Safety Assessment of YieldGard® Insect-Protected Corn Event MON 810

Executive Summary

Using modern biotechnology, Monsanto has developed insect-protected YieldGard® corn, event MON 810, that produces the naturally occurring *Bacillus thuringiensis* (*Bt*) protein, Cry1Ab. YieldGard corn is protected from feeding damage by the European corn borer (ECB, *Ostrinia nubilalis*), the southwestern corn borer (SWCB, *Diatraea grandiosella*) and the pink borer (*Sesamia cretica*).

The Cry1Ab protein produced in YieldGard corn binds to specific receptors in the midgut of sensitive insects, but does not affect mammals or insects that lack those receptors. Therefore, the Cry1Ab protein has selective toxicity to specific lepidopteran insects but is harmless to humans, fish, wildlife and beneficial insects that can help control other pests. *Bt* proteins have been used safely for nearly 40 years in microbial insecticides.

The safety of the Cry1Ab protein in YieldGard corn has been thoroughly evaluated. These tests confirm that the protein is present at very low levels in the grain and food; is rapidly degraded in simulated gastric fluids; shows no similarity to known allergens; and shows no harmful effects to animals when fed at very high levels.

Compositional analyses of the grain and forage of YieldGard corn grown in various environments over several years show that the levels of the key nutrients in YieldGard corn are comparable to the levels found in conventional corn hybrids. In addition, the safety of the feed produced from YieldGard corn has been confirmed through animal feeding trials. These studies show that animals perform in a comparable manner when fed biotechnology-derived and conventional corn products. YieldGard corn plants are equivalent to other corn varieties in disease susceptibility and other agronomic and morphological characteristics. YieldGard corn improves grain quality by reducing insect damage in ears, one of the main pathways by which mold infects grain, and reduces losses caused by some grain pests during storage.

The environmental assessment shows that YieldGard corn does not harm agriculturally beneficial insects, including honeybees, ladybird beetles, green lacewings or predatory insects and spiders. YieldGard corn is unlikely to have any significant impact on Monarch butterfly populations due to limited exposure**.** In addition, the Cry1Ab protein produced in YieldGard corn is rapidly degraded in soil and has no effect on soil invertebrates, including earthworms and collembola.

These studies demonstrate that the Cry1Ab protein is safe to humans, animals, non-target organisms and beneficial insects, and that the forage and grain of YieldGard corn plants are as safe and nutritious as conventional corn varieties.

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Introduction

Using modern biotechnology, Monsanto has developed insect-protected YieldGard® corn, event MON 810, that produces the naturally occurring *Bacillus thuringiensis* (*Bt*) protein, Cry1Ab. YieldGard corn is protected from feeding damage by the European corn borer (ECB, *Ostrinia nubilalis*), the southwestern corn borer (SWCB, *Diatraea grandiosella*) and the pink borer (*Sesamia cretica*).

European corn borer (ECB), *Ostrinia nubilalis* (Hubner), is an economically important pest of corn that has spread throughout the major corn-growing regions of the United States and Europe (Dicke and Guthrie, 1988). This insect typically produces one to three generations per year. Insect damage to the corn plant includes leaf feeding by the first generation; stalk tunneling by the first and second generations; and leaf sheath, collar feeding and ear damage by the second and third generations. Chemical control is ineffective once the borers have tunneled into the plant due to the inaccessibility of the chemical to the insect pest. Yield losses due to ECB damage are estimated to be 3 to 7% per borer per plant, which causes annual losses from $37 to $172 per hectare of corn (Sanders *et al.*, 1998).

The benefits of planting insect-protected corn include: 1) a reliable means to control these corn pests; 2) control of target insects while maintaining beneficial species; 3) reduced use of chemical insecticides (Rice and Pilcher, 1999); 4) reduced applicator exposure to chemical pesticides; 5) fit with integrated pest management (IPM) and sustainable agricultural systems; 6) reduced fumonisin mycotoxin levels in corn kernels (Munkvold *et al.*, 1999; Masoero *et al.*, 1999); and 7) no additional labor or machinery requirements, allowing both large and small growers to maximize hybrid yields.

The development of corn transformation methodology (Fromm *et al.*, 1990) created the opportunity to protect corn plants from insect feeding damage using genes isolated from the bacterium *Bacillus thuringiensis*. The *cry1Ab* coding sequence (Höfte and Whiteley, 1989) was isolated from the *Bacillus thuringiensis* var. *kurstaki (Btk)* HD-1 strain present in DIPEL®, the leading microbial insecticide in agricultural use. Several laboratories have developed transgenic corn plants producing proteins of the Cry1A class in corn (Hill *et al.*, 1995; Armstrong *et al.*, 1995). YieldGard insect-protected corn varieties derived from event MON 810 were commercialized in the United States in 1997, and since then, more than 32 million acres of these varieties have been planted in the United States.

The food, feed and environmental safety assessment data generated for YieldGard corn event MON 810 have been used to obtain the necessary regulatory approvals in key corn producing countries and in countries likely to import insect-protected corn. These food, feed, and environmental safety assessments include: product characterization consisting of molecular analysis of the inserted DNA, protein characterization and determination of protein levels in corn tissues; protein safety evaluation; compositional analysis of food components to establish substantial equivalence to commercial varieties; and environmental assessment to assure that there will be no deleterious effects on the environment (Sanders *et al.*, 1998). In addition to the studies conducted by Monsanto,

numerous peer-reviewed articles have been published which support the a conclusion of minimal risk for YieldGard corn varieties containing the Cry1Ab protein (Pilcher *et al.,* 1997; Aulrich *et al.,* 1998; Daenicke *et al.,* 1999).

Molecular Characterization of YieldGard Corn

YieldGard insect-protected corn event MON 810 was produced by microprojectile bombardment of embryogenic corn tissue (Armstrong *et al.,* 1991) with plasmid PV-ZMBK07 (Figure 1). The PV-ZMBK07 plasmid contains the *cry1Ab* coding sequence, which expresses the insecticidally active Cry1Ab protein. The *cry1Ab* coding sequence from *Bacillus thuringiensis* subsp. HD-1 (Fischhoff *et al.,* 1987) was modified to increase the levels of the Cry1Ab protein in plants. The enhanced cauliflower mosaic virus (CaMV) 35S promoter (Kay *et al.,* 1987; Odell *et al.,* 1985) and hsp70 maize intron (Rochester *et al.,* 1986) regulate the expression of the *cry1Ab* coding sequence. The 3' nontranslated region of the nopaline synthase (NOS) gene, isolated from the Ti plasmid of *Agrobacterium tumefaciens*, terminates transcription and directs polyadenylation of the messenger RNA (mRNA) (Fraley *et al.,* 1983). The plasmid also contains the neomycin phosphotransferase (*nptII*) coding sequence (Beck *et al.,* 1982), that encodes a bacterial selectable marker that was used to identify transformed corn cells during the development process.

Southern blot analysis of corn event MON 810 demonstrated that a single functional copy of the *cry1Ab* coding sequence was integrated into the corn genome. The *nptII* coding sequence was not integrated during transformation. The *cry1Ab* coding sequence is inherited in the expected Mendelian pattern and is transmitted through pollen, which demonstrates stable integration into the nuclear genome. The integrity of the insert has been maintained during extensive breeding into commercial corn hybrids.

Cry1Ab Protein Levels in YieldGard Corn Plants

Levels of the Cry1Ab protein in various corn tissues were estimated to define the amount of active ingredient present in MON 810 seed; to calculate expected exposure levels to nontarget organisms and humans; to support the effective dose insect resistance management strategy; and to demonstrate stability of the encoded protein during breeding. Cry1Ab protein levels were measured on samples from four different field trials: 1994 and 1995 trials in the U.S. and 1995 and 1996 trials in Europe. A direct double antibody sandwich enzyme-linked immunosorbent assay, ELISA, was developed and validated to quantify the levels of Cry1Ab protein in various plant tissues. The Cry1Ab protein levels in tissues collected from plants of YieldGard corn event MON 810 have been consistent across several years of evaluation in the U.S. and Europe (Table 1). The consistency of Cry1Ab protein levels through years of breeding supports the stability of the insert, an important component of product performance. The Cry1Ab protein levels are sufficient to provide effective protection from both first and second generation ECB feeding damage throughout the growing season (Gianessi and Carpenter, 1999).

Table 1. Cry1Ab Protein Levels in YieldGard Corn Plants (µg/g fwt tissue)

Plant tissue	Parameter	1994 US[1] (6 sites)	1995 US (5 sites)	1995 EU[1] (4 sites)	1996 EU (3 sites)
Leaf[2]	Mean	9.35	8.95	8.60	12.15
	Std. Dev.	1.03	2.17	0.74	3.86
	Range	7.93-10.34	5.21-10.61	7.59-9.39	7.77-15.06
Forage/ whole plant[3]	Mean	4.15	3.34	4.80	4.88
	Std. Dev.	0.71	1.09	0.75	0.52
	Range	3.65-4.65	2.31-4.48	4.11-5.56	4.32-5.34
Grain[2]	Mean	0.31	0.57	0.53	0.41
	Std. Dev.	0.09	0.21	0.12	0.06
	Range	0.19-0.39	0.39-0.91	0.42-0.69	0.35-0.46
Overseason leaf[4]					
(1st)	Mean	9.78			
(2nd)	Mean	8.43			
(3rd)	Mean	4.91			

[1]US is United States; EU is European Union.
[2]The mean was calculated from the analyses of plant samples from each field site.
[3]For the 1994 US trials, values represent the analysis of whole plants; for the remaining trials, values represent the analysis of forage tissue. Whole plants were collected two weeks after pollination; forage samples were collected at the soft dough or early dent stage. Means were determined from the analysis of plant samples from one site in the US and all sites in the EU. A plant sample was a pool of two individual plants.
[4]Mean of a pooled leaf sample collected at two week intervals, from V4 stage until pollination, at one site.

Safety Assessment of the Cry1Ab Protein in YieldGard Corn

Safety assessment of the Cry1Ab protein includes protein characterization, digestion in simulated gastric and intestinal fluids, acute oral toxicity in mice, amino acid sequence comparison to known toxins and allergens, and effects on nontarget organisms. Due to the extremely low levels of Cry1Ab protein produced in corn, it was necessary to produce sufficient quantities of Cry1Ab protein by bacterial fermentation in *E. coli* to conduct safety studies. The physicochemical and functional equivalence of the protein produced in *E. coli* with that produced in YieldGard corn has been demonstrated, justifying the use of the *E. coli*-produced Cry1Ab protein in safety assessment studies (Lee *et al.,* 1995).

Cry1Ab Protein Mode of Action and Specificity

The mode of action of *Bacillus thuringiensis* delta-endotoxins such as the Cry1Ab protein have been studied extensively and reviewed (Gill *et al.,* 1992; English and Slatin, 1992; Yamamoto and Powell, 1993; Knowles, 1994; Dean *et al.,* 1996). The Cry1Ab protein must be ingested by the susceptible insect to produce an insecticidal effect (Huber and Lüthy, 1981). Following ingestion, Cry1-type toxins (mol. wt. ~134 kD) are solubilized and proteolytically processed to the active toxic core protein (mol. wt. ~63 kD). After

traversing the insect midgut peritrophic membrane, Cry1-type toxins selectively bind to specific receptors localized on the brush border midgut epithelium (Hofmann *et al.*, 1988a and b). Cation-specific pores are formed, disrupting midgut ion flow, which cause gut paralysis and death of the susceptible insect.

The Cry1Ab protein is insecticidal only to Lepidopteran insects. Thus, in a host range study it was determined that seven of eighteen insects species were sensitive to the Cry1Ab and Cry1Ac proteins, with all seven insect species being lepidopterans (Macintosh *et al.*, 1990). This specificity is directly attributable to the presence of Cry1A specific receptors in the target insects (Hofmann *et al.*, 1988a; Van Rie *et al.*, 1990).

There are no receptors for the protein delta-endotoxins of *Bacillus thuringiensis* subspecies on the surface of mammalian intestinal cells; therefore, humans are not susceptible to these proteins (Sacchi *et al.*, 1986; Hofmann *et al.*, 1988b; Noteborn *et al.*, 1995). In addition to the lack of receptors for the *Bt* proteins, the absence of adverse effects in humans is further supported by numerous reviews on the safety of the *Bt* proteins and the long history of safe use of microbial *Bt* products (Ignoffo, 1973; Shadduck, 1983; Siegel and Shadduck, 1989; McClintock *et al.*, 1995).

Digestion of Cry1Ab Protein in Simulated Gastric and Intestinal Fluids
The trypsin-resistant core of the Cry1Ab protein (mol. wt. ~63 kD) was used in the simulated digestion study because this is the insecticidally active form of the Cry1Ab protein (Huber and Lüthy, 1981). In gastric fluid, which contains the enzyme pepsin, the Cry1Ab protein is degraded rapidly; thus, more than 90% of the Cry1Ab protein is degraded within two minutes of incubation in simulated gastric fluids, as assessed by western blot analysis. Cry1Ab protein bioactivity, as measured by insect bioassay, also dissipated readily; 74 to 90% of the Cry1Ab activity dissipated within two minutes of incubation in simulated gastric fluids, the earliest time point measured. To put the rapid degradation of the Cry1Ab protein in the simulated gastric system into perspective, approximately 50% of solid food has been estimated to empty from the human stomach within two hours, while liquid empties in approximately 25 minutes (Sleisenger and Fordtran, 1989). In intestinal fluid, which contains trypsin and other proteases, the Cry1Ab trypsin-resistant core protein did not degrade substantially after 19.5 hours of incubation as expected, as assessed by both western blot analysis and insect bioassay. The tryptic core of this and other *Bacillus thuringiensis* insecticidal proteins are widely known to be relatively resistant to digestion by serine proteases like trypsin, a major protease in intestinal fluid (Bietlot *et al.*, 1989).

Lack of Acute Oral toxicity in Mice
An acute oral toxicity study was performed with the Cry1Ab protein in mice. An acute oral study was considered appropriate since toxic proteins are only known to exert acute effects (Sjoblad *et al.*, 1992). The Cry1Ab protein was administered orally by gavage to three groups of ten male and female mice; additionally, one group of mice was dosed with a vehicle control lacking the Cry1Ab the protein. The targeted doses of Cry1Ab protein administered to mice were 0, 400, 1000, and 4000 mg/kg. A mice control group was dosed with Bovine serum albumin (BSA) at 4000 mg/kg. At the time of sacrifice, 7 days after

dosing, there were no statistically significant differences in mortality, body weights, cumulative body weight or total food consumption between the BSA control groups and Cry1Ab protein-treated groups. Results from this study demonstrated that the Cry1Ab protein is, as expected, not acutely toxic to mammals. The highest dose of the Cry1Ab protein tested in the mouse oral toxicity study is ~20 million times the expected dietary intake for human consumption.

Lack of Sequence Similarity of Cry1Ab Protein to Known Protein Toxins
Another approach to assess potential toxic effects of proteins introduced into plants is to compare the amino acid sequence of the protein to that of known toxic proteins. Homologous proteins derived from a common ancestor are likely to share function. Therefore, it is undesirable to introduce DNA which encodes for a protein that is homologous to a toxin. Published criteria (Doolittle, 1990) using the degree of amino acid similarity between proteins were used to assess whether the Cry1Ab protein is homologous to known toxins. Based on these criteria, it was determined that the Cry1Ab protein does not show meaningful amino acid sequence similarity when compared to known protein toxin sequences listed in the PIR, EMBL, SwissProt and GenBank protein databases, with the exception of other Cry proteins.

Lack of Homology of Cry1Ab Protein to Known Allergens
The most important factor to consider in assessing allergenic potential is whether the source of the gene being introduced into plants is allergenic (FDA, 1992; Metcalfe *et al.,* 1996). *Bacillus thuringiensis,* the source of the *cry1Ab* gene, has no history of causing allergy. In nearly 40 years of commercial use, there have been no documented cases of allergenicity to *Bacillus thuringiensis,* including occupational allergy associated with manufacture of products containing *Bacillus thuringiensis* (McClintock *et al.,* 1995).

In addition, the biochemical profile of the Cry1Ab protein provides a basis for allergenic assessment when compared with known protein allergens. Protein allergens must be stable to the peptic and tryptic digestion and the acid conditions of the digestive system if they are to pass through the intestinal mucosa to elicit an allergenic response. Another significant factor contributing to the allergenicity of proteins is their high concentration in foods that elicit an allergenic response (Taylor *et al.,*1987; Taylor, 1992; Taylor *et al.,* 1992; and Metcalfe *et al.,* 1996). The physiochemical properties of the Cry1Ab protein are clearly distinct from these characteristics of known allergens.

A comparison of the amino acid sequence of an introduced protein with the amino acid sequences of known allergens is also a useful indicator of allergenic potential (Metcalfe *et al.,* 1996). This reference defines an immunologically relevant sequence comparison test for similarity between the amino acid sequence of the introduced protein and known allergens as a match of at least eight contiguous identical amino acids.

The amino acid sequences of the 219 allergens present in public domain genetic databases (GenBank, EMBL, PIR, and SwissProt) have been searched for overall similarity to the amino acid sequence of Cry1Ab protein using the FASTA computer program (Pearson and

Lipman, 1988). No immunologically significant sequences or overall allergen homologies (Doolittle, 1990) were identified in the Cry1Ab protein (Metcalfe *et al.*, 1996). It was concluded that: (1) the *cry1Ab* gene introduced into corn does not encode a known allergen, and (2) the introduced protein does not share any immunologically significant amino acid sequences with known allergens.

In summary, the Cry1Ab protein shows no amino acid sequence homology to known protein toxins, other than other Cry proteins, and is rapidly degraded with loss of insecticidal activity under conditions that simulate mammalian digestion. There were no indications of toxicity as measured by treatment-related adverse effects in mice administered Cry1Ab protein by oral gavage. The *cry1Ab* gene was not derived from an allergenic source, and the Cry1Ab protein does not possess immunologically relevant sequence similarity with known allergens or possess the characteristics of known protein allergens. These studies support the safety of Cry1Ab protein and are fully consistent with the extensive history of safe use for the Cry1Ab protein that has high selectivity for insects, with no deleterious effects on other types of organisms such as mammals, fish, birds or invertebrates (EPA, 1988; McClintock *et al.*, 1995).

Compositional Analysis and Nutritional Assessment of YieldGard Corn

The design of a food and feed safety assessment program for a genetically engineered crop requires detailed understanding of the uses of the crop and crop products in animal and human nutrition. Approximately 80% of all corn grown in the United States is used as feed by producers of livestock, poultry and fish (NCGA, 2000). Corn is also processed into a plethora of food and industrial products that include items such as corn flakes, soft drinks, jelly beans, baby foods, tooth paste, crayons, baby diapers, cosmetics and pharmaceuticals (NCGA, 2000). Corn is palatable, readily digested by humans and by monogastric and ruminant animals, and is one of the best sources of metabolizable energy among the grains (Wright, 1988). Corn is a major food and feed source worldwide and, therefore, extensive data have been generated to demonstrate the food and feed safety of YieldGard insect-protected corn.

New corn varieties developed by traditional breeding are typically selected for yield potential, with little or no measurements of nutritional parameters. Corn feed is supplemented with protein, minerals and vitamins to meet the nutrient requirements of animals. Food safety can be demonstrated by confirming that the new food is substantially equivalent (*e.g.*, as safe as) to the conventional food. The establishment of substantial equivalence is an important component of a food safety assessment (OECD, 1993; WHO, 1995; FAO/WHO, 1996). Compositional analyses were completed that demonstrate that YieldGard is compositionally substantially equivalent to other commercial corn varieties. Compositional analyses were performed on grain harvested from the 1994 U.S. and 1995 E.U. field trials (Tables 2-5) and forage harvested from the 1995 E.U. field trials (Table 6). The compositional values of YieldGard corn event MON 810 were compared to that of the control line, as well as to published literature values.

The compositional parameters measured on grain samples included proximates (protein, fat, ash, crude fiber, neutral detergent fiber, acid detergent fiber and moisture), amino acids, fatty acids, calcium, phosphorus and tocopherol (vitamin E). Carbohydrate values were determined by subtracting the sum of the percent protein, fat, ash and moisture values from 100%. Forage samples were analyzed for proximates.

The values of the proximate parameters measured in grain (Table 2) were all within the range of published literature values (Jugenheimer, 1976; Watson, 1987). There were no statistically significant differences between the control and event MON 810 for the proximate parameters protein, fat, ash, neutral detergent fiber, acid detergent fiber and carbohydrates. Statistically significant differences were observed for crude fiber in the 1994 U.S. trials and for moisture in 1995 E.U. trials. The difference for moisture is unlikely to be of nutritional significance since moisture content is typically dependent on the drying period employed at each location. The crude fiber content of event MON 810 was ~8% higher than that of the control line. This small difference is unlikely to be of biological significance since it is well within the range of literature values.

The results from the amino acid composition analysis of grain are summarized in Table 3. The values for 15 of 18 amino acids were within the range of published literature values (Watson, 1982). The values for cystine, histidine and glutamic acid were slightly higher than the published literature range for both event MON 810 and the control line. This is most likely due to differences in analytical methodology. In the grain from 1994 U.S. trials, there were no statistically significant differences between event MON 810 and the control line for 10 of 18 amino acids. However, the values for eight amino acids (cystine, tryptophan, histidine, phenylalanine, alanine, proline, serine and tyrosine) were significantly higher in MON 810 versus the control line. In the grain from 1995 E.U. field trials, there were no statistically significant differences for 16 of 18 amino acids. However, methionine and tryptophan levels were significantly lower in MON 810 compared to the control grain. The few differences measured were minor, and except for tryptophan not consistent across multiple years and geography. Tryptophan values in MON 810 grain were significantly higher than those in control grain from 1994 U.S. trials but significantly lower in control grain from 1995 E.U. trials. Therefore, these inconsistent differences are unlikely to be of biological significance.

The fatty acid composition for the grain of corn event MON 810 and the control line are summarized in Table 4. Ten fatty acids, for which the measured values were near or below the limit of detection of the assay (arachidonic, caprylic, capric, eicosadienoic, eicosatrienoic, heptadecenoic, lauric, myristic, myristoleic, and pentadecanoic) were excluded from statistical evaluation. Also, the data for four fatty acids (palmitoleic, arachidic, eicosenoic and behenic) are not shown because their values were low (< 0.4% of total fatty acids) and there was very little difference between MON 810 and control line values. There were no statistically significant differences observed for the five fatty acids in the grain samples analyzed from the 1994 U.S. field trials. There were no statistically significant differences for four of the five fatty acids in grain from the 1995 E.U. trials. Palmitic acid was statistically significantly higher in MON 810 grain (~3%)

compared to control grain. This small difference is unlikely to be of biological significance since it was not consistent across multiple-year data and was within published literature values.

The values for the mineral components, calcium and phosphorus, and tocopherol (vitamin E) are reported in Table 5. The values for phosphorus and vitamin E were within the published literature values; however, calcium values for both MON 810 and control, were below the published literature range. This may be attributed to differences in analytical methods with older procedures subject to interferences from elements such as phosphorus (Sidhu et al., 2000). The calcium level in MON 810 was statistically significantly higher than the control line in 1994 U.S. trials, but showed no statistical differences in 1995 E.U. trials. These inconsistent results suggest that the difference observed in 1994 U.S. trials is unlikely to be biologically meaningful. There were no statistically significant differences in the phosphorus values between MON 810 and the control line.

The results of proximate analyses of forage collected from the 1995 E.U. field trials are presented in Table 6. There were no statistically significant differences in the values for fat, ash, neutral detergent fiber, acid detergent fiber, carbohydrate and dry matter content between MON 810 and the control line. There was a statistically significant increase in protein level in the forage of MON 810 compared to the control line. However, this is unlikely to be biologically meaningful since it was within the range of historical conventional control values for forage.

To further demonstrate the nutritional equivalence of YieldGard corn hybrids, numerous animal feed performance studies have been completed on YieldGard and other *Bt* corn hybrids. The results of these studies demonstrate that animals perform in a comparable manner when fed *Bt* corn and conventional corn products. More specifically, chickens fed *Bt* corn showed no differences in growth or feed efficiency compared to chickens fed conventional corn products (Aulrich *et al.,* 1998); no differences in feed intake, milk yield, milk composition or udder health were found between lactating dairy cows fed *Bt* corn and conventional green chop (Faust and Miller, 1997); no difference was reported in digestibility in cattle fed *Bt* and conventional corn silage (Daenicke *et al.,* 1999); and, in the first year of a two-year study involving beef cows grazing on *Bt* and conventional cornstalks, no difference was reported in performance (Russell and Petersen, 1999).

The grain and forage compositional data confirm that corn line MON 810 is substantially equivalent to the parental hybrid as well as traditional corn hybrids. Processing is unlikely to alter the compositional components of corn and, therefore, products derived from corn grain will also be substantially equivalent to and as safe as current corn-derived products. Animal feed performance studies further confirm that YieldGard and other *Bt* corn varieties are as wholesome and nutritious as conventional corn.

Grain Quality

YieldGard corn has been demonstrated to improve the quality of corn grain, which further helps to ensure food and feed safety. Researchers have confirmed that YieldGard corn

reduces insect damage in corn ears, one of the main pathways by which *Fusarium moniliforme* mold infects grain (Munkvold *et al.,* 1997). *Fusarium* produces fumonisin, a class of mycotoxins hazardous to animals and humans that can cause equine leukoencephalomalacia and porcine pulmonary edema syndrome and has been linked to esophageal cancer in humans (Sobek and Munkvold, 1999). Research conducted by Iowa State University and the U.S. Department of Agriculture on corn produced in the mid-western U.S. corn belt from 1995 to 1998 shows that Cry1Ab YieldGard corn hybrids reduce ear rot and fumonisin levels in grain by as much as 90 to 93 percent compared to non-*Bt* hybrids (Munkvold *et al.,* 1997; Munkvold *et al.,* 1999; Masoero *et al.,* 1999).

YieldGard corn has also been demonstrated to improve grain quality by reducing losses from some stored grain pests susceptible to the Cry1Ab protein. Kentucky State University researchers (Sedlacek *et al.,* 1999) found that YieldGard corn reduced survival of the larvae of Indian meal moth and the Angoumois grain moth by approximately 80 percent. In addition, surviving insects produced 70 to 80 percent fewer eggs, thus reducing the spread of these pest populations.

Environmental Impact of YieldGard Corn

There is extensive information on the lack of non-target effects from microbial preparations of *B.t.k.* strains containing the Cry1Ab protein (Melin and Cozzi, 1990). The full length Cry1Ab protein encoded by the *cry1Ab* gene used to produce YieldGard insect-protected corn plants, and the insecticidally active core protein produced in the insect gut following ingestion, are identical to the respective full length and trypsin-resistant core Cry1Ab proteins contained in microbial formulations that have been used safely for nearly 40 years. The *B.t.k.* Cry1A proteins are extremely selective for the lepidopteran insects (Dulmage, 1981; Klausner, 1984; Aronson *et al.,* 1986; Whiteley and Schnepf, 1986; MacIntosh *et al.,* 1990), bind specifically to receptors on the mid-gut of lepidopteran insects (Wolfersberger *et al.,* 1986; Hofmann *et al.,* 1988a; Hofmann *et al.,* 1988b; Van Rie *et al.,* 1989; Van Rie *et al.,* 1990) and have no deleterious effect on beneficial/non-target insects, including predators and parasitoids of lepidopteran insect pests or honeybee (*Apis mellifera*) (Cantwell *et al.,* 1972; Krieg and Langenbruch, 1981; Flexner *et al.,* 1986; EPA, 1988; Vinson, 1989; and Melin and Cozzi, 1990).

To confirm and expand on the results produced for the microbial products which contain the same Cry1A protein as produced in YieldGard corn, the potential impact of the Cry1Ab protein on non-target organisms was assessed on several representative organisms. These studies were conducted with the trypsin-resistant core of the Cry1Ab protein because this is the insecticidally-active portion of the Cry1Ab protein. The non-target insect species included larvae and adult honey bee (*Apis mellifera* L.), a beneficial insect pollinator; green lacewing larvae (*Chrysopa carnea*), a beneficial predatory insect; Hymenoptera (*Brachymeria intermedia*), a beneficial parasite of the housefly; the ladybird beetle (*Hippodamia convergens*), a beneficial predaceous insect; and earthworms *(Eisenia fetida).* Leaf material of MON 810 plants was used for the Collembola *(Folsomia candida)* non-target soil organism study. Due to the potential exposure of aquatic invertebrates to corn

pollen containing the Cry1Ab protein, a toxicity test was also performed with *Daphnia magna*. The results of these non-target organism studies showed that the mortality of non-Lepidoptera insect species and three other representative organisms exposed to the Cry1Ab protein did not differ significantly from control mortality.

In addition to these studies, no acute detrimental effects were observed for three predator species (*Coleomegilla maculata, Orius insidiosus* and *Chrysoperla carnea*) exposed to pollen of plants expressing the Cry1Ab protein (Pilcher *et al.*, 1997). No effects were observed when *Folsomia candida* and *Oppia nitens* were fed cotton leaf material containing the Cry1Ac and Cry1Ab proteins (Yu *et al.*, 1997). Also, in 1994 field experiments, there were no differences observed between corn plants expressing the Cry1Ab protein and control plants in oviposition, and in predation/parasitism of the European corn borer by natural enemies (Orr and Landis, 1997). These results demonstrate the safety of the Cry1Ab protein to non-target organisms.

In a recent laboratory study, Monarch butterfly larvae have been reported to be susceptible to the Cry1Ab protein found in *Bt* corn pollen (Losey *et al.* 1999). In response to this article, several researchers presented their findings at Monarch Butterfly Research Symposia and workshops in 1999 and 2000. To better understand the impact of *Bt*-pollen on Monarch butterflies, five areas of research were discussed: 1) monarch biology, 2) pollen movement, 3) pollen levels necessary to affect larvae, 4) milkweed distribution, and 5) the impact of weather. The studies demonstrated that exposure of monarch and black swallowtail butterflies to pollen under field conditions is very low (Sears *et al*, 2000a; Wraight *et al*, 2000). The researchers concluded that: 1) Monarch migration and *Bt* pollen shed may not coincide, 2) Monarchs prefer to lay their eggs on milkweed plants not surrounded by corn plants, 3) corn pollen is heavy and does not travel far from corn fields (>90% is deposited within 5 meters of the field perimeter; Sears *et al*, 2000a), 4) YieldGard pollen on leaves, at densities up to 1100 grains/cm^2, had no effect on larval weight or survivorship (Hellmich *et al*., 2000a), and 5) the density of milkweeds is highest along roadsides rather than along corn fields. Results from field studies conducted in 2000 found that Monarch larvae survived and developed normally on milkweed plants within Bt cornfields during the period of pollen shed (Anderson *et al*., 2000; Hellmich *et al*., 2000b; Sears *et al*., 2000b). These results support the conclusion that *Bt* pollen is unlikely to have any significant impact on Monarch butterfly populations.

In addition to assessing the potential effect of the Cry1Ab protein on non-target organisms, a study was conducted to confirm the expected rapid degradation of the Cry1Ab protein in soil. Corn plant tissues remaining after harvest may be tilled into the soil or remain on the soil surface (no till), depending upon agricultural practices following harvest. The degradation rate of the Cry1Ab protein was assessed by measuring the decrease in insecticidal activity of YieldGard corn tissue incubated in soil. The Cry1Ab protein, as a component of corn tissue, had an estimated DT_{50} (time to 50% reduction of bioactivity) and DT_{90} (time to 90% reduction of bioactivity) of 1.6 and 15 days, respectively (Sims and Holden, 1996). This measured rate of degradation in soil is

comparable to that reported for the *Btk* protein in genetically modified cotton (Palm *et al.*, 1994) and to the degradation rate reported for microbial *Bt* products (West *et al.*, 1984; West, 1984; and Pruett *et al.*, 1980). This rapid degradation further supports the lack of deleterious effects on non-target soil organisms.

In a recent publication (Crecchio and Stotzky, 1998), it has been reported that isolated *Bt* proteins can bind to clay particles and humic acids in artificial soil mixes in a way that could lead to reduced breakdown of the *Bt* protein while still retaining some of the *Bt* proteins' insecticidal activity. The authors implied that *Bt* protein produced in transgenic crops could enter the soil either through tillage or processes like exudation, and bind to particles in the soil and thereby accumulate to levels that might be toxic to soil organisms. However, these claims are contradicted by data which show that Cry1Ab and other *Bt* proteins expressed in YieldGard corn plants are rapidly degraded in soil (see above). These data, combined with the specificity of *Bt* proteins, and the absence of adverse ecological effects support the conclusion that *Bt* proteins are unlikely to have an adverse impact on the soil organisms.

Entomologists have observed that insect populations adapt to insecticides if those insecticides are not managed correctly. Integrated pest management was developed as a result of industry experiences with chemical insecticides. To address similar concerns for insect protected transgenic crops, Monsanto instituted a YieldGard corn insect resistance management (IRM) plan, co-incident with the 1997 U.S. commercial launch of this product. To delay the onset of insect resistance and maximize the sustainability of YieldGard corn, the IRM plan included the following: 1) expanding the knowledge of insect biology and ecology; 2) effective dose product which kills nearly all of the resistant heterozygote insects; 3) refuges to support populations of Cry1Ab susceptible insects; 4) monitoring for any incidents of pesticide resistance and implementing a containment plan; 5) employment of integrated pest management practices that encourage ecosystem diversity and multiple tactics for insect control; 6) grower education to assure implementation of these strategies; and 7) development of products with alternative modes of action. Since the implementation of this plan, target insect species have shown no change in the sensitivity or resistance to the Cry1Ab protein.

The environmental assessment shows that YieldGard corn does not harm agriculturally beneficial insects, including honeybees, ladybird beetles, green lacewings or predatory insects and spiders. YieldGard corn is unlikely to have any significant impact on Monarch butterfly populations due to limited exposure**.** In addition, the Cry1Ab protein produced in YieldGard corn is rapidly degraded in soil and has no effect on soil invertebrates, including earthworms and collembola.

Summary

YieldGard corn plants have demonstrated effective control of the targeted insect pests in corn field trials since 1993. The DNA insert in YieldGard corn event MON 810 containing the *cry1Ab* gene has been crossed into commercial corn inbreds to produce hybrids of

superior agronomic performance with protection against lepidopteran insects. Detailed food, feed and environmental safety assessments confirm the safety of this product and support the regulatory approval of YieldGard insect-protected corn event MON 810. The analyses included: 1) detailed molecular characterization of the introduced DNA; 2) safety assessment of the expressed Cry1Ab protein; 3) compositional analysis of corn grain and forage; and 4) environmental impact assessment of the corn plants. These studies demonstrate that the Cry1Ab protein is safe to humans, animals, non-target organisms, and beneficial insects, and that the forage and grain of YieldGard corn plants are as safe and nutritious as conventional corn varieties.

Information and data contained within this document have been provided to regulatory authorities for review. Regulatory review continues as we update regulatory files and make submissions to additional countries globally.

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Figure 1. Plasmid used to produce YieldGard corn event MON 810

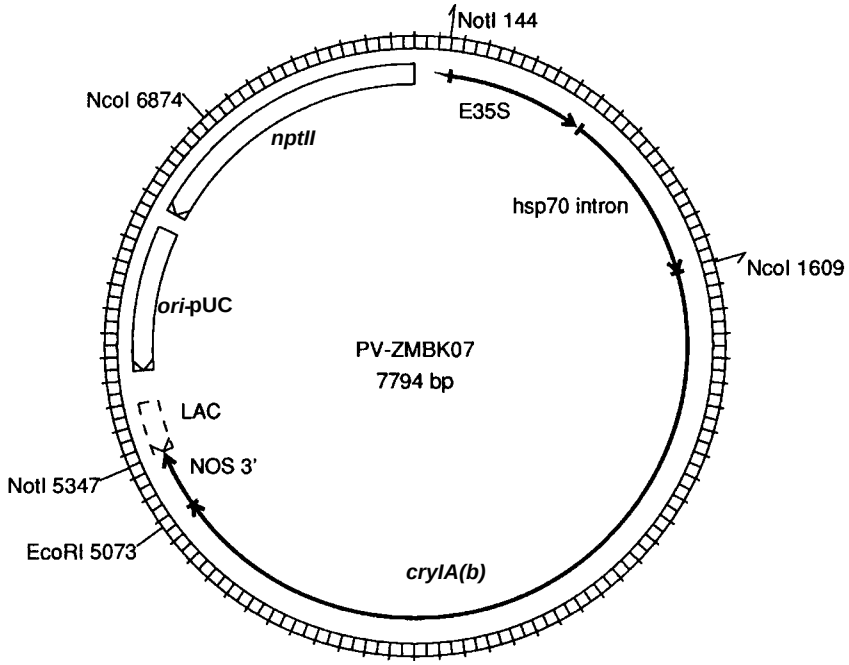

Table 2. Summary of Proximate Analysis of Corn Grain

	1994 U.S.		1995 E.U.		
	Mean[a]	(Range[b])	Mean[c]	(Range)[d]	
Component	MON 810	MON 818[e]	MON 810	MON 820[e]	Literature Range
Protein[f]	13.1	12.8	11.5	10.8	6.0-12.0[g]
	(12.7-13.6)	(11.7-13.6)	(10.5-12.2)	(9.0-11.8)	9.7-16.1[h]
Fat[f]	3.0	2.9	3.0	3.0	3.1-5.7[g]
	(2.6-3.3)	(2.6-3.2)	(2.8-3.3)	(2.4-3.3)	2.9-6.1[h]
Ash[f]	1.6	1.5	1.4	1.4	1.1-3.9[g]
	(1.5-1.7)	(1.5-1.6)	(1.3-1.5)	(1.2-1.6)	
Crude Fiber[f]	2.6[j]	2.4	N.A.[i]	N.A.	2.0-5.5[k]
	(2.5-2.8)	(2.3-2.5)			
Neutral Detergent Fiber[f]	N.A.	N.A.	12.1	12.4	8.3-11.9[g]
			(10.7-13.9)	(9.6-15.3)	
Acid Detergent Fiber[f]	N.A.	N.A.	3.4	3.9	3.3-4.3[g]
			(2.7-4.1)	(3.1-5.3)	
Carbohydrate[f]	82.4	82.7	84.1	84.9	not reported
	(81.8-82.9)	(81.7-83.8)	(83.1-84.8)	(83.7-86.3)	
Moisture %	12.4	12.0	13.3[j]	12.1	7-23[g]
	(11.0-14.4)	(10.6-14.2)	(12.1-15.2)	(11.6-12.3)	

a: Value reported is mean of six samples, one from each field site.
b: Range denotes the lowest and highest individual values across six sites for each line.
c: Value reported is mean of four samples, one from each field site.
d: Range denotes the lowest and highest individual values across four sites for each line.
e: Control corn line in the trial.
f: Percent dry weight of sample.
g: Watson, 1987.
h: Jugenheimer, 1976.
i: N.A., not analyzed.
j: Values are statistically significant from the control line values at the 95% confidence level.
k: Watson, 1982.

Table 3. Amino Acid Composition of Corn Grain[a]

	1994 U.S. Mean[b] (Range)[c]		1995 E.U. Mean[d] (Range)[e]		
Amino Acid	MON 810	MON 818[f]	MON 810	MON 820[f]	Literature[g] Range
Methionine	1.7	1.7	1.4[h]	1.5	
	(1.6-1.9)	(1.6-1.7)	(1.4-1.5)	(1.4-1.7)	1.0-2.1
Cystine	2.0[h]	1.9	1.9	2.1	
	(1.9-2.1)	(1.8-2.0)	(1.9-2.1)	(1.9-2.4)	1.2-1.6
Lysine	2.8	2.8	2.9	3.1	
	(2.5-2.9)	(2.7-2.9)	(2.7-3.1)	(2.6-3.5)	2.0-3.8
Tryptophan	0.6[h]	0.6	0.5[h]	0.6	
	(0.5-0.7)	(0.4-0.6)	(0.4-0.5)	(0.5-0.7)	0.5-1.2
Threonine	3.9	3.8	3.7	3.7	
	(3.7-4.4)	(3.7-3.9)	(3.6-3.7)	(3.3-3.8)	2.9-3.9
Isoleucine	3.7	3.8	3.8	3.9	
	(3.3-4.1)	(3.6-4.0)	(3.4-4.3)	(3.7-4.3)	2.6-4.0
Histidine	3.1[h]	2.9	3.0	3.1	
	(2.9-3.3)	(2.8-3.0)	(2.9-3.0)	(2.9-3.2)	2.0-2.8
Valine	4.5	4.6	4.7	4.8	
	(4.1-4.9)	(4.3-4.8)	(4.4-4.9)	(4.4-4.9)	2.1-5.2
Leucine	15.0	14.5	14.5	14.2	
	(14.1-16.7)	(13.8-15.0)	(13.9-15.3)	(13.3-15.3)	7.8-15.2
Arginine	4.5	4.5	3.9	4.1	
	(4.1-4.7)	(4.2-4.7)	(3.6-4.1)	(3.8-4.3)	2.9-5.9
Phenylalanine	5.6[h]	5.4	5.6	5.6	
	(5.4-6.1)	(5.2-5.6)	(5.4-5.9)	(5.3-6.0)	2.9-5.7
Glycine	3.7	3.7	3.5	3.6	
	(3.4-4.0)	(3.5-3.8)	(3.4-3.7)	(3.2-3.9)	2.6-4.7

[a:] Values are expressed as percent of total protein.
[b:] Value reported is mean of six samples, one from each field site.
[c:] Range denotes the lowest and highest individual values across six sites for each line.
[d:] Value reported is mean of four samples, one from each field site.
[e:] Range denotes the lowest and highest individual values across four sites for each line.
[f:] Control line in the trial.
[g:] Watson, 1982. Values are percent of total protein [10.1% total protein (Nx6.25)].
[h:] Values are statistically significant from the control line values at the 95% confidence level.

Table 3. Amino Acid Composition of Corn Grain[a] (cont'd.)

	1994 U.S. Mean[b] (Range)[c]		1995 E.U. Mean[d] (Range)[e]		
Amino Acids	MON 810	MON 818[f]	MON 810	MON 820[f]	Literature Range[g]
Alanine	8.2[h]	7.8	8.2	8.1	
	(7.8-8.9)	(7.5-8.0)	(7.9-8.4)	(7.5-8.6)	6.4-9.9
Aspartic Acid	7.1	6.6	7.1	6.9	
	(6.4-8.2)	(6.3-6.8)	(6.9-7.3)	(6.4-7.3)	5.8-7.2
Glutamic Acid	21.9	21.1	21.3	20.9	
	(20.4-24.4)	(20.1-21.6)	(20.8-21.8)	(19.5-22.1)	12.4-19.6
Proline	9.9[h]	9.6	9.7	9.7	
	(9.7-10.5)	(9.4-9.8)	(9.5-9.9)	(9.2-10.1)	6.6-10.3
Serine	5.5[h]	5.2	5.5	5.3	
	(5.3-5.9)	(5.1-5.4)	(5.4-5.6)	(4.9-5.5)	4.2-5.5
Tyrosine	4.4[h]	4.0	4.0	4.0	
	(4.1-4.8)	(3.9-4.1)	(3.9-4.2)	(3.7-4.3)	2.9-4.7

[a:] Values are expressed as percent of total protein.
[b:] Value reported is mean of six samples, one from each field site.
[c:] Range denotes the lowest and highest individual values across six sites for each line.
[d:] Value reported is mean of four samples, one from each field site.
[e:] Range denotes the lowest and highest individual values across four sites for each line.
[f:] Control line in the trial.
[g:] Watson, 1982. Values are percent of total protein [10.1% total protein (Nx6.25)].
[h:] Values are statistically significant from the control line values at the 95% confidence level.

Table 4. Fatty Acid Composition of Corn Grain[a]

	1994 U.S. Mean[b] (Range)[c]		1995 E.U. Mean[d] (Range)[e]		
Fatty Acids	MON 810	MON 818[f]	MON 810	MON 820[f]	Literature Range[g]
Palmitic (16:0)	10.5	10.5	10.5[h]	10.3	
	(10.2-11.1)	(10.2-10.7)	(10.3-10.8)	(9.9-10.7)	7-19
Stearic (18:0)	1.9	1.8	1.5	1.5	
	(1.7-2.1)	(1.8-1.9)	(1.4-1.7)	(1.4-1.6)	1-3
Oleic (18:1)	23.2	22.8	22.0	22.4	
	(21.5-25.4)	(21.6-23.9)	(21.0-22.9)	(21.8-23.5)	20-46
Linoleic (18:2)	62.6	63.0	64.0	64.0	
	(59.5-64.7)	(61.8-64.6)	(63.3-64.6)	(62.7-65.1)	35-70
Linolenic (18:3)	0.8	0.9	1.1	1.0	
	(0.7-0.9)	(0.8-0.9)	(1.0-1.1)	(1.0-1.1)	0.8-2

a: Value of fatty acid is % of total lipid. Fatty acids not listed were below the limit of detection of the assay.
b: Value reported is mean of six samples, one from each field site.
c: Range denotes the lowest and highest individual values across six sites for each line.
d: Value reported is mean of four samples, one from each field site.
e: Range denotes the lowest and highest individual values across four sites for each line.
f: Control line in the trial.
g: Watson, 1982.
h: Values are statistically significant from the control line at the 95% confidence level.

Table 5. Tocopherol, Calcium and Phosphorus in Corn Grain[a]

	1994 U.S. Mean[b] (Range)[c]		
	MON 810	MON 818	Literature Range[d]
Tocopherol (Vit E) mg/kg	10.4	10.9	
	(9.7-11.3)	(9.9-12.1)	3.0-12.1
Calcium %	0.0036[e]	0.0033	
	(0.0033-0.0039)	(0.0029-0.0037)	0.01-0.1
Phosphorus %	0.358	0.348	
	(0.334-0.377)	(0.327-0.363)	0.26-0.75

a: Values on a dry weight basis.
b: Value reported is mean of six samples, one from each field site.
c: Range denotes the lowest and highest individual values across six sites for each line.
d Watson, 1982.
e: Value is significantly different from control, MON 818, at the 95% confidence level.

Table 6. Summary of Proximate Analysis of Forage

Component	1995 E.U. Mean[a] (Range)[b] MON 810	MON 820[c]	Historical Range[d]
Protein[e]	7.3[f]	6.1	
	(5.7-8.4)	(4.8-7.4)	4.8-8.4
Fat[e]	1.4	1.8	
	(1.3-1.7)	(1.4-2.1)	1.4-2.1
Ash[e]	3.2	3.4	
	(3.1-3.6)	(2.9-4.4)	2.9-5.1
Neutral Detergent Fiber[e]	38.4	41.5	
	(36.9-41.4)	(39.9-43.3)	39.9-46.6
Acid Detergent Fiber[e]	24.7	27.3	
	(22.6-27.2)	(25.6-29.2)	21.4-29.2
Carbohydrate[e]	88.0	88.8	
	(86.9-89.8)	(88.0-89.1)	84.6-89.1
Dry Matter %	30.0	28.7	
	(28.7-32.4)	(26.5-31.3)	26.5-31.3

a: Value reported is mean of four samples, one from each field site.
b: Range denotes the lowest and highest individual values across four sites for each line.
c: Values are average of two sets of measurements. MON 820 is the control maize line.
d: Range for control lines planted in Monsanto Co. field trials conducted between 1993 and 1995 (Sidhu et al., 2000).
e: Percent dry weight of sample.
f: Value is statistically significant from the control line at the 95% confidence level.