A Review of the Environmental Safety of the Cry34Ab1 and Cry35Ab1 Proteins

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INTRODUCTION

This document provides a comprehensive review of the information and data relevant to the environmental risk assessment of two proteins encoded by genes isolated from *Bacillus thuringiensis*, Cry 34Ab1 and Cry35Ab1, and presents a summary statement about the environmental safety of these proteins when produced in the genetically engineered (GE) maize event DAS-59122-7 (*Zea mays*, L.). All sources of information reviewed herein are publicly available and include dossiers presented to regulatory authorities, decision documents prepared by regulatory authorities, product descriptions prepared by product developers, and peer-reviewed literature.

Environmental risk assessments related to the planting of GE crops are conducted on a case-by-case basis, taking into account the biology of the plant, the characteristics of the transgenes and the encoded proteins, the phenotype conferred by the transgenes, the intended uses of the crop, and the nature of the receiving environment into which the plant will be introduced. These assessments, which consider both hazard and exposure, typically involve comparisons to an untransformed parental line or closely related isolines (Craig, Tepfer, Degrassi, and Ripandelli, 2008; OECD, 2007). The goal of these comparisons is the identification of potential risk the GE plant might present beyond those already accepted for similar, non-GE plants grown in the environment. The consequences of these risks, if any, are then evaluated.

Regulatory approvals[1] for the environmental release and food and feed use of GE maize event DAS-59122-7, present singly in a maize variety or stacked with other GE events, have been issued in three countries: Canada (in 2005), Japan (in 2006), and the United States (in 2005) (See ILSI, GM Crop Database, http://cera-gmc.org/index.php?action=gm_crop_database).

ORIGIN AND FUNCTION OF THE CRY34Ab1 AND CRY35Ab1 PROTEINS

Bacillus thuringiensis and the Cry34Ab1 and Cry35Ab1 Insecticidal Proteins

Bacillus thuringiensis (Bt) is a rod-shaped, gram-positive bacterium that produces long-lived endospores. Although considered a soil bacterium, Bt is ubiquitous in the environment. It has been extensively studied and used commercially for many years as an agricultural pesticide, due to its ability to synthesize a large number of proteins with activity against a wide variety of crop pests (Hofte and Whiteley, 1989; OECD, 2007; Schnepf *et al.*, 1998; van Frankenhuyzen, 2009).

Preparations of natural isolates of Bt bacteria were first used as commercial insecticides in France in 1938, and since that time, the use of Bt preparations as insecticides has become commonplace around the world. *Bacillus thuringiensis* has been registered for use in the United States since 1961 (USEPA, 1998), and various Bt preparations have been registered in Australia, Canada, and the European Union (APVMA, 2013; DGSANCO, 2013; Health Canada, 2008; Kumar, Sharma, and Malik, 1996; Schnepf *et al.*, 1998). Because these preparations are derived from cultured cells of Bt bacteria, they contain a complex mixture of all the pesticidal proteins produced by the Bt strain used. Several hundred pesticidal proteins have been isolated from Bt cultures (Crickmore *et al.*, 2012), and these proteins display tremendous variety in sequence diversity, mode of action, and target specificity (Hofte and Whiteley,

1 Regulations may require periodic renewal of pesticide registrations. For example, the current status of USEPA registrations can be found at http://www.epa.gov/oppbppd1/biopesticides/pips/pip_list.htm.

Key words

Cry34, Cry35, insecticidal crystalline proteins, binary toxin, *Bacillus thuringiensis*, insect resistance, genetically engineered, environmental risk assessment

1989; OECD, 2007; Pigott and Ellar, 2007; Schnepf et al., 1998; Vachon, Laprade, and Schwartz, 2012; van Frankenhuyzen, 2009). They include antifungal compounds, ß-exotoxins, Cyt (cytolytic) proteins, vegetative insecticidal proteins (Vip) and the δ-endotoxins, a group that includes the insecticidal Cry (crystalline) proteins (Hofte and Whiteley, 1989; OECD, 2007; Schnepf *et al.*, 1998). These proteins may interact with each other to influence the toxicity and activity spectrum of individual bacterial preparations (OECD, 2007; Schnepf *et al.*, 1998).[2]

In 2002, industry scientists reported the isolation of a novel proteinaceous substance from crystalline parasporal inclusion bodies of several Bt strains (Ellis *et al.*, 2002; Narva *et al.*, 2000). It was discovered that this substance was toxic to western corn rootworm (*Diabrotica virgifera virgifera*) (WCR) but not to European corn borer (*Ostrinia nubilalis*) (ECB), corn earworm (*Helicoverpa zea*), or black cutworm (*Agrotis ipsilon*). Further analysis revealed that the toxin was actually composed of two proteins, one of approximate mass of 44 kDa and the other with a mass between 13 and 14 kDa. The two proteins were encoded by adjacent genes in a single operon, with the gene encoding the smaller protein located upstream of the gene for the larger protein, separated by a spacer of approximately 100 bp. While neither of the proteins had significant sequence homology to known Cry insecticidal Bt proteins, the 44 kDa protein had approximately 26 to 29% sequence homology to insecticidal Cry proteins from *B. sphaericus*[3] having activity against mosquitoes (Ellis *et al.*, 2002; Jones *et al.*, 2007; Schnepf *et al.*, 2005).

Recombinant Bt strains producing only the 14 kDa or the 44 kDa protein were not insecticidal to WCR, leading to the conclusion that the two proteins make up a binary toxin (Ellis *et al.*, 2002).[4] When tested on southern corn rootworm larvae (*Diabrotica undecimpunctata howardi*) (SCR), the 14 kDa protein alone inhibited insect growth, but its toxicity against SCR was synergized by the 44 kDa protein. Relatively small amounts of the 44 kDa protein appear to be necessary for synergism, for example, a 9:1 ratio of 14 kDa:44 kDa is sufficient for high SCR mortality. However an optimal ratio has not been identified (Ellis *et al.*, 2002; Herman *et al.*, 2002).

The 14 kDa and 44 kDa proteins are classified as Cry δ-endotoxins and have been designated Cry34Ab1 and Cry35Ab1, respectively (Crickmore *et al.*, 2012). The genes encoding the proteins, *cry*34Ab1 and *cry*35Ab1,[5] have been cloned into *Pseudomonas fluorescens* for heterologous expression (Gao *et al.*, 2004; Huang, Badger, Haney, and Evans, 2007). The biological activity of the binary form of the toxin, when produced in *P. fluorescens*, was comparable to that of the toxin that was produced in GE maize plants (Gao *et al.*, 2004; Huang *et al.*, 2007; Moellenbeck *et al.*, 2001; USEPA, 2005a). The proteins, whether produced in *P. fluorescens* or maize, had the same molecular weight, immunogenic recognition, and N-terminal sequences. In addition, neither of the proteins are glycosylated in *P. fluorescens* or in maize, even though both proteins contain N-glycolsylation sites (Gao *et al.*, 2004). Cry34Ab1 readily undergoes C-terminal truncation in the midgut of sensitive insects, which reduces its molecular weight to 40 kDa, but the truncation does not inhibit the synergistic effects of Cry35Ab1 on the toxicity of Cry34Ab1; in fact, the truncated version of Cry35Ab1 has a stronger potentiation effect on the toxicity of Cry34Ab1 (Gao *et al.*, 2004).

The binary toxin has a narrow activity spectrum, being toxic primarily to larvae of coleopteran species. Larvae of WCR, SCR, and Northern Corn Rootworm (*Diabrotica barberi*) are the most sensitive. Therefore, genes encoding the binary toxin are used, either alone or in combination with genes for other *Diabrotica*-active Bt toxins, in maize varieties likely to be grown in regions where rootworm predation causes significant crop losses (Baum *et al.*, 2004).

Mechanism of Cry34Ab1/Cry35Ab1 Insecticidal Activity

Like other Cry insecticidal proteins, the binary toxin causes cell damage in the midgut of susceptible insects through the creation of ion channels, resulting in membrane destabilization. This effect is significantly enhanced under acidic conditions, such as those typical for coleopteran guts, as opposed to the alkaline conditions found in the lepidopteran larval midgut (Masson *et al.*, 2004; Moellenbeck *et al.*, 2001). Pores can be created by either the Cry34Ab1 or by the Cry35Ab1 protein acting alone, but mixtures of the two proteins result in pores that remain open longer, causing greater membrane destabilization. In addition, the 40 kDa truncated version of the Cry35Ab1 protein was more effective at pore formation than the native 44 kDa version. The conversion of the native protein to the truncated version has an optimal pH of 5.5-6.0, similar to the midgut pH of coleopteran larvae, and it is thought that the truncation process may be crucial for full activity of the binary toxin. The lepidopteran midgut provides a more alkaline environment, and this difference may be a factor in the selectivity of the binary toxin (Masson *et al.*, 2004). Although the precise mechanism by which the binary toxin forms pores and destabilizes membranes is unknown, it appears to involve a different mechanism than the one as-

2 The activity spectrum of sprays prepared from a particular Bt culture is due to a combination of the multiple toxins produced by the bacterium as well as the qualities of the bacterial cells, which can have an impact of the selectivity and host range of a particular preparation (Schnepf *et al*., 2005; Tabashnik, 1992). Therefore, the activity spectrum of sprays made from Bt bacterial cultures may differ from the activity spectrum of individual Bt proteins produced by a GE plant (OECD, 2007).

3 *Bacillus sphaericus* has been renamed *Lysinibacillus sphaericus* (See Ahmed *et al*. 2007. *International Journal of Systematic and Evolutionary Microbiology* 57(5): 1117-25.

4 In this paper the terms "binary toxin", "Cry34Ab1 and Cry35Ab1", and "Cry34Ab1/Cry35Ab1" will be used interchangeably.

5 Further research has revealed that the Cry34Ab1/Cry35Ab1 binary toxin is a member of a family of toxins produced by various strains of *B. thuringiensis* found in Asia, Australia, and North and South America (Jones *et al*., 2007). However, of the toxins discovered so far within this family, the Cry34Ab1/Cry35Ab1 binary toxin has the highest level of pesticidal activity against WCR (Baum *et al*., 2004; Schnepf *et al*., 2005).

sociated with Cry3Bb1, another Bt insecticidal protein active against WCR (Gassmann, Petzold-Maxwell, Keweshan, and Dunbar, 2011; Masson *et al.*, 2004).

Expression of Cry34Ab1/Cry35Ab1 in Insect-Resistant GE Maize

Transgene expression levels in a GE plant can be influenced by several factors related to the genetic transformation process, including the types of promoter and terminator sequences employed, as well as the chromosomal location where the transgene has been incorporated into the genome. Expression levels may also be influenced by the type of tissue sampled, the age of the plant at the time the sample was taken, and the environmental conditions under which the plant was growing. Data from enzyme-linked immunosorbent assays (ELISA), showing levels of Cry34Ab1 and Cry35Ab1 protein expression in GE maize DAS-59122-7 have been made available in publicly accessible regulatory submissions and decision documents associated with regulatory authorization processes at the national, supranational, and member state levels (BBAC, 2009; CFIA, 2005a; EFSA, 2008, 2009a, 2009b; FSANZ, 2005; USDA, 2005). Samples were collected from several tissue types, at multiple growth stages, and from plants grown in several different locations to produce data representative of the typical range of protein expression for both of the proteins. Table 2 presents the highest reported values of Cry34Ab1/Cry35Ab1 expression in GE maize plants containing DAS-59122-7 alone or when DAS-59122-7 is stacked with other events in the same plant. Protein expression data may be used to estimate the potential exposure of various organisms in the environment to Cry34Ab1/Cry35Ab1 when GE maize event DAS-59122-7 producing these proteins is cultivated. Currently available protein expression data for *Cry*34Ab1/*Cry*35Ab1 by DAS-59122-7 and by DAS-59122-7 stacked with other GE events are presented in Annex I. In some cases when the GE maize plants contained *cry*34Ab1/*cry*35Ab1 as well as a gene for herbicide tolerance (pat or epsps), protein expression data were collected from plants that had been treated with the appropriate herbicide, glufosinate or glyphosate, as well as from plants grown in the same location but not sprayed, to determine whether the herbicide had any effect on protein concentrations.

Table 1. Highest reported protein concentration of Cry34Ab1 and Cry35Ab1 in GE maize DAS-59122-7 and stacks of DAS-59122-7 tissues.

Tissue	ng Cry34Ab1/mg dry weight (growth stage)	ng Cry35Ab1/mg dry weight (growth stage)
Leaf	302 (R4 – kernel contents are doughy)	126 (R4 – kernel contents are doughy)
Grain	117 (Seed maturity)	3.7 (Seed maturity)
Root	102 (Maturity)	15.4 (V9 – collar of 4th leaf is visible)
Pollen	87.2 (Anthesis)	0.15 (Anthesis)
Whole Plant	88 (Maturity)	18.1 (R1 – silks become visible)

Modifications to the genes encoding Cry34Ab1 and Cry35Ab1 in GE maize

To produce GE insect-resistant maize DAS-59122-7, cry34Ab1 and cry35Ab1 genes were isolated from B. thuringiensis Berliner strain PS149B1 (Ellis *et al.*, 2002). Synthetic versions of the genes were created in which the DNA sequences had been modified to reflect codon preference in maize, for optimal protein expression (Murray, Lotzer, and Eberle, 1989). The amino acid sequences of the resulting Cry34Ab1 and Cry35Ab1 proteins were identical to the sequences of the native bacterial proteins. Transcription of the cry34Ab1 and cry35Ab1 genes was directed by the ubiquitin promoter and the wheat peroxidase promoter, respectively. Transcription termination was directed by the 35S terminator (CFIA, 2005a; EFSA, 2008; FSANZ, 2005; Health Canada, 2006; USDA, 2005; USEPA, 2005a, 2005b, 2010; USFDA, 2004).[6]

NON-TARGET ORGANISMS TESTING AND IMPACTS OF EXPOSURE TO THE CRY34Ab1 AND CRY35Ab1 PROTEIN

The Cry34Ab1/Cry35Ab1 binary toxin has insecticidal properties against certain coleopteran insect species when expressed in GE maize DAS-59122-7. The toxin targets root-feeding coleopteran insect pests, thereby reducing feeding damage (Baum *et al.*, 2004; Ellis *et al.*, 2002; Gao *et al.*, 2004; Herman *et al.*, 2002; Kaiser-Alexnat, Büchs, and Huber, 2009; Oppert, Ellis, and Babcock, 2010; Schnepf *et al.*, 2005; Storer, Babcock, and Edwards, 2006). Organisms in the environment that are not pests of maize but are directly or indirectly exposed to the binary toxin are called non-target organisms (NTOs). Direct exposure occurs when NTOs feed on living crop tissues expressing the binary toxin or on crop residues, either above or below ground. Indirect exposure results from the predation by one organism on another organism that has had direct exposure to the binary toxin. The potential for harm to NTOs from exposure to the binary toxin has been considered in risk assessments conducted by several regulatory authorities (CFIA, 2005a; EFSA, 2008; USDA, 2005; USEPA, 2005a, 2010). Data collected from field trials of GE maize producing the binary toxin and submitted to regulators have established that the Cry34Ab1/Cry35Ab1 proteins are active specifically against the subset of coleopteran pests which feed on maize roots and are harmless to vertebrate species and other NTOs (CFIA, 2005a; EFSA, 2008; FSANZ, 2005; Health Canada, 2006; USDA, 2004, 2005; USEPA, 2005a, 2010).

Assessments of the potential impacts to NTOs, and the regulatory decisions informed by the assessments, have been grounded in the well-documented and long history of evaluation of classical insecti-

6 The DNA sequence used in the original transformation process, which resulted in the isolation of event DAS-59122-7, also contained the *pat* gene, which confers tolerance to glufosinate-ammonium herbicides. For a full discussion of the environmental safety of the PAT protein, please see "A Review of the Environmental Safety of the PAT Protein" (CERA, 2011).

cidal formulations including microbial formulations of *B. thuringiensis* (Romeis *et al.*, 2008, 2013; Sanvido *et al.*, 2012). Assessments of impacts to NTOs include the critical review of data submitted by the product developer to demonstrate that NTOs exposed to the binary toxin, either directly or indirectly, are not harmed. The NTO risk assessment typically begins with a determination of the organisms that are likely to be directly or indirectly exposed to the binary toxin. Particular consideration is often given to NTOs having beneficial environmental functions, such as pollinators or the natural enemies of agricultural pests. Regulatory authorities may give special attention to NTOs that have been designated as threatened or endangered species or species of recognized cultural value. These species, or valid surrogates of these species, are then tested to determine if exposure to the binary toxin could cause significant adverse impacts.

The "tiered" approach for assessing the impacts of chemical pesticides on NTOs has been used effectively for many years, and tiered testing has also been determined by scientists and regulators to be appropriate for the assessment of potential impacts of GE crops on NTOs (Duan, Lundgren, Naranjo, and Marvier, 2009; Dutton, Romeis, and Bigler, 2003; EFSA, 2006; Garcia-Alonso *et al.*, 2006; Raybould, 2006; Romeis *et al.*, 2008, 2013; USEPA, 2007, 2011). Early tier studies generally involve the exposure of NTOs or surrogate species to high concentrations of the pesticide, under controlled laboratory conditions. These studies identify those species that are significantly affected by the pesticide. Such effects, when found, may require further analysis at a higher tier level. Early tier tests also identify NTOs that are unaffected by the pesticidal protein and for which higher tier testing is unnecessary. Higher level tier testing may also be appropriate when the results of early tier tests are inconclusive. Testing at higher tiers typically involves increasing levels of complexity and increasingly realistic assay conditions (EFSA, 2006; Garcia-Alonso *et al.*, 2006; Romeis *et al.*, 2008; USEPA, 2007, 2011).

Routes of Environmental Exposure

In addition to direct contact with the GE maize plant, regulatory authorities may consider other routes of potential exposure to the Cry34Ab1/Cry35Ab1 binary toxin: exposure to the toxin in pollen, exposure to toxin deposited in the soil by decomposing plant material, and exposure to predator species consuming herbivores that have been feeding on the GE maize plants (CFIA, 2005a; EFSA, 2007, 2008; FSANZ, 2005; Health Canada, 2006; USDA, 2004, 2005; USEPA, 2005a, 2010).

Exposure to Cry35Ab1 via pollen is limited by the low expression levels of that protein in the pollen of varieties that have been authorized for environmental release by regulators (see Table 2 and Annex II). Expression of Cry34Ab1 in pollen is comparable to levels found in whole maize plants, but exposure to Cry34Ab1 via pollen is limited by the rapidly decreasing density of pollen deposition with increasing distance from the source plant (JBCH, 2006a). (See Annex I for expression level data in the pollen of approved varieties.) In addition, data submitted to regulatory authorities indicate that the binary toxin is quickly degraded once released from decomposing plant tissue and is not likely to persist or accumulate in the soil environment (USDA, 2004, 2005; USEPA, 2005b, 2010).

Ecotoxicological Testing of Cry34Ab1 and Cry35Ab1 on Non-Target Organisms

Ecotoxicological testing of Cry34Ab1 and Cry35Ab1 on NTOs has been conducted on a variety of well-characterized test organisms that are typically used for ecotoxicological testing of chemical pesticides, and the data from these tests have been evaluated by regulatory authorities in the course of performing risk assessments for the environmental release of GE maize varieties. Although some biologically signficant exposure may occur within a short distance of crop fields, regulatory authorities have generally requested data only for impacts of the binary toxin on representative pollinator species, *i.e.*, honey bees. In addition, the specificity of the binary toxin to Coleoptera, as well as evidence suggesting the potential for exposure from the soil, has led regulators to require the testing of representative soil dwelling arthropod species. Some regulatory authorities also require data to be collected on non-arthropod, soil-dwelling species, such as earthworms, to demonstrate that there are no significant impacts to these species from exposure to the binary toxin. Test organisms have included *Apis mellifera* (honeybee); *Orius insidious* (minute pirate bug); *Coleoptera: Coccinella septempunctata*, *Hippodamia convergens*, and *Coleomegilla maculata* (ladybird beetle) *Chrysoperla carnea* (green lacewing); *Nasonia vitripennis* (parasitic wasp); *Folsomia candida* (springtail); *Daphnia magna*; and *Eisenia foetida* (earthworm) (Balog, Szenasi, Szekeres, and Palinkas, 2011; CFIA, 2005a; FSANZ, 2005; Health Canada, 2006; USDA, 2004, 2005; USEPA, 2005a, 2010). Test organisms were exposed to levels of Cry34Ab1 and Cry35Ab1 representing worst case scenario exposure estimates based on the highest observed tissue concentrations of the binary toxin in GE maize plants, ranging from 0.15 – 302 ng/mg dry tissue weight (See **Table 2**). None of the test organisms showed a significant response to the binary toxin (CFIA, 2005a; FSANZ, 2005; Health Canada, 2006; USDA, 2004, 2005; USEPA, 2005a, 2010). Additionally, vertebrate toxicological testing and nutritional equivalence testing has been conducted on *Mus musculus* (mouse); *Oncorhynchus mykiss* (rainbow trout); *Gallus domesticus* (chicken); and *Rattus rattus* (Sprague-Daley rat)(CFIA, 2005a; EFSA, 2007; FSANZ, 2005; Health Canada, 2006; Malley *et al.*, 2007; USDA, 2004, 2005; USEPA, 2005b, 2010).[7] See Table 2.

The results from Tier 1 tests indicate that no higher tier testing is necessary from a regulatory standpoint, because no adverse effects

7 The USEPA issued a conditional registration of event DAS-59122-7, but required the applicant to perform two additional tests, using *Orius insidiosus* (minute pirate bug) and a carabid (ground beetle) (USEPA, 2005a).

Table 2. Summary of ecotoxicological studies of Cry34Ab1 and Cry35Ab1 on non-lepidopteran non-target organisms (Malley *et al.*, 2007; USDA, 2004)

Species	Method of Exposure	Results	Comments
Apis mellifera (honeybee)	Cry34/35Ab1 pollen from event TC5639	$NOEC_{pollen}$ = 2 mg/larvae (0.056 µg Cry34/35Ab1 ICP†/larvae) $NOEC_{ICP}$ = 20 µg/larvae	
	Microbially produced Cry34Ab1 protein (54% pure) and Cry35Ab1 protein (37% pure)	$NOEC_{Cry34Ab1}$ = 3.2 µg/larvae $NOEC_{Cry35Ab1}$ = 2.4 µg/larvae	
Chrysoperla carnea (green lacewing)	Microbially produced Cry34Ab1 protein (54% pure) and Cry35Ab1 protein (37% pure)	NOEC > 280 µg a.i./mL $LC_{50\ Cry34Ab1}$ > 160 µg a.i./mL $NOEC_{Cry34Ab1}$ > 160 µg a.i./mL $LC50_{Cry35Ab1}$ > 120 µg a.i./mL $NOEC_{Cry35Ab1}$ > 120 µg a.i./mL	
Coleomegilla maculata (twelvespotted ladybird beetle)	Microbially produced Cry34Ab1 protein (54% pure) and Cry35Ab1 protein (37% pure)	LC_{50} > 902 µg a.i./g NOEC > 902 µg a.i./g $LC_{50\ Cry34Ab1}$ > 900 µg a.i./g $NOEC_{Cry34Ab1}$ > 900 µg a.i./g $LC_{50\ Cry35Ab1}$ > 2 µg a.i./g $NOEC_{Cry35Ab1}$ > 2 µg a.i.	Weight reduction reported
	Homozygous inbred pollen mixed 1:1 with ground corn earworm eggs	NOEC >58.52 µg a.i./g $NOEC_{Cry34Ab1}$ > 58.5 µg a.i./g $NOEC_{Cry35Ab1}$ > 0.02 µg a.i./g	No effect on mortality, weight, or development
Daphnia magna	Microbially produced Cry34Ab1 protein (54% pure) and Cry35Ab1 protein (37% pure)	EC_{50} > 100 mg a.i./L NOEC > 100 mg a.i./L $LC_{50\ Cry34Ab1}$ > 57 mg a.i./L $NOEC_{Cry34Ab1}$ > 57 mg a.i./L $LC_{50\ Cry35Ab1}$ > 43 mg a.i./L $NOEC_{Cry35Ab1}$ > 43 mg a.i./L	
Eisenia fetida (earthworm)	Microbially produced Cry34Ab1 protein (54% pure) and Cry35Ab1 protein (37% pure)	LC_{50} > 25.4 mg a.i./kg dry soil NOEC > 25.4 mg a.i./kg dry soil $LC_{50\ Cry34Ab1}$ > 6.4 mg a.i./kg dry soil $NOEC_{Cry34Ab1}$ > 6.4 mg a.i./kg dry soil $LC_{50\ Cry35Ab1}$ > 19.0 mg a.i./kg dry soil $NOEC_{Cry35Ab1}$ > 19.0 mg a.i./kg dry soil	
Folsomia candida (Collembola)	Microbially produced Cry34Ab1 protein (54% pure) and Cry35Ab1 protein (37% pure)	LC_{50} > 12.7 mg a.i./kg diet NOEC > 12.7 mg a.i./kg diet $LC_{50\ Cry34Ab1}$ > 3.2 mg a.i./kg diet $NOEC_{Cry34Ab1}$ > 3.2 mg a.i./kg diet $LC_{50\ Cry35Ab1}$ > 9.5 mg a.i./kg diet $NOEC_{Cry35Ab1}$ > 9.5 mg a.i./kg diet	
Gallus domesticus (chicken)	Feed consisting of 60% maize, event DAS-15344	LC_{50} > 25.1 ng a.i./mg diet NOEC > 25.1 ng a.i./mg diet $LC_{50\ Cry34Ab1}$ > 23 ng a.i./mg diet $NOEC_{Cry34Ab1}$ > 23 ng a.i./mg diet $LC_{50\ Cry35Ab1}$ > 2.1 ng a.i./mg diet $NOEC_{Cry35Ab1}$ > 2.1 ng a.i./mg diet	No effects on mortality, weight gain, feed efficiency, or carcass yields
Hippodamia convergens (ladybird beetle)	Microbially produced Cry34Ab1 protein (54% pure) and Cry35Ab1 protein (37% pure)	LC_{50} ICP > 280 µg a.i./mL NOEC > 280 µg a.i./mL $LC_{50\ Cry34Ab1}$ > 160 µg a.i./mL $NOEC_{Cry34Ab1}$ > 160 µg a.i./mL $LC_{50\ Cry35Ab1}$ > 120 µg a.i./mL $NOEC_{Cry35Ab1}$ > 120 µg a.i./mL	
Mus musculus (mouse)	Microbially produced Cry34Ab1 protein (54% pure) and Cry35Ab1 protein (37% pure)	$LD_{50\ Cry34Ab1}$> 2700 mg a.i./kg $LD_{50\ Cry35Ab1}$> 1850 mg a.i./kg $LD_{50\ Cry34/35Ab1}$> 2000 mg a.i./kg	
Nasonia vitripennis (parasitic wasp)	Microbially produced Cry34Ab1 protein (54% pure) and Cry35Ab1 protein (37% pure)	LC_{50} > 280 µg a.i./mL NOEC > 280 µg a.i./mL $LC_{50\ Cry34Ab1}$ > 160 µg a.i./mL $NOEC_{Cry34Ab1}$ > 160 µg a.i./mL $LC_{50\ Cry35Ab1}$ > 120 µg a.i./mL $NOEC_{Cry35Ab1}$ > 120 µg a.i./mL	

continued on page 6

Table 2 (cont'd)

Species	Method of Exposure	Results	Comments
Oncorhynchus mykiss (rainbow trout)	Microbially produced Cry34Ab1 protein (54% pure) and Cry35Ab1 protein (37% pure)	LC_{50} > 100 mg a.i./kg diet NOEC > 100 mg a.i./kg diet $LC_{50\ Cry34Ab1}$ > 25 mg a.i./kg diet $NOEC_{Cry34Ab1}$ > 25 mg a.i./kg diet $LC_{50\ Cry35Ab1}$ > 75 mg a.i./kg diet $NOEC_{Cry35Ab1}$ > 75 mg a.i./kg diet	
Rattus rattus (Sprague Daley rats)	90-day subchronic assay; feed consisting of 35% maize, event DAS-59122-7		No adverse diet-related differences observed

† ICP = Insecticidal Crystalline Protein

were noted;[8] however, as discussed below, studies of the effects of the binary toxin on natural populations of NTOs have been performed.

Field Studies of Cry34Ab1/Cry35Ab1 on Non-Target Organisms

Regulatory authorities have considered the potential impact of the binary toxin on natural populations of NTOs and determined that adverse effects on NTOs are unlikely for several reasons. First, the binary toxin has a narrow spectrum of pesticidal activity. Second, Tier I laboratory assays, employing a range of invertebrate species present in maize agricultural ecosystems, or surrogates for those species, have shown that the binary toxin causes no significant observable effects in these species. Third, Tier I studies have also demonstrated that the binary toxin has no observable effect on representative vertebrate and aquatic species. Fourth, the levels of the binary toxin used in these Tier I assays were much higher than those measured in GE maize tissues growing in the field. Fifth, field studies of corn varieties producing the binary toxin show no significant adverse effects on rove beetles, a beneficial, non-target arthropod (Balog *et al.*, 2011) and no effect on black cutworm (*Agrotis ipsilon*) (USDA, 2004). Sixth, when compared to insect control via the binary toxin, traditional insect control using chemical pesticides significantly alters species diversity and harms non-target species. Together, these findings indicate that the binary toxin is unlikely to have adverse effects on natural populations of organisms, except for the target coleopteran crop pests (Balog *et al.*, 2011; CFIA, 2005a; EFSA, 2008; FSANZ, 2005; Health Canada, 2006; USDA, 2004, 2005; USEPA, 2005a, 2010).

ESTABLISHMENT AND PERSISTENCE IN THE ENVIRONMENT OF MAIZE PLANTS EXPRESSING CRY34Ab1 AND CRY35Ab1

Biology of the Plant Species

The biology of the non-GE plant species in the receiving environment is typically the starting point for environmental risk assessments of GE plants (OECD, 2003, 2007). Information about the biology of the non-GE plant can be used to assess whether a GE variety of the plant may become weedy, invasive, or otherwise harmful to the environment. It can also provide details on significant interactions between the plant and other organisms that may be important when considering potential harms. By considering the biology of the host plant, a risk assessor can identify potential hazards that may be associated with the expression of the novel protein (*e.g.*, Cry34Ab1 or Cry35Ab1) and then be able to assess the likelihood of these hazards. For example, whether the plant is an annual or perennial species or whether the plant is self pollinated or wind pollinated can bear on the assessment of the likelihood of the GE plant establishing and persisting outside of cultivation (EFSA, 2006; OECD, 1992, 2003, 2007).

Phenotypic Data

Information about the phenotype of GE plants expressing Cry34Ab1 and Cry35Ab1 is collected from laboratory, greenhouse, and field trial studies and is presented in regulatory submissions to (1) identify any intentional changes to the phenotype that might impact the environmental safety of the plant and (2) to identify any unintended changes to the biology of the plant that might impact environmental safety. Phenotypic data in regulatory submissions and peer reviewed publications have focused on characteristics of the plant that might contribute to its survival or persistence (*i.e.*, potential weediness), or that negatively affect agricultural performance (*e.g.*, disease susceptibility and yield data) (CFIA, 2005a; JBCH, 2006a; USDA, 2004, 2005; USEPA, 2005a, 2010). The phenotypic observations take into account the desired phenotype resulting from the transgenic trait, in this case insect predation resistance mediated by Cry34Ab1 and Cry35Ab1. Some of the collected data are quantitative (*e.g.*, plant height or percent seed germination) while other data are qualitative and observational (*e.g.*, symptoms of disease susceptibility). Statistically significant differences between GE maize plants expressing the binary toxin and controls were observed, but these differences were not consistent among the field trial locations and fell within the reported range for non-GE maize varieties (CFIA, 2005a; JBCH, 2006a; USDA, 2004, 2005; USEPA, 2005a, 2010). Collectively, regulators have determined that the phenotypic data do not support the hypothesis that the expression of the binary toxin had any unintended impact on the gross morphology or phenotypic characteristics

8 Conducting field studies is considered case-by-case, based on the level of potential hazard and exposure, and goals may be adjusted as information and experience accumulate (USEPA, 2007).

of maize plants, besides conferring resistance to coleopteran insect pests (CFIA, 2005a; JBCH, 2006a; USDA, 2004, 2005; USEPA, 2005a, 2010).

Weediness in Agricultural Environments

Maize is not generally regarded as a weed, possessing few of the characteristics that increase the likelihood of a plant to become a weed, such as seed dormancy, shattering, and competitiveness (Baker, 1974; Carpenter *et al.*, 2002; JBCH, 2006a; OECD, 2003; Raybould *et al.*, 2011; USDA, 2004, 2005; USEPA, 2005a, 2010). There are no data indicating that expression of the binary toxin results in altered seed dormancy, over-wintering capacity, or other characteristics that would alter the prevalence of volunteer maize in subsequent growing seasons (Carpenter *et al.*, 2002; JBCH, 2006a; OECD, 2003; Raybould *et al.*, 2011; USDA, 2004, 2005; USEPA, 2005a, 2010). Following-season maize volunteers producing the binary toxin would not be expected to present any management difficulty and can be dealt with in the same manner as conventional volunteers of maize.

Weediness in Non-Agricultural Environments

The primary mechanisms by which the binary toxin may be introduced into a non-agricultural environment are through the movement of propagules outside of cultivated areas and gene flow from the GE plant to a naturalized population of sexually compatible relatives (Lee and Natesan, 2006). Risk assessments for GE maize expressing the binary toxin have considered the potential impacts associated with both types of movement (Carpenter *et al.*, 2002; JBCH, 2006a; OECD, 2003; Raybould *et al.*, 2011; USDA, 2004, 2005; USEPA, 2005a, 2010). As a result of extensive selective breeding, commercial maize varieties are severely restricted in their ability to persist in non-agricultural environments without human intervention, and maize is not considered to be an invasive or aggressive weed outside of agricultural systems (Carpenter *et al.*, 2002; JBCH, 2006a, 2006b; OECD, 2003; USDA, 2004, 2005; USEPA, 2005a, 2010). Agronomic data show that the binary toxin does not have a significant impact on traits associated with weediness (Carpenter *et al.*, 2002; JBCH, 2006a; OECD, 2003; USDA, 2004, 2005; USEPA, 2005a, 2010). Although release from natural control factors (including insect herbivores) has been offered as a partial explanation for the success of invasive species (Blumenthal, 2005; Keane and Crawley, 2002; Mack, 1996; Mason, Braun, Warwick, Zhu, and Stewart, 2004), regulatory decisions have determined that it is unlikely that resistance to coleopteran pests would allow maize producing the binary toxin to become invasive in non-agricultural environments (Carpenter *et al.*, 2002; JBCH, 2006a, 2006b; OECD, 2003; USDA, 2004, 2005; USEPA, 2005a, 2010).

Movement of the Transgene to Sexually Compatible Relatives

The movement of transgenes from a GE plant to its wild relatives is pollen mediated, and the production of reproductively viable hybrids depends on several factors: whether the pollen donor is self-pollinated, the physical and temporal proximity of the GE plants to sexually compatible species, pollen mobility and viability, and the presence of appropriate pollinators. Maize is predominantly wind pollinated and does not have sexually compatible relatives that are considered invasive (Carpenter *et al.*, 2002; OECD, 2003). Maize freely hybridizes with wild teosintes, but gene introgression is thought to be limited (Baltazar, De Jesús Sánchez-Gonzalez, De la Cruz-Larios, and Schoper, 2005; Castillo-Gonzalez and Goodman, 1997; OECD, 2003). Wild teosinte populations are limited to Mexico, Guatemala, and a single population in Nicaragua, and while teosinte is considered a serious weed by some farmers in Mexico, it is used as a forage plant by other farmers and is considered a culturally significant species (González and Corral, 1997; Mondragon-Pichardo and Vibrans, 2005). Crosses between teosinte and GE maize expressing Cry34Ab1 and Cry35Ab1 are not expected to occur more frequently than those between teosinte and traditionally bred maize varieties (Carpenter *et al.*, 2002; USDA, 2004, 2005; USEPA, 2005a, 2010).

COMPOSITIONAL ANALYSIS OF MAIZE PLANTS EXPRESSING CRY34Ab1 AND CRY35Ab1

A compositional analysis is required in many regulatory approval processes for GE plants intended to be used in food or feed. Compositional data can be used to identify unintended changes in the crop due to the presence of the transgene. The analysis typically compares the GE plant to the untransformed parent line or a closely related isoline, and the analytes measured depend on the crop and its intended uses. The analysis may use plants grown in a variety of locations over the course of more than one year, because local environmental conditions may impact nutritional composition even in conventionally bred varieties. The goal of the analysis is to verify that the values obtained for the GE plant are within the range observed in traditional varieties grown under comparable conditions.

Seed from GE maize expressing Cry34Ab1 and Cry35Ab1 has undergone proximate analysis to determine levels of crude protein, crude fat, fiber, moisture, and ash. In addition, levels of select minerals, fatty acids, and amino acids have been determined. Some crop plants produce toxins or anti-nutritive compounds, and levels of these compounds are also measured to determine whether the presence of the transgenes has inadvertently resulted in elevated production of these substances. Maize is known to produce the anti-nutritive compounds phytic acid, raffinose, and trypsin inhibitor (OECD, 2002), and levels of these substances were determined. Data from publicly available sources are summarized in Annex II. All differences noted between the GE maize varieties analyzed and the comparator varieties were within the normal range of variation, and these differences were deemed irrelevant to environmental safety (CFIA, 2005a; EFSA, 2008, 2009b; Health Canada, 2006; USDA, 2004, 2005; USEPA, 2005a, 2010).

CONCLUSION

The Cry34Ab1 and Cry35Ab1 proteins produced by insect-resistant GE maize plants are derived from the common soil bacterium *Bacillus thuringiensis* and are specifically toxic to Coleoptera. Toxicity testing with a range of representative non-target organisms produced NOEC values at concentrations significantly higher than the expected environmental concentrations of Cry34Ab1 or Cry35Ab1. Field data suggest that cultivation of GE maize plants expressing the binary toxin does not affect the abundance of non-target arthropods. The binary toxin in plants can be toxic to non-target Coleoptera, but regulatory risk assessments for approved products have concluded that the risk is low, due to the lack of exposure to the toxin in the environment, especially when compared to other insect-control practices. The weight of evidence from analyses of phenotypic and compositional data demonstrates that Cry34Ab1 and Cry35Ab1 expression in approved maize varieties do not alter the gross physiology of the crop plants and indicates that these plants are not more likely to become weedy or invasive than conventional maize varieties.

REFERENCES

APVMA. (2013). Record of Approved Active Constituents. *Public Chemical Registration Information System - PUBCRIS*. Retrieved January 24, 2013, from http://services.apvma.gov.au/PubcrisWebClient/welcome.do

Baker, H. (1974). The evolution of weeds. *Annual Review of Ecology and Systematics*, 5, 1-24.

Balog, A., Szenasi, A., Szekeres, D., and Palinkas, Z. (2011). Analysis of soil dwelling rove beetles (Coleoptera: Staphylinidae) in cultivated maize fields containing the Bt toxins, Cry34/35Ab1 and Cry1F x Cry34/35Ab1. *Biocontrol Science & Technology*, 21(3-4), 293-297.

Baltazar, B. M., De Jesús Sánchez-Gonzalez, J., De la Cruz-Larios, L., and Schoper, J. B. (2005). Pollination between maize and teosinte: an important determinant of gene flow in Mexico. *Theoretical and Applied Genetics*, 110(3), 519-526. doi:10.1007/s00122-004-1859-6

Baum, J. A., Chu, C., Rupar, M., Brown, G. R., Donovan, W. P., Huesing, J. E., Ilagan, O., *et al*. (2004). Binary Toxins from *Bacillus thuringiensis* active against the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. *Applied and Environmental Microbiology*, 70(8), 4889-4898. doi:10.1128/AEM.70.8.4889

BBAC. (2009). *Advice of the Belgian Biosafety Advisory Council on the application EFSA/GMO/UK/2005/21 from Pioneer Hi-Bred under Regulation (DC) No. 1829/2003. Regulation.* Brussels, Belgium.

Blumenthal, D. (2005). Interrelated causes of plant invasion. Science, 310(October), 243–244.

Carpenter, J., Felsot, A., Goode, T., M. Hammig, Onstad, D., Sankula., S., Hammig, M., *et al.* (2002). *Comparative Environmental Impacts of Biotechnology-derived and Traditional Soybean, Corn, and Cotton Crops. Crops*. Ames, IA: Council for Agricultural Science and Technology. Retrieved from http://oregonstate.edu/instruct/bi430-fs430/Documents-2004/7B-MIN TILL AG/CAST-ComparEnvImpactGMOCrops-2002.pdf

Castillo-Gonzalez, F., and Goodman, M. M. (1997). Research on gene flow between improved maize and landraces. *In:* J. A. Serratos, M. C. Willcox and F. Castillo (Eds.), *Gene Flow Among Maize Landraces , Improved Maize Varieties , and Teosinte: Implications for Transgenic Maize*. El Batan, Mexico: CIMMYT.

CERA. (2011, October). A review of the environmental safety of the PAT protein. doi:10.1051/ebr/2012004

CFIA. (2005a). *Determination of the Safety of Dow AgroSciences Canada Inc. and Pioneer Hi-Bred Production Inc.'s Insect Resistant and Glufosinate-Ammonium Herbicide Tolerant Corn (*Zea mays *L .) Line 59122*.

CFIA. (2005b). *Determination of the Safety of Dow AgroSciences Canada Inc . and Pioneer Hi-Bred Production Inc.'s Insect Resistant and Glufosinate-Ammonium Herbicide Tolerant Corn (* Zea mays *L .) Line 59122*.

Craig, W., Tepfer, M., Degrassi, G., and Ripandelli, D. (2008). An overview of general features of risk assessments of genetically modified crops. *Euphytica*, 164(3), 853–880. Springer Netherlands. doi:10.1007/s10681-007-9643-8

Crickmore, N., Zeigler, D. R., Schnepf, E., Van Rie, J., Lerechus, D., Baum, J., Bravo, A., *et al.* (2012). *Bacillus thuringiensis* toxin nomenclature. Retrieved January 24, 2013, from http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/

DGSANCO. (2013). EU Pesticides Database. Brussels, Belgium. Retrieved January 24, 2013, from http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=activesubstance.selection

Duan, J. J., Lundgren, J. G., Naranjo, S., and Marvier, M. (2009). Extrapolating non-target risk of Bt crops from laboratory to field. *Biology Letters*, 6(1), 74-77. doi:10.1098/rsbl.2009.0612

Dutton, A., Romeis, J., and Bigler, F. (2003). Assessing the risks of insect resistant transgenic plants on entomophagous arthropods: Bt-maize expressing Cry1Ab as a case study. *Agriculture*, 48(6), 611-636. Springer Netherlands. doi:10.1023/A:1026313719424

EFSA. (2006). *Guidance Document of the Scientific Panel on Genetically Modified Organisms for the Risk Assessment of Genetically Modified Plants and Derived Food and Feed*. Parma, Italy. Retrieved from http://www.efsa.europa.eu/en/scdocs/doc/gmo_guidance_derived_feed_food.pdf

EFSA. (2007). *Opinion of the Scientific Panel on Genetically Modified Organisms on an application (Reference EFSA-GMO-NL-2005-12) for the placing on the market of insect-resistant genetically modified maize 59122, for food and feed uses, import and processing* (pp. 1-4). Parma, Italy.

EFSA. (2008). *Scientific Opinion of the Panel on Genetically Modified Organisms on application (Reference EFSA-GMO-UK-2005-20) for the placing on the market of the insect-resistant and herbicide-tolerant genetically modified maize 59122 x NK603, for food and feed use. Analysis*. Retrieved from http://www.efsa.europa.eu/en/scdocs/doc/s874.pdf

EFSA. (2009a). Scientific Opinion: Application (Reference EFSA-GMO-UK-2005-21) for the placing on the market of the insect-resistant and herbicide-tolerant genetically modified maize 59122 x 1507 x NK603 for food and feed uses, import and processing. *The EFSA Journal*, 1050, 1-32. Retrieved from http://www.efsa.europa.eu/en/efsajournal/doc/1050.pdf

EFSA. (2009b). Scientific Opinion: Application (Reference EFSA-GMO-NL-2005-15) for the placing on the market of the insect-resistant and herbicide-tolerant genetically modified maize 1507 x 59122, for food and feed uses, import and processing. *The EFSA Journal*, 1074, 1-28. Retrieved from http://www.efsa.europa.eu/en/efsajournal/doc/1050.pdf

Ellis, R. T., Stockhoff, B. A., Stamp, L., Schnepf, H. E., Schwab, G. E., Knuth, M., Russell, J., *et al.* (2002). Novel *Bacillus thuringiensis* binary insecticidal crystal proteins active on western corn rootworm, *Diabrotica virgifera virgifera* LeConte. *Applied and Environmental Microbiology*, 68(3), 1137-1145. doi:10.1128/AEM.68.3.1137

FSANZ. (2005). *Final Assessment Report: Application A543 Food Derived from Insect-Protected, Glufosinate Ammonium-Tolerant Corn, Line 59122-7*. Retrieved from http://www.foodstandards.gov.au/_srcfiles/A543 GM corn FAR_FINAL.pdf

Gao, Y., Schafer, B. W., Collins, R. A., Herman, R. A., Xu, X., Gilbert, J. R., Ni, W., et al. (2004). Characterization of Cry34Ab1 and Cry35Ab1 insecticidal crystal proteins expressed in transgenic corn plants and *Pseudomonas fluorescens*. *Journal of Agricultural and Food Chemistry,* 52(26), 8057-8065.

Garcia-Alonso, M., Jacobs, E., Raybould, A., Nickson, T. E., Sowig, P., Willekens, H., Van Der Kouwe, P., *et al*. (2006). A tiered system for assessing the risk of genetically modified plants to non-target organisms. *Environmental Biosafety Research*, 5(02), 57-65. Retrieved from http://journals.cambridge.org/abstract_S1635792206000182

Gassmann, A. J., Petzold-Maxwell, J. L., Keweshan, R. S., and Dunbar, M. W. (2011). Field-evolved resistance to Bt maize by western corn rootworm. (P. Meyer, Ed.) *PloS One*, 6(7), e22629. Public Library of Science. doi:10.1371/journal.pone.0022629

González, J. de J. S., and Corral, J. A. R. (1997). Teosinte distribution in Mexico. *In*: J. A. Serratos, M. C. Wilcox, and F. Castillo (Eds.), *Gene Flow Among Maize Landraces , Improved Maize Varieties , and Teosinte: Implications for Transgenic Maize*. El Batan, Mexico: CIMMYT. Retrieved from http://repository.cimmyt.org/xmlui/handle/123456789/577?show=full

Health Canada. (2006). Bacillus thuringiensis (B.t.) *Cry34/35/Ab1 insect resistant, glufosinate-tolerant transformation corn event DAS-59122-7*. Retrieved from http://www.hc-sc.gc.ca/fn-an/gmf-agm/appro/nf-an125decdoc-eng.php

Health Canada. (2008). Bacillus thuringiensis. *Re-Evaluation Decision Document*. Retrieved from http://www.hc-sc.gc.ca/cps-spc/pubs/pest/_decisions/rvd2008-18/index-eng.php

Herman, R. A., Scherer, P. N., Young, D. L., Mihaliak, C. A., Meade, T., Woodsworth, A. T., Stockhoff, B. A., *et al*. (2002). Binary insecticidal crystal protein from *Bacillus thuringiensis*, strain PS149B1:effects of individual protein components and mixtures in laboratory bioassays. *Journal of Economic Entomology*, 95(3), 635-639. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12076012

Hofte, H., and Whiteley, H. R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Reviews*, 53(2), 242-255. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=372730&tool=pmcentrez&rendertype=abstract

Huang, K.-X., Badger, M., Haney, K., and Evans, S. L. (2007). Large scale production of *Bacillus thuringiensis* PS149B1 insecticidal proteins Cry34Ab1 and Cry35Ab1 from *Pseudomonas fluorescens*. *Protein Expression and Purification*, 53(2), 325-330.

JBCH. (2006a). *Outline of the Biological Diversity Risk Assessment Report: Maize resistant to Coleoptera and tolerant to glufosinate herbicide*. Retrieved from http://www.bch.biodic.go.jp/download/en_lmo/DAS_59122_7enRi.pdf

JBCH. (2006b). *Cotton resistant to Lepidoptera, and tolerant to glufosinate herbicide and glyphosate herbicide (cry1F, cry1Ac, pat, cp4 epsps,* Gossypium hirsutum *L.)(281 x 3006 x 1445, OECD UI: DAS-24236-5 x DAS-21023-5 x MON-1445-2)*. Tokyo.

Jones, G. W., Nielsen-Leroux, C., Yang, Y., Yuan, Z., Dumas, V. F., Monnerat, R. G., and Berry, C. (2007). A new Cry toxin with a unique two-component dependency from *Bacillus sphaericus*. *FASEB Journal*, 21(14), 4112-20. doi:10.1096/fj.07-8913com

Kaiser-Alexnat, R., Büchs, W., and Huber, J. (2009). Studies on the proteolytic processing and binding of Bt toxins Cry3Bb1 and Cry34Ab1/Cry35Ab1 in the midgut of Western corn rootworm (*Diabrotica virgifera virgifera* LeConte). *Insect Pathogens and Insect Parasitic Nematodes Bulletin*, 45, 235-238.

Keane, R. M., and Crawley, M. J. (2002). Exotic plant invasions and the enemy release hypothesis. *Trends in Ecology & Evolution*, 17(4), 164-170.

Kumar, A. P., Sharma, R. P., and Malik, V. S. (1996). The insecticidal proteins of *Bacillus thuringiensis*. *Advances in Applied Microbiology*, 42, 1-43.

Lee, D., and Natesan, E. (2006). Evaluating genetic containment strategies for transgenic plants. *Trends in Biotechnology*, 24(3), 109. Retrieved from http://www.sciencedirect.com/science/article/B6TCW-4J6WNXK-4/2/f6bf81ff4bce3a0ae-01d93e0566c5463

Mack, R. N. (1996). Predicting the identity and fate of plant invaders: Emergent and emerging approaches. *Biological Conservation*, 78, 107-121.

Malley, L. A., Everds, N. E., Reynolds, J., Mann, P. C., Lamb, I., Rood, T., Schmidt, J., *et al*. (2007). Subchronic feeding study of DAS-59122-7 maize grain in Sprague-Dawley rats. *Food and Chemical Toxicology*, 45(7), 1277-1292.

Mason, P., Braun, L., Warwick, S. I., Zhu, B., and Stewart, C. N. (2004). Transgenic Bt-producing *Brassica napus*: *Plutella xylostella* selection pressure and fitness of weedy relatives. *Environmental Biosafety Research*, 2(2003), 263-276. doi:10.1051/ebr

Masson, L., Schwab, G., Mazza, A., Brousseau, R., Potvin, L., and Schwartz, J.-L. (2004). A novel *Bacillus thuringiensis* (PS149B1) containing a Cry34Ab1/Cry35Ab1 binary toxin specific for the western corn rootworm *Diabrotica virgifera virgifera* LeConte forms ion channels in lipid membranes. *Biochemistry*, 43(38), 12349-12357.

Moellenbeck, D. J., Peters, M. L., Bing, J. W., Rouse, J. R., Higgins, L. S., Sims, L., Nevshemal, T., *et al*. (2001). Insecticidal proteins from *Bacillus thuringiensis* protect corn from corn rootworms. *Nature Biotechnology*, 19(7), 668-672. doi:10.1038/90282

Mondragon-Pichardo, J., and Vibrans, H. (2005). Ethnobotany of the Balsas teosinte. *Maydica*, 50, 123-128.

Murray, E. E., Lotzer, J., and Eberle, M. (1989). Codon usage in plant genes. *Nucleic Acids Research*, 17(2), 12-18.

Narva, K. E., Schnepf, H. E., Knuth, M., Pollard, M. R., Cardineau, G., and Schwab, G. E. (2000). Pesticidal Proteins. *United States Patent*. United States Patent Office.

OECD. (1992). *Recombinant DNA Safety Considerations. Assessment*. Paris: Organization for Economic Cooperation and Development. Retrieved from http://www.oecd.org/LongAbstract/0,3425,en_2649_34537_40986856_1_1_1_1,00.html

OECD. (2003). *Consensus Document on the Biology of* Zea mays *subsp.* mays *(Maize). Development*. Paris: OECD Biosafety Publications. Retrieved from http://www.oecd.org/officialdocuments/displaydocumentpdf?cote=env/jm/mono(2003)11&doclanguage=en

OECD. (2007). *Consensus Document on Safety Information on Transgenic Plants Expressing* Bacillus thuringiensis - *Derived Insect Control Proteins. North*. Paris.

Oppert, B., Ellis, R. T., and Babcock, J. (2010). Effects of Cry1F and Cry34Ab1/35Ab1 on storage pests. *Journal of Stored Products Research*, 46(3), 143-148. Elsevier Ltd. doi:10.1016/j.jspr.2010.01.003

Pigott, C. R., and Ellar, D. J. (2007). Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiology and Molecular Biology Reviews*, 71(2), 255-81. doi:10.1128/MMBR.00034-06

Raybould, A. (2006). Problem formulation and hypothesis testing for environmental risk assessment of genetically modified crops. *Environmental Biosafety Research*, 5, 119-126. doi:10.1051/ebr

Raybould, A., Higgins, L. S., Horak, M. J., Layton, R. J., Storer, N. P., De La Fuente, J. M., and Herman, R. A. (2011). Assessing the ecological risks from the persistence and spread of feral populations of insect-resistant transgenic maize. *Transgenic Research*, 21(3), 655-664. doi:10.1007/s11248-011-9560-4

Romeis, J., Bartsch, D., Bigler, F., Candolfi, M. P., Gielkens, M. M. C., Hartley, S. E., Hellmich, R. L., *et al*. (2008). Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. *Nature Biotechnology*, 26(2), 203-208. Nature Publishing Group. Retrieved from http://dx.doi.org/10.1038/nbt1381

Romeis, J., Raybould, A., Bigler, F., Candolfi, M. P., Hellmich, R. L., Huesing, J. E., and Shelton, A. M. (2013). Deriving criteria to select arthropod species for laboratory tests to assess the ecological risks from cultivating arthropod-resistant genetically engineered crops. *Chemosphere*, 90(3), 901-909. Elsevier Ltd. doi:10.1016/j.chemosphere.2012.09.035

Sanvido, O., Romeis, J., Gathmann, A., Gielkens, M., Raybould, A., and Bigler, F. (2012). Evaluating environmental risks of genetically modified crops: ecological harm criteria for regulatory decision-making. *Environmental Science & Policy*, 15(1), 82-91. doi:10.1016/j.envsci.2011.08.006

Schnepf, H. E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R., *et al*. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews*, 62(3), 775-806. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=98935&tool=pmcentrez&rendertype=abstract

Schnepf, H. E., Lee, S., Dojillo, J., Burmeister, P., Fencil, K., Morera, L., Nygaard, L., *et al*. (2005). Characterization of Cry34/Cry35 binary insecticidal proteins from diverse *Bacillus thuringiensis* strain collections. *Applied and Environmental Microbiology*, 71(4), 1765-1774. American Society for Microbiology.

Storer, N. P., Babcock, J. M., and Edwards, J. M. (2006). Field measures of western corn rootworm (Coleoptera: Chrysomelidae) mortality caused by Cry34/35Ab1 proteins expressed in maize event 59122 and implications for trait durability. *Journal of Economic Entomology*, 99(4), 1381-1387.

Tabashnik, B. E. (1992). Evaluation of synergism among *Bacillus thuringiensis* toxins. *Applied and Environmental Microbiology*, 58(10), 3343-3346. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=183101&tool=pmcentrez&rendertype=abstract

USDA. (2004). A*pplication for the Determination of Nonregulated Status for B.t. Cry34/35Ab1 Insect-Resistant Glufosinate-Tolerant Corn: Corn Line 59122*. Retrieved from http://www.aphis.usda.gov/brs/aphisdocs/03_35301p.pdf

USDA. (2005). *Approval of Mycogen Seeds/Dow AgroSciences LLC and Pioneer Hi-Bred International, Inc. (Request 03-353-01p) Seeking a Determination of Nonregulated Status for Bt Cry34Ab1/35Ab1 Insect Resistant, Glufosinate Tolerant Corn Line 59122-7*. Washington, DC. Retrieved from http://www.aphis.usda.gov/brs/aphisdocs2/03_35301p_com.pdf

USEPA. (1998). *Reregistration Eligibility Decision (RED)* Bacillus thuringiensis. Washington, DC.

USEPA. (2005a). Bacillus thuringiensis *Cry34Ab1 and Cry35Ab1 Proteins and the Genetic Material Necessary for Their Production in Event DAS-59122-7 Corn*. Retrieved from http://www.docstoc.com/docs/7874905/Bt-corn-Event-DAS-59262-7-with-Cry34Ab1--Cry35Ab1-(Herculex-RW)-(PDF)

USEPA. (2005b). *Pesticides: Regulating Pesticides Bacillus thuringiensis Cry-34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (plasmid insert PHP 17662) in Event DAS-59122-7 corn.* Washington DC. Retrieved from http://www.epa.gov/oppbppd1/biopesticides/ingredients_keep/factsheets/factsheet_006490.htm

USEPA. (2007). *White Paper on Tier-Based Testing for the Effects of Proteinaceous Insecticidal Plant-Incorporated Protectants on Non-Target Arthropods for Regulatory Risk Assessments. Environmental Protection* (p. 36). Washington DC. Retrieved from http://www.epa.gov/pesticides/biopesticides/pips/non-target-arthropods.pdf

USEPA. (2010). Bacillus thuringiensis *Cry34Ab1 and Cry35Ab1 Proteins and the Genetic Material Necessary for Their Production (PHP17662 T-DNA) in Event DAS-59122-7 Corn (OECD Unique Identifier: DAS-59122-7)*. Retrieved from http://www.epa.gov/oppbppd1/biopesticides/pips/cry3435ab1-brad.pdf

USEPA. (2011). Harmonized Test Guidelines. Retrieved from http://epa.gov/ocspp/pubs/frs/home/guidelin.htm

USFDA. (2004). *Biotechnology Consultation Note to the File BNF NO. 000081*. Retrieved from http://www.fda.gov/Food/Biotechnology/Submissions/ucm155622.htm

Vachon, V., Laprade, R., and Schwartz, J.-L. (2012). Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: A critical review. *Journal of Invertebrate Pathology*, 111(1), 1-12. Elsevier Inc. doi:10.1016/j.jip.2012.05.001

Van Frankenhuyzen, K. (2009). Insecticidal activity of *Bacillus thuringiensis* crystal proteins. *Journal of Invertebrate Pathology*, 101(1), 1-16. doi:10.1016/j.jip.2009.02.009

ANNEX I: SUMMARY OF CRY34Ab1/CRY35Ab1 EXPRESSION DATA

The tables that follow present summary data from decision documents prepared by regulatory authorities, and regulatory submissions. Whenever possible, the data and accompanying statistics are presented as they appeared in the cited document to facilitate cross-referencing. Additional information on collection and sampling methodologies can be found in the referenced sources.

Table I.1. Maximum and minimum mean expression levels of Cry34Ab1 and Cry35Ab1 in DAS-59122-7 corn

	Minimum Mean ng/mg tissue dry weight	Maximum Mean ng/mg tissue dry weight
Cry34Ab1	29.2 (sprayed* hybrid stalk)	232 (sprayed hybrid leaf)
Cry35Ab1	0.01 (sprayed hybrid pollen)	85.3 (non-sprayed hybrid leaf)

* "sprayed" treatments received two sequential applications of glufosinate ammonium herbicide.

Table I.2. Expression levels in grain (ng/mg tissue dry weight)(EFSA, 2008, 2009a, 2009b)

	59122 x NK603[1]		59122 x 1507[2]		59122 x 1507 x NK603		59122	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Cry34Ab1	52	34 – 76	52	28 – 88	48	23 – 70	41	30 – 51
Cry35Ab1	2.8	2.1 – 3.7	2.3	1.7 – 3.1	2.1	1.4– 2.9	2.2	1.3 – 3.2

1 Parental line NK603 conferred glyphosate tolerance (CP4 EPSPS).
2 Parental line 1507 conferred glufosinate tolerance and insect resistance (PAT and Cry1F).

Table I.3. Summary of expression levels of Cry34Ab1 protein in DAS-59122-7 corn grain harvested at maturity

	Mean (ng/mg dry dry weight)	Standard Deviation	Range (ng/mg dry weight)[1]	Number of Samples[2]
Non-GM control	0	0	0-0	6/6
GM hybrid unsprayed	49.7	16.2	28.9-84.8	30/0
GM hybrid sprayed	61.1	19.4	30.9-117	30/0
GM inbred	51.7	11.5	38.6-78.3	15/0

1 The limit of quantitation (LOQ) for Cry34Ab1 for grain was 0.072 ng/mg dry weight.
2 Number of samples = the number samples analyzed/the number of samples below the LOQ.

Table I.4. Summary of expression levels of Cry35Ab1 protein in DAS-59122-7 corn grain harvested at maturity

	Mean (ng/mg dry dry weight)	Standard Deviation	Range (ng/mg dry weight)[1]	Number of Samples[2]
Non-GM control	0	0	0-0	6/6
GM hybrid unsprayed	0.99	0.33	0.48-1.58	30/0
GM hybrid sprayed	0.92	0.30	0.50-1.61	30/0
GM inbred	1.10	0.54	0-1.83	15/2

1 The limit of quantitation (LOQ) for Cry34Ab1 for grain was 0.072 ng/mg dry weight.
2 Number of samples = the number samples analyzed/the number of samples below the LOQ.

Table I.5. Mean expression levels of Cry34Ab1 and Cry35Ab1 in tissues from a hybrid maize line containing event DAS-59122-7 (USEPA, 2005a, 2010)

Tissue	Cry34Ab1 (ng/mg tissue dry weight ± standard deviation)*	Cry35Ab1 (ng/mg tissue dry weight ± standard deviation)*
Leaf	50±8 - 220±38	41±7 - 85±19
Root	37±9 - 50±20	3±2 - 8±8
Whole Plant	32±16 - 77±10	7±2 - 14±2
Pollen	74±7	0.02±0.04
Stalk	33±4	10±2
Forage	53±10	12±3
Grain	50±16	1±0.3

* Ranges reflect the range of means at different growth stages.

Table I.6. Summary of expression levels of Cry34Ab1 protein measured in tissues collected from corn hybrid 59122 (event DAS-59122-7) (USDA, 2004)

Growth Stage	Tissue	Mean of Cry34Ab1 (ng/mg Tissue Dry Weight)	Standard Deviation	Range of Cry34Ab1 (ng/mg Tissue Dry Weight)[1]	Number of Samples[2]
V9[3]	Leaf	49.5	7.79	37.0 - 81.4	30
	Root	38.8	8.28	24.6 - 56.3	18
	Whole Plant	31.5	15.5	8.67 - 51.9	6
R1[4]	Leaf	80.6	12.4	59.1 - 103	30
	Pollen	74.4	6.57	62.9 - 87.2	30
	Stalk	32.9	4.14	25 - 40.6	30
	Root	36.8	8.54	23.3 - 52.1	30
	Whole Plant	45.4	13.5	35 - 71.9	6
R4[5]	Leaf	220	37.5	143 - 302	18
	Root	49.1	9.23	33.3 - 67.3	18
	Forage	53.1	19.1	30.5 - 82.6	6
Maturity[6]	Grain	49.7	16.2	28.9 -- 84.8	30
R6[7]	Leaf	163	83.6	4.26 - 296	18
	Root	49.7	19.6	25.7 - 102	18
	Whole Plant	76.5	10.3	60.5 - 88	6

1 Sample limit of quantitation (LOQ) for Cry34Ab1: 0.18 ng/mg dry weight for leaf; 0.26 ng/mg dry weight for pollen; 0.12 ng/mg dry weight for stalk; 0.99 ng/mg dry weight for root; and 0.072 ng/mg dry weight for grain and whole plant tissues.
2 All samples measured were above LOQ
3 Growth stage when the collar of the fourth leaf becomes visible.
4 Growth stage when silks become visible.
5 Growth stage when the material within the kernel produces a doughy consistency. This stage can occur as early as 24 days after pollination.
6 Typical harvest maturity for grain.
7 Maturity, the typical harvest maturity for grain.

Table I.7. Summary of expression levels of Cry35Ab1 protein measured in tissues collected from corn hybrid 59122 (event DAS-59122-7) (USDA, 2004)

Growth Stage	Tissue	Mean of Cry34Ab1 (ng/mg Tissue Dry Weight)	Standard Deviation	Range of Cry34Ab1 (ng/mg Tissue Dry Weight)[1]	Number of Samples[2]
V9[3]	Leaf	40.7	7.29	29.7 – 55.1	30
	Root	8.06	2.98	4.08 – 15.4	18
	Whole Plant	7.36	2.19	4.13 – 10.1	6
R1[4]	Leaf	52.2	12.9	29.2 – 80.8	30
	Pollen	0.02	0.04	0 – 0.15	30
	Stalk	10.0	2.26	5.64 – 14.2	30
	Root	5.08	1.57	2.49 – 8.85	30
	Whole Plant	12.3	3.54	9.02 – 18.1	6
R4[5]	Leaf	85.3	18.9	61.1 – 126	18
	Root	3.50	0.85	1.74 – 5.76	18
	Forage	12.4	2.77	8.44 – 16.4	6
Maturity[6]	Grain	0.99	0.33	0.48 – 1.58	30
R6[7]	Leaf	54.4	22.2	1.41 – 77.3	18
	Root	3.10	2.43	0.72 – 10.6	18
	Whole Plant	13.9	1.91	10.7 – 16.4	6

1 Sample limit of quantitation (LOQ) for Cry34Ab1: 0.18 ng/mg dry weight for leaf; 0.26 ng/mg dry weight for pollen; 0.12 ng/mg dry weight for stalk; 0.99 ng/mg dry weight for root; and 0.072 ng/mg dry weight for grain and whole plant tissues.
2 All samples measured were above LOQ
3 Growth stage when the collar of the fourth leaf becomes visible.
4 Growth stage when silks become visible.
5 Growth stage when the material within the kernel produces a doughy consistency. This stage can occur as early as 24 days after pollination.
6 Typical harvest maturity for grain.
7 Maturity, the typical harvest maturity for grain.

Table I.8. High-end exposure estimates (HEEE)[1] for expression of Cry34/35Ab1 proteins. The HEEE are based upon expression values obtained from corn plants not sprayed with glufosinate-ammonium and plants sprayed with glufosinate-ammonium. (USDA, 2004)

	Cry34Ab1				Cry35Ab1				Sum of Cry34 and Cry35
Tissue (Growth Stage)	**Mean**	**Std. Dev.**	**HEEE[a]**	**N**	**Mean**	**Std. Dev.**	**HEEE**	**N**	**HEEE**
Leaf (V9)	45.93	7.56	47.19	60	36.27	9.07	37.79	60	84.98
Whole Plant (V9)	39.41	22.76	48.31	12	7.75	1.92	8.50	12	56.81
Root (V9)	38.48	8.72	40.37	36	8.05	2.80	8.65	36	49.02
Pollen (R1)	74.27	6.09	75.29	60	0.02	0.03	0.03	60	75.32
Stalk (R1)	31.03	4.62	31.81	60	8.64	2.54	9.06	60	40.87
Root (R1)	40.58	10.43	42.32	60	5.24	1.58	5.50	60	47.83
Leaf (R1)	76.24	1.257	78.34	60	54.51	14.30	56.91	60	135.25
Whole Plant (R1)	46.48	14.23	52.05	12	12.66	4.27	14.34	12	66.38
Forage (R4)	46.58	24.23	56.06	12	13.25	2.89	14.38	12	70.44
Root (R4)	57.98	22.00	62.75	36	3.70	1.02	3.93	36	66.68
Leaf (R4)	226.44	39.52	235.03	36	83.01	18.39	87.00	36	322.03
Grain (R6, Harvest)	55.39	18.3	58.51	60	0.95	0.31	1.01	60	59.52
Whole Plant, Including Grain (R6, Senescence)	86.30	18.01	93.35	12	15.38	2.93	16.52	12	109.87
Whole Plant, Excluding Grain (R6, Senescence)			109.03[2]						132.53[3]
Root (R6, Senescence)	54.04	20.83	58.56	36	3.33	2.18	3.80	36	62.37
Leaf (R6, Senescence)	149.52	81.93	167.31	36	53.20	23.26	58.25	36	225.57

1 High-end exposure estimate = Mean + ($t_{0.1,\ \text{upper tail},\ n-1}$ x Std. Dev.)/$n^{1/2}$.
2 Grain g dw/A = ((235 bu/A – 235 bu/A x (1-0.85 dry weight)) x 56 lb/bu) x 1 g/0.002205 lb = 5073016. Stover g dw/A = Grain g dw/A x 1/0.45 = 11273369. Protein in stover µg/g dw = (Protein in Whole Plant, Including Grain (R6, Senescence) x Sum of Grain+Stover g dw/A. Protein in Grain (R6, Harvest) x Grain g dw/A) x 1/Stover g dw/A = (93.35 x 16346384 – 58.51 x 5073016) x 1/11273369 = 109.03.
3 (109.87 x 16346384 – 59.52 x 5073016) x 1/11273369 = 132.53.

Table I.9. Average Cry34Ab1 and Cry35Ab1 protein produced in DAS-59122-7 corn (CFIA, 2005b)

	Leaf, all growth stages	**Root, all growth stages**	**Stalk**	**Grain**	**Pollen**	**Forage**
Cry34Ab1	54.9-266.4[1]	35.4-43.7	49	36.4	64.7	97.7
Cry35Ab1	23.3-97.1	5.3-15.5	19.3	2.0	0.06	28.1

1 All values expressed as nanograms protein per milligram dry weight tissue.

ANNEX II: SUMMARY OF COMPOSITIONAL ANALYSES OF GE MAIZE EXPRESSION CRY34Ab1 AND CRY35Ab1

Table II.1. Summary of proximate and fiber analysis in DAS-59122-7 corn grain (across 6 sites) (FSANZ, 2005)

Analyte[1]	Literature Range	Means[2]		
		DAS-59122-7 unsprayed	DAS-59122-7 sprayed	Control
Crude protein	6-16.1	10.0*	10.3*	9.61
Crude fat	1.2-18.8	4.69	4.62	4.49
Crude fibre	1.6-5.5	2.3	2.2	2.3
Acid detergent fibre	1.82-11.3	3.5	3.6	3.5
Neutral detergent fibre	3.0-22.6	10.8	11.2*	10.3
Ash	0.62-6.28	1.55*	1.6*	1.42
Carbohydrate[3]	63.3-89.8	83.8	83.5*	84.5

1 Percent dry weight
2 Least square means
3 Carbohydrate=100% - % protein -% fat -% ash
* Statistically significant difference between DAS-59122-7 grain and Control grain (P<0.05)

Table II.2. Summary of proximates and fiber analysis for DAS-59122-7 and control forage (across 6 sites) (FSANZ, 2005)

Analyte[1]	Literature Range	Means[2]		
		DAS-59122-7	Control	Standard Error
Crude protein	3.14 - 15.9	6.45	6.27	0.097
Crude fat	0.37 - 6.7	2.73	2.68	0.068
Crude fiber	19 - 42	24.0	23.7	0.237
Acid detergent fiber	16.1 - 41.0	31.7	31.1	0.363
Neutral detergent fiber	20.3 - 63.7	49.4	49.4	0.388
Ash	1.3 - 10.5	5.60*	5.13	0.103
Carbohydrate[3]	66.9 - 94.5	85.2*	85.9	0.210

1 Percent dry weight
2 Least square means
3 Carbohydrate=100% - % protein -% fat -% ash
* Statistically significant difference between DAS-59122-7 grain and Control grain (P<0.05)

Table II.3. Summary of mineral analysis of DAS-59122-7 corn grain (across 6 sites) (FSANZ, 2005)

Analyte[1]	Literature Range	Means[2]		
		DAS-59122-7 unsprayed	DAS-59122-7 sprayed	Control
Calcium	0.002-0.1	0.00278*	0.00286*	0.00227
Phosphorus	0.21-0.75	0.299	0.308*	0.266
Copper	0.000085-0.001	0.000112	0.000104	0.000118
Iron	0.0001-0.01	0.00199	0.00225	0.00194
Magnesium	0.08-1.0	0.117	0.123	0.108
Manganese	0.00007-0.0054	0.000648	0.000686*	0.000577
Potassium	0.28-0.72	0.352	0.362	0.332
Sodium	0.0-0.15	0.000427	0.000367	0.000378
Zinc	0.00065-0.0037	0.00183	0.00179	0.00163

1 Percent dry weight
2 Least square means
* Statistically significant difference between DAS-59122-7 grain and Control grain (P<0.05)

Table II.4. Summary of fatty acid analysis of DAS-59122-7 corn grain (across 6 sites) (FSANZ, 2005)

Analyte[1]	Literature Range	Means[2]		
		DAS-59122-7 unsprayed	DAS-59122-7 sprayed	Control
Palmitic acid	6.51-19	11.5*	11.7	12.1
Stearic acid	0-4.17	1.39*	1.40*	1.57
Oleic acid	18.6-46	22.8	23.1	23.3
Linoleic acid	34-70	63.0*	62.4	61.7
Linolenic acid	0-2.0	1.14	1.15*	1.07

1 Percent dry weight
2 Least square means
* Statistically significant difference between DAS-59122-7 grain and Control grain (P<0.05)

Table II.5. Summary of amino acid analysis in DAS-59122-7 corn grain (across 6 sites) (FSANZ, 2005)

Analyte[1]	Literature Range	Means[2]		
		DAS-59122-7 unsprayed	DAS-59122-7 sprayed	Control
Methionine	0.1-0.46	0.20	0.19	0.19
Cysteine	0.08-0.32	0.23	0.22	0.22
Lysine	0.05-0.55	0.28	0.29	0.28
Tryptophan	0.04-0.13	0.06*	0.06	0.06
Threonine	0.21-0.58	0.38	0.41*	0.37
Isoleucine	0.19-.071	0.34*	0.35*	0.33
Histidine	0.15-0.40	0.26*	0.28*	0.25
Valine	0.21-0.85	0.46*	0.48*	0.45
Leucine	0.43-2.41	1.33*	1.38*	1.28
Arginine	0.22-0.64	0.29*	0.30*	0.28
Phenylalanine	0.04-0.83	0.56*	0.59*	0.54
Glycine	0.24-0.50	0.35	0.36*	0.33
Alanine	0.37-1.20	0.82	0.83*	0.80
Aspartic acid	0.37-0.95	0.69	0.70*	0.66
Glutamic acid	0.89-3.04	2.03	2.08*	1.97
Proline	0.43-1.46	0.96*	0.98*	0.91
Serine	0.24-0.91	0.51	0.54*	0.50
Tyrosine	0.11-0.79	0.24*	0.26*	0.21

1 Percent dry weight
2 Least square means
* Statistically significant difference between DAS-59122-7 grain and Control grain (P<0.05)

Table II.6. Summary of vitamin analysis of DAS-59122-7 corn grain (across 6 sites) (FSANZ, 2005)

Analyte[1]	Literature Range	Means[2]		
		DAS-59122-7 unsprayed	DAS-59122-7 sprayed	Control
Beta-carotene	1.0, 2.5[3]	7.62	7.74	6.87
Vitamin B1	1.0-8.6	5.45	5.93	5.77
Vitamin B2	0.25-16.5	ND[4]	ND	ND
Folic acid	0.147-1.209	0.593*	0.603	0.634
Vitamin E[5]	1.5-6.87	6.59*	6.60*	5.65

1 Percent dry weight
2 Least square means
3 Only 2 reference values were available
4 ND=not detected
5 Measured as α-tocopherol
* Statistically significant difference between DAS-59122-7 grain and Control grain (P<0.05)

Table II.7. Summary of secondary metabolites and anti-nutrients of DAS-59122-7 corn grain (across 6 sites) (FSANZ, 2005)

Analyte[1]	Literature Range	Means[2]		
		DAS-59122-7 unsprayed	DAS-59122-7 sprayed	Control
Secondary metabolites				
Inositol	NR[3]	0.022	0.022	0.021
Raffinose	0.08-0.31	0.13	0.13	0.12
Furfural	NR	ND[4]	ND	ND
P-Coumaric acid	0.003-0.058	0.014	0.014	0.015
Ferulic acid	0.02-0.37	0.177	0.176	0.182
Antinutrients				
Phytic acid	0.29-1.29	0.877	0.798	0.798
Trypsin inhibitor (TIU/g)	1.1-7.18	2.82	2.84	2.84

1 Percent dry weight
2 Least square means
3 NR=not reported
4 ND=not detected