinus virginianus	Bobwhite quail	Galliformes: Phasianidae	Wild bird
Mus musculus	Mouse	Rodentia: Muridae	Wild mammals
Oncorhynchus mykiss	Rainbow trout	Salmoniformes: Salmonidae	Farmed fish

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there are pests of maize in these families), and therefore, the beetle species chosen for testing were taken from non-target taxa common in maize fields: Coccinellidae, Carabidae and Staphylinidae (Steffey et al. 1999).

The remaining test species were chosen as representatives of taxonomic and functional groups that may be exposed to mCry3A via consumption of MIR604 maize tissues, or through consumption of prey that has fed on MIR604 maize.

4.2 Test design considerations

All species tested have been used in safety evaluations for pesticides, and therefore protocols and testing guidelines setting out samples sizes, statistical power, validity criteria and endpoints were available. Apart from the fish study, all species were exposed via a microbial test substance; mCry3A purified from a recombinant *Escherichia coli* expression system was chosen in preference to plant material containing mCry3A in order that the species could be exposed to concentrations of mCry3A in excess of those in the field (see section 2, above). Biochemical and biological tests showed microbial mCry3A to be a suitable surrogate for plant-expressed mCry3A: both proteins had the predicted molecular weight of 67.7 kDa and cross-reacted with the same mCry3A antibody; neither protein showed post-translational glycosylation; and the proteins showed similar LC_{50} values against WCRW. Fish were exposed via feed prepared from MIR604 grain; the feed was prepared to maximize exposure to mCry3A (see section 3.4, above).

The earthworm, honeybee, bobwhite quail, mouse and fish studies were carried out exactly to the pesticide-testing guidelines for these species. The ladybird, flower bug, ground beetle and rove beetle studies were modified substantially from the pesticide protocols. First, these species had to be exposed to the test substance orally rather than topically; this necessitated the development of artificial diets that would maintain bioactivity of mCry3A while permitting normal development of the species. In the non-target insect studies, and in the fish study, fresh diet was supplied daily to maximize exposure to bioactive mCry3A. Secondly, exposure times were longer than the pesticide tests. Finally, in some cases more sensitive endpoints were evaluated; for example, larval development and mortality, rather than adult mortality in the ground beetle study. Diets, exposure times and endpoints are summarized in table 2; more details of the experimental methods to test ladybird, flower bug, ground beetle and rove beetle are given by Stacey et al. (2006).

In all studies, a negative control group was exposed to a diet identical to that of the treatment group except that the mCry3A test substance was omitted. For the study to be valid, the mortality in the negative control group had to be less than a certain value: 10–30% depending on the species as set by the relevant guideline. In the studies of insects, the test species were exposed to diets containing an insect growth regulator as a toxic reference substance; for the study to be valid, mortality in these positive control groups had to be above 50%. The sensitivity of the earthworms was assessed by determining the LC_{50} of 2-chloracetamide. All studies were carried out under international codes of Good Laboratory Practice.

The treatment and negative control diets from all non-target invertebrate studies, apart from the honeybee, the soils used in the earthworm study and the feeds used in the fish study were analysed by ELISA to determine the concentration of mCry3A. In addition, these materials were analysed by Western blot to determine the intactness of mCry3A. The diets from the flower bug and rove beetle studies and the soil from the earthworm study were also analysed by first-instar Colorado potato beetle bioassays to test for bioactivity of mCry3A. It was not felt necessary to analyse the honeybee diet as the bees were provided with fresh diet daily, and mCry3A is known to be stable in aqueous solution. No analysis was necessary for the bobwhite quail and mouse studies as mCry3A was supplied as a single dose via gavage.

4.3 Test results

In all studies, the negative control mortality was below the validity criterion. For studies with positive controls, the positive control mortality or reduction in reproduction exceeded the validity criterion. The earthworm sensitivity to 2-chloracetamide was within the guideline.

With one exception, there were no statistically significant differences between the mCry3A treatment

Table 2. *Summary of test methods used to assess the toxicity of mCry3A to non-target organisms*

Test organism	Life stage	Route of exposure to mCry3A	Duration	Guideline or protocol*
Coleomegilla	Second instar	Aphids dipped in solution of microbial test substance	24 days	Schmuck et al. 2000
Orius	Nymph	Microbial test substance incorporated into liver-based diet	21 days	Bakker et al. 2000
Poecilus	2-day-old larvae	Solution of microbial test substance injected into blowfly pupae	30 days	Heimbach 1998
Aleochara	Adult	Microbial test substance incorporated into minced beef diet	11 weeks	Grimm et al. 2000
Eisenia	Adult	Microbial test substance incorporated into artificial soil	14 days	OECD no. 207
Apis	Eggs and brood	Microbial test substance in sucrose solution	26 days	Oomen et al. 1992
Colinus	Juvenile	Single dose of microbial test substance by gavage	14 days	US EPA OPP no. 71-1
Mus	Young adult	Single dose of microbial test substance by gavage	14 days	US EPA OPP no. 81-1
Oncorhychus	Juvenile	Fish feed made from MIR604 grain	28 days	OECD no. 215

US EPA OPP, United States Environmental Protection Agency Office of Pesticide Programs; OECD, Organisation for Economic Co-operation and Development.
*Non-target arthropod tests following protocols in the literature also followed the US EPA Microbial Pesticide Test Guidelines where relevant.

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and the negative control groups in any study. The exception was the time to adult emergence in the ladybird study; this was 9.48 days in the mCry3A treatment group and 9.80 days in the negative control group ($P = 0.05–0.01$ using a t-test for unmatched pairs). This was not considered an adverse effect for the purposes of risk assessment because the difference is small (approximately 3%) and shorter development times are unlikely to be harmful if this effect were found in the field. The positive and negative control validity criteria were met in all studies.

Analyses revealed intact mCry3A in all treatment diets; bioassays of the flower bug and rove beetle diets and the earthworm study soil showed that intactness was correlated with bioactivity. The concentrations of mCry3A detected by ELISA were therefore taken to be the minimum value for the NOEC or NOAEC of mCry3A for the species tested. The NOEC for the honeybee study was taken as the nominal concentration of 50 μg mCry3A/g diet. The results of the toxicity studies and diet analyses are summarized in table 3.

5 Risk assessment

The risk assessment for non-target organisms exposed to MIR604 maize is based on a test of the hypothesis that mCry3A is not harmful at the EEC (section 2, above). If the hypothesis is not falsified after rigorous testing, the risk assessment provides high confidence that MIR604 maize poses minimal risk (is safe) to non-target organisms (e.g. Raybould 2006).

The results of the hazard studies and the exposure estimates can be combined to provide a rigorous test of the hypothesis of no harm at the EEC from estimates of the hazard quotient (HQ). One definition of the HQ is the ratio of the exposure concentration (or dose) divided by the minimum concentration (or dose) above which adverse effects are observed (Kelly and Roy-Harrison 1998). In the case of MIR604 maize, if the worst-case EEC or DDD divided by the NOEC or the NOEL (the worst-case HQ) is less than or equal to 1, the risk hypothesis is corroborated and minimal risk to non-target organisms is demonstrated with high confidence.

Table 4 shows that the worst-case HQ is below 1 for all species tested apart from ladybird and the fish. It should be remembered that the NOECs and NOELs in the studies were the only concentration or dose tested, and therefore the HQ are maxima. The ladybird study did not show any adverse effects, but the artificial diet did not give exposure above the EEC; therefore the hypothesis that mCry3A is not harmful at the worst-case EEC is not falsified, it is simply not corroborated with the same rigour as the other studies. In the case of the fish, the worst-case EEC was taken as the concentration of mCry3A in the diet and therefore the worst-case HQ is ≤1 by definition. For the other species, the worst-case HQ is below 1 by at least a factor of 2.5, and in some cases the HQ is below 1 by at least 3 orders of magnitude.

The HQs based on realistic EECs or DDDs, which may be more suitable than worst-case exposures for protecting populations, are also given in table 4. In all

Table 3. *Summary of the results of ecotoxicology studies on mCry3A*

Test organism	Endpoint	Concentration or dose*	Study result
Coleomegilla	Pre-imaginal and adult mortality; development time	9 μg mCry3A/g aphids	NOAEC ≥9 μg mCry3A/g aphids[†]
Orius	Pre-imaginal mortality	50 μg mCry3A/g diet	NOEC ≥50 μg mCry3A/g diet
Poecilus	Adult emergence	12 μg mCry3A/g diet	NOEC ≥12 μg mCry3A/g diet
Aleochara	Reproduction	50 μg mCry3A/g diet	NOEC ≥50 μg mCry3A/g diet
Eisenia	Mortality	250 μg mCry3A/g soil	NOEC ≥250 μg mCry3A/g soil
Apis	Brood development	50 μg mCry3A/g diet	NOEC ≥50 μg mCry3A/g diet
Colinus	Mortality and feeding	652 mg mCry3A/kg bw	NOEL ≥652 mg mCry3A/kg bw
Mus	Mortality and various histological variables	2377 mg mCry3A/kg bw	NOEL ≥2377 mg mCry3A/kg bw
Oncorhychus	Mortality and growth rate	0.09 μg mCry3A/g diet	NOEC ≥0.09 μg mCry3A/g diet

bw, body weight; NOEC, no observable adverse effect concentration; NOAEC, no observable adverse effect concentration; NOEL, no observable adverse effect level.
*Concentrations confirmed by analysis of diet except for honeybee study, for which nominal concentration is used as mCry3A is stable in aqueous solutions.
[†]Statistically significant difference in development time from larva to adult. Not considered an adverse effect – see text for details.

Table 4. *Hazard quotients (HQ = exposure/NOEC or NOEL) for representative indicator species exposed to mCry3A*

Test organism	Worst-case exposure	Realistic exposure	NOEC or NOEL	Worst-case HQ	Realistic HQ
Coleomegilla	10.14 μg mCry3A/g leaves	2.03 μg mCry3A/g diet	≥9 μg mCry3A/g	≤1.127	≤0.226
Orius	10.14 μg mCry3A/g leaves	2.03 μg mCry3A/g diet	≥50 μg mCry3A/g	≤0.203	≤0.041
Poecilus	4.55 μg mCry3A/g roots	0.15 μg mCry3A/g soil	≥12 μg mCry3A/g	≤0.379	≤0.013
Aleochara	4.55 μg mCry3A/g roots	0.15 μg mCry3A/g soil	≥50 μg mCry3A/g	≤0.091	≤0.003
Eisenia	4.55 μg mCry3A/g roots	0.15 μg mCry3A/g soil	≥250 μg mCry3A/g	≤0.018	≤0.001
Apis	0.21 μg mCry3A /g pollen	0.11 μg mCry3A /g pollen	≥50 μg mCry3A/g	≤0.004	≤0.002
Colinus	0.54 mg mCry3A/kg bw	0.27 μg mCry3A/g bw	≥652 mg mCry3A/kg bw	≤0.001	≤0.001
Mus	0.51 mg mCry3A/kg bw	0.37 μg mCry3A/g bw	≥2377 mg mCry3A/kg bw	≤0.001	≤0.001
Oncorhychus	0.09 μg mCry3A/g feed	0.013 μg mCry3A/g feed	≥0.09 μg mCry3A/g diet	≤1.000	≤0.144

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cases the realistic HQ is below 1, indicating corroboration of the risk hypothesis. Again, it should be remembered that the HQs are maxima as no adverse effects were observed in any study. As the risk hypothesis of no effect of mCry3A at the EEC has not been falsified after rigorous testing, there is high confidence that MIR604 maize poses minimal risk to non-target organisms.

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Author's address: Alan Raybould, Syngenta, Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, UK. E-mail: alan.raybould@syngenta.com

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