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Safety and Advantages of *Bacillus thuringiensis*-Protected Plants to Control Insect Pests

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Plants modified to express insecticidal proteins from *Bacillus thuringiensis* (referred to as *Bt*-protected plants) provide a safe and highly effective method of insect control. *Bt*-protected corn, cotton, and potato were introduced into the United States in 1995/1996 and grown on a total of approximately 10 million acres in 1997, 20 million acres in 1998, and 29 million acres globally in 1999. The extremely rapid adoption of these *Bt*-protected crops demonstrates the outstanding grower satisfaction of the performance and value of these products. These crops provide highly effective control of major insect pests such as the European corn borer, southwestern corn borer, tobacco budworm, cotton bollworm, pink bollworm, and Colorado potato beetle and reduce reliance on conventional chemical pesticides. They have provided notably higher yields in cotton and corn. The estimated total net savings to the grower using *Bt*-protected cotton in the United States was approximately $92 million in 1998. Other benefits of these crops include reduced levels of the fungal toxin fumonisin in corn and the opportunity for supplemental pest control by beneficial insects due to the reduced use of broad-spectrum insecticides. Insect resistance management plans are being implemented to ensure the prolonged effectiveness of these products. Extensive testing of *Bt*-protected crops has been conducted which establishes the safety of these products to humans, animals, and the environment. Acute, subchronic, and chronic toxicology studies conducted over the past 40 years establish the safety of the microbial *Bt* products, including their expressed insecticidal (Cry) proteins, which are fully approved for marketing. Mammalian toxicology and digestive fate studies, which have been conducted with the proteins produced in the currently approved *Bt*-protected plant products, have confirmed that these Cry proteins are nontoxic to humans and pose no significant concern for allergenicity. Food and feed derived from *Bt*-protected crops which have been fully approved by regulatory agencies have been shown to be substantially equivalent to the food and feed derived from conventional crops. Nontarget organisms exposed to high levels of Cry protein are virtually unaffected, except for certain insects that are closely related to the target pests. Because the Cry protein is contained within the plant (in microgram quantities), the potential for exposure to farm workers and nontarget organisms is extremely low. The Cry proteins produced in *Bt*-protected crops have been shown to rapidly degrade when crop residue is incorporated into the soil. Thus the environmental impact of these crops is negligible. The human and environmental safety of *Bt*-protected crops is further supported by the long history of safe use for *Bt* microbial pesticides around the world.

Key Words: **Cry proteins; *Bacillus thuringiensis;* insect-protected crops.**

INTRODUCTION

Microbial *Bacillus thuringiensis* (*Bt*)-based products have been used commercially for almost 40 years by growers, including organic growers, to control selected insect pests (Baum *et al.*, 1999). More recently, the gene(s) encoding the insecticidal proteins in these *Bt* microbial products have been cloned (Schnepf and Whiteley, 1981) and introduced and expressed in genetically modified plants (Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987; Perlak *et al.*, 1990) to enable plants to protect themselves against insect damage. This review describes: (1) what *Bt*-protected plants are; (2) why *Bt*-protected plants were developed; (3) the advantages of using *Bt*-protected crops; and (4) the food, feed, and environmental safety of *Bt*-protected plants and plant products. The review will also address many of the concerns which have been raised relative to the use and safety of *Bt*-protected plants both by summarizing the extensive published literature on *Bt* microbial products and by providing additional data which has been developed on *Bt*-protected plants and plant products. This information will hopefully enable a more science-based discussion on the risks, the safety, and the usefulness of these products to farmers, to the environment, and to society.

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WHAT ARE *Bt*-PROTECTED PLANTS?

Plants which are modified to produce an insecticidal protein from *Bt* are known as *Bt*-protected plants. *Bt* is a ubiquitous gram-positive soil bacterium that forms crystalline protein inclusions during sporulation (Hofte and Whitely, 1989). The inclusion bodies consist of proteins (referred to as Cry proteins) which are selectively active against a narrow range of insects and, as a class of proteins, are effective against a wide variety of insect pests. Cry proteins are produced as protoxins that are proteolytically activated upon ingestion (Hofte and Whitely, 1989). Cry proteins bind to specific sites (i.e., receptors) in the midgut cells of susceptible insects and from ion-selective channels in the cell membrane (English and Slatin, 1992). The cells swell due to an influx water which leads to cell lysis and ultimately the death of the insect (Knowles and Ellar, 1987).

Many *Bt* strains, which contain mixtures of up to six or eight different Cry proteins, have been widely used as microbial pesticides since 1961. These products currently account for about 1 to 2% of the global insecticide market (Baum *et al.*, 1999). *Bt* microbial products have, and continue to be, the preferred insect control choice for organic growers. Cry protein-encoding genes were an obvious choice for plant expression as a means to protect crops against insect pests. In 1981, the first *cry* gene was cloned and expressed in *Escherichia coli* (Schnepf and Whiteley, 1981) followed a few years later by the production of the first genetically modified *Bt*-protected tomato, tobacco, and cotton plants (Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987; Perlak *et al.*, 1990).

Today, *Bt*-protected potato, cotton, and corn have been commercialized in the United States and one or more of these products are marketed in Argentina, Australia, Canada, China, France, Mexico, Portugal, Romania, South Africa, Spain, and Ukraine (James, 1998, 1999). These plants express one of several Cry proteins for the control of lepidopteran or coleopteran insect pests (Table 1). Several other *Bt*-protected crops are under development. With more than 100 *cry* genes described (Crickmore *et al.*, 1998) and dozens of plants transformed to produce Cry proteins, there is significant potential for expanding the role of *Bt*-mediated plant protection. The next generation of *Bt*-protected plants will contain multiple *cry* genes, thereby providing growers with a product that offers a broader spectrum of pest control and reduced susceptibility for insects to develop resistance.

WHY DEVELOP *Bt*-PROTECTED PLANTS?

Bt-protected plants meet the key criteria for developing a new pest control product: technical feasibility, need, efficacy, and safety. *Bt*-protected crops offer the promise of safe and effective insect control. Based on the extensive safety database and the almost 40-year history of safe use of microbial *Bt* products, *Bt* products are considered reduced risk insecticides and typically have a special status with regulatory agencies. These factors, in combination with the intense need for better pest control methods and the environmental benefits of reducing reliance on chemical insecticides, made *Bt*-protected crops an obvious choice for product development.

TABLE 1
***Bt*-Protected Crops Fully Approved in the United States**

Crop	Cry protein	Pest(s) controlled	Date of first introduction
Potato	Cry3A	Colorado potato beetle	1995
Cotton	Cry1Ac	Tobacco budworm, cotton bollworm, pink bollworm	1996
Corn	Cry1Ab	European corn borer, southwestern corn borer, corn earworm	1996
Corn	Cry1Ac	European corn borer, southwestern corn borer	1997

Source: EPA (1995a,b,c; 1996b, 1997).

Technical Feasibility

Until recently, the technical means to produce *Bt*-protected plants were not available. Now, however, the combination of plant cell tissue culture and modern molecular methods allows for a greater diversity of traits, including *Bt* genes, to be efficiently introduced and deployed in plants for insect control. Because they are proteins and the difficulty of expressing this class of protein in plants has been overcome (Perlak *et al.*, 1991), *Bt* proteins are now relatively straightforward to produce in plants. Thousands of *Bt* strains have been identified worldwide, which provides a tremendous diversity of genes and potential proteins. Collectively, these strains offer a rich source of *cry* genes, providing the building blocks for the development of numerous products to control a diversity of insect pests.

Need

Growers sustain billions of dollars in crop loss or reduced yield due to pests which have the potential to be controlled by Cry proteins (Gianessi and Carpenter, 1999). In cases such as European corn borer, stalk damage caused by second generation borers which have entered the inside of the corn stalks is difficult to control with externally applied pesticides. In addition, important chemical insecticides, such as synthetic pyrethroids used on cotton to control budworm, are losing their effectiveness due to the onset of pest resistance (Smith, 1999). Therefore, there is a need for cost-effec-

tive, environmentally acceptable, low-risk pest control tools for growers, such as *Bt*-protected plants.

Efficacy

The Cry protein-based efficacy of microbial *Bt* products is well established. *Bt kurstaki* strain HD1 was commercialized in 1961. This strain has long been an industry standard, being widely used to control several important lepidopteran pests. The efficacy of the *Bt* HD1 strain results largely from the presence of four Cry proteins: Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa. The *cry1Ab* and *cry1Ac* genes in the *Bt* HD1 strain are the prototypes for the genes currently expressed in corn and cotton. Deployment of Cry proteins in plants offers several opportunities to improve efficacy compared to microbial delivery systems. Unlike externally applied microbial *Bt* products, the efficacy of plant-produced Cry proteins is not affected by application timing and accuracy or by subsequent rain wash-off and sunlight inactivation. *Bt*-protected plants produce sufficient quantities of Cry protein to ensure effective insect control. These attributes and the cost savings offered by these products have contributed to the rapid adoption of *Bt*-protected plants by growers.

Safety

Several characteristics, inherent to *Bt*-protected plants, provide these products with a degree of safety that is unmatched by any other pest control product. First, proteins as a class are generally not toxic to humans and animals, nor are they likely to bioaccumulate in fatty tissue or to persist in the environment like some halogenated chemical pesticides. Proteins which are toxic to humans and animals have been well studied and are readily identified in short-term laboratory studies with surrogate species (Sjoblad *et al.*, 1992). Second, Cry proteins exhibit a high degree of specificity for the target and closely related insect species and must be ingested to be effective. The Cry proteins have no contact activity. Each Cry protein affects relatively few insect species and then, only when ingested by early larval instars; later instars are generally less sensitive. Third, the potential for human and nontarget exposure to Cry proteins is extremely low. Unlike pesticides applied to leaves, Cry proteins are contained within the plant tissue in microgram quantities and are produced at low levels in the pollen. In addition to these inherent safety factors, product safety has been established by an extensive safety database on and experience with microbial *Bt* products (McClintock *et al.*, 1995; EPA, 1988, 1998a,b). In addition, the safety of the Cry protein produced in each *Bt*-protected plant product has been individually confirmed with specific safety studies. (The safety of both the Cry proteins in the microbial *Bt* products and the *Bt*-protected plant products will be discussed in detail below.) Microbial *Bt* products have enjoyed a history of safe use around the world for approximately 40 years.

TABLE 2
Acreage Planted with *Bt*-Protected Crops in the United States (1998 and 1999)

Crop	Number of acres 1998 (millions)	Percentage of total acres	Number of acres 1999 (millions)	Percentage of total acres
Field corn	14.4	18	18	23
Cotton	2.3	17	4	28
Potato	0.05	4	0.05	4

Source: James (1998, 1999).

ADVANTAGES OF USING *Bt*-PROTECTED CROPS

During the 5 years since their commercial introduction, growers have rapidly adopted *Bt*-protected crops as an effective tool to enhance high yield sustainable agriculture. Total planted acreage in the United States for *Bt*-protected cotton, corn, and potato exceeded 16 million acres in 1998 (Gianessi and Carpenter, 1999), comprising 17 and 18% of the total corn and cotton acreage, respectively (Table 2). According to reports by James (1997, 1998, 1999), the global acres of *Bt*-protected plants has increased from approximately 10 million acres in 1997 to 20 million acres in 1998 and 29 million acres in 1999. The benefits of decreased pest management costs, increased yields, and greater crop production flexibility are responsible for the rapid adoption of these crops (Marra *et al.*, 1998; Culpepper and York, 1998). The Economic Research Service of the U.S. Department of Agriculture reports (Klotz-Ingram *et al.*, 1999) that the use of certain *Bt* crops is associated with "significantly higher yields" and "fewer insecticide treatments for target pests."

A recent study conducted by the U.S. National Center for Food and Agricultural Policy (Gianessi and Carpenter, 1999) examined the impact of planting *Bt*-protected crops. The authors concluded that: "rapid adoption of this technology is directly tied to benefits of greater effectiveness in pest control technology and very competitive cuts in farmer's costs." Gianessi and Carpenter (1999) reported that *Bt* cotton created an estimated $92 million in additional value in the United States in 1998. In summary, the benefits of using *Bt*-protected crops include the following: (A) reduced chemical insecticide treatments for target pests; (B) highly effective pest control; (C) increased crop yields; (D) supplemental pest control by preserving or enhancing populations of beneficial organisms; and (E) reduced levels of fungal toxin.

Reduced Insecticide Treatments

The adoption of *Bt*-protected plants has led to significant reductions in chemical insecticide use. Plantings of *Bt*-protected cotton in 1996 helped Alabama growers use the least amount of insecticides on cotton since the 1940s (Smith, 1997). In 1998, an estimated 2 million pounds less chemical insecticide was used for bollworm/budworm control in six key cotton-producing states compared to 1995 usage (Table 3). Following the introduction of *Bt*-protected cotton in 1996, a total average of 2.4 insecticide applications were made to control budworm/bollworm across all cotton-producing states (Williams, 1997). Pre-1996 insecticide use was significantly higher (2.9 to 6.7 applications) in the six states where the *Bt* cotton has been most widely adopted (Williams, 1999). During the 3 years in which *Bt*-protected cotton has been planted, the number of insecticide treatments for budworm/bollworm in these states fell to an overall average of 1.9 applications (Table 4). The reduced number of insecticide treatments corresponds to a 12% decline in the total pounds of chemical insecticides applied. Of course, some insecticide applications may be necessary to control those insects which are not controlled by the specific *Bt* protein expressed in the plant.

Comparable surveys of cotton growers in Australia during 1998–1999 also showed substantial reductions in insecticide use following the introduction of *Bt*-protected cotton. Depending on the growing region, reductions in chemical insecticide use varied from 27–61%, with an average of 43% reduction. This corresponded to 7.7 fewer insecticide sprays on the *Bt*-protected cotton than on conventional cotton fields.

In China, insecticide reductions associated with *Bt*-protected cotton have been even greater (Xia *et al.*, 1999). In 4 years of testing, the use of insecticides has decreased by 60–80% compared with chemical insecticide use in conventional cotton. In countries like India with tropical agricultural systems that have heavy pest insect pressure, and consequent high insecticide use, insecticide use reduction should be comparable to the reductions observed in China.

TABLE 3

Cotton Bollworm/Budworm Insecticide Use Reductions after the Introduction of *Bt*-Protected Cotton (1995 Usage Compared to 1998 Usage—AR, AZ, LA, MS, TX)

Insecticide	Use of Pesticide Active Ingredient (1000s Pounds)
Amatraz (Ovasyn)	−42
Cyfluthrin (Baythroid)	−35
Cypermethrin (Ammo)	−81
Deltamethrin (Decis)	+11
Esfenvalerate (Asana)	−19
Lambdacyhalothrin (Karate)	−58
Methomyl (Lannate)	−156
Profenofos (Curacron)	−1014
Spinosad (Tracer)	+19
Thiodicarb (Larvin)	−665
Tralomethrin (Scout)	−4
ζ-Cypermethrin (Fury)	+1
Total	−2044

Source: Gianessi and Carpenter (1999).

TABLE 4

Number of Insecticide Treatments in Cotton for Bollworm/Budworm before (1995) and after (1996–1998) the Introduction of *Bt*-Protected Cotton

State	1995	1996	1997	1998
Alabama	6.7	0.1	0.5	1.4
Arizona	2.9	1.7	0.9	0.4
Florida	5.7	1.1	1.0	2.0
Georgia	3.4	1.7	2.5	1.5
Louisiana	4.7	3.9	3.2	3.5
Mississippi	5.7	2.2	2.5	2.5

Source: Williams (1999).

The reduction in insecticide use associated with the introduction of *Bt*-protected corn is more difficult to assess. Infestations of the primary target pest, European corn borer, vary widely from year to year. Insecticides used for corn borer control may also be needed to control other pests that are less susceptible to *Bt*. Nevertheless, 30% of the growers planting *Bt* corn in 1997 indicated they did so to eliminate insecticides for controlling European corn borer (Gianessi and Carpenter, 1999). Corn acres treated with the five chemical insecticides recommended for control of European corn borer declined 7% in 1998. For analytical purposes, Gianessi and Carpenter (1999) assumed that about one-third of the decline (2.5%) was due to the introduction of *Bt*-protected corn; thus chemical insecticide was estimated to be reduced on at least 2 million acres in 1998. Rice (1998) projected that corn insecticide use would be reduced by 1.2 million pounds if 80% of the corn acres were planted with *Bt*-protected corn.

Thus far, the market penetration of *Bt*-protected potato has been modest (4%). Because growers must apply insecticides to control other pests, the reduction in pesticide use has been relatively minor (Gianessi and Carpenter, 1999). Growers using *Bt*-protected potatoes in 1997 averaged one less insecticide application than growers using non-*Bt*-protected potatoes. However, the recent approval of potatoes that resist both the Colorado potato beetle and the plant viruses led U.S. Environmental Protection Agency officials to state their expectation that widespread use of this product would significantly reduce the current high use of insecticides to control aphids that vector the potato virus (Gianessi and Carpenter, 1999).

Plant-deployed *Bt* provides growers with “built in”

TABLE 5
Percentage of Cotton Insect Pests Killed by *Bt*-Protected Cotton in Research Plots

Pest species	Percentage of control
Tobacco budworm	95
Pink bollworm	99
Cotton bollworm (pre-bloom)	90
Cotton bollworm (blooming)	70

Source: Halcomb *et al.* (1996).

pest protection and also greatly reduces the need to transport, mix, apply, and dispose of externally applied chemical pesticides. The risk of misuse, ineffective timing of applications, and worker exposure to pesticide is virtually eliminated. Of course, because the Cry protein does not protect against all pests, supplemental applications of external pesticides may be required even on *Bt* crops to control those pests not controlled by the specific Cry protein produced.

Highly Effective Pest Control

Most European and southwestern corn borer larvae that attempt to feed on *Bt*-protected corn are only able to make a slight scar on the corn leaf and die within 72 h. *Bt* corn hybrids express Cry protein in all plant parts throughout the season and provide essentially 100% protection from European and southwestern corn borer. A survey by Weinzierl *et al.* (1997) found only two corn borer survivors on about 325 acres of YieldGard corn surveyed in 1998.

Bt-protected cotton provides effective control of tobacco budworm and pink bollworm and moderate control of cotton bollworm. Efficacy ratings range from 70 to 99% for these pests (Table 5). The first to fourth instars of budworm and pink bollworm are highly susceptible to Cry protein, whereas the fifth instars have greatly reduced sensitivity (Halcomb *et al.*, 1996).

Bt potatoes are protected throughout the season from all stages of Colorado potato beetle (Perlak *et al.*, 1993). No supplemental insecticide applications are needed to control this pest in potato.

Higher Crop Yields

Bt crop protection translates to significant yield increases. Annual corn loss due to European corn borer fluctuates widely, 33 to 300 million bushels per year (USDA, 1975). In 1997, *Bt*-protected corn was planted on 4 million acres (USDA, 1998) and European corn borer infestation was typical to heavy. That year, *Bt* corn provided a yield premium of almost 12 bushels per acre (Gianessi and Carpenter, 1999). One year later, European corn borer infestation was extremely light and *Bt*-protected corn was planted on 14 million acres. Yet, U.S. farmers that planted *Bt* corn still realized a yield increase of 4.3 bushels per acre or a total increase of 60 million bushels.

In 1995, the year prior to the introduction of *Bt*-protected cotton in the United States, the average yield loss due to tobacco budworm and cotton bollworm approached 4% with the loss reaching 29% in Alabama (Gianessi and Carpenter, 1999). Three years later, *Bt* cotton accounted for 17% of the total U.S. cotton crop and over 90% of the cotton grown in Alabama (Gianessi and Carpenter, 1999). Reduced crop damage on this acreage led to an increase in total lint yield of 85 million pounds. Based on an estimate of $40 per acre net advantage in the United States, Gianessi and Carpenter (1999) projected that the farmers planting *Bt*-protected cotton experienced an overall net benefit of more than $92 million in 1998. Values for Bollgard cotton in other world areas are similar or greater than in the United States.

James (1999) estimated that *Bt* cotton and corn growers in the United States and Canada generated $133 million and $124 million, respectively, in value in 1997, whereas Falck-Zepeda *et al.* (1999) estimated that *Bt* cotton created a $190.1 million increase in world surplus in 1997. As for *Bt*-protected potatoes, their introduction has not yet had a significant impact on overall yield.

Supplemental Pest Control by Beneficial Organisms

Cry proteins generally have little or no effect on natural insect predators and parasites, as indicated by laboratory and field studies conducted with lady beetles, green lacewing, damsel bugs, big-eyed bugs, parasitic wasps, and other arthropods (for example, Dogan *et al.*, 1996; Amer *et al.*, 1999). This allows beneficial organisms to survive in *Bt*-protected crops where the beneficial insects can help control secondary pests. Secondary pests can often become a problem when predator and parasite populations are reduced by conventional broad-spectrum insecticides. As was previously observed in research plots (Feldman *et al.*, 1992; Reed *et al.*, 1993), beneficial arthropods alone kept aphids below damaging levels in commercial NewLeaf Plus potato fields which had not been treated to control aphids. Beneficial insects and spiders were more abundant in these fields (Fig. 1). This appears to provide an additional benefit of preventing economic outbreaks of spider mites (Fig. 2). Similarly, use of *Bt* cotton in China, with a concomitant reduction in insecticide use, resulted in an average increase of 24% in the number of insect predators over what was found in conventional cotton fields (Xia *et al.*, 1999). Thus, to the extent that *Bt* crops require fewer applications of externally applied insecticides, populations of beneficial organisms are more likely to be preserved, which result in less crop damage, requirement for fewer chemical insecticides, and the potential for higher yields.

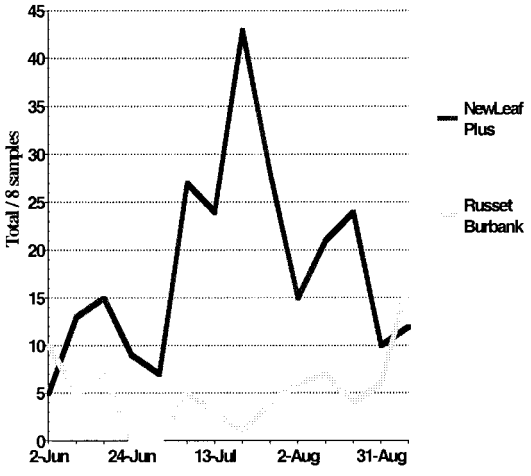

FIG. 1. Populations of predators and parasites collected from samples in NewLeaf Plus fields and comparison Russet Burbank fields in Ephrata, WA, over time in 1998 (Reibe, unpublished).

Reduced Levels of Fungal Toxins

Corn borers feeding on stalk and ear tissue cause damage to the developing grain, which enables spores of the toxin-producing fungi *Fusarium* to germinate. The spores germinate and the fungus proliferates, leading to ear and kernel rot and producing increased levels of the fumonisin family of mycotoxins. Fumonisins are fungal toxins that produce death and morbidity in horses and swine (Norred, 1993) and have been linked in epidemiological studies to high rates of esophageal and liver cancer in African farmers (Marasas *et al.*, 1988). Because the Cry1Ab protein virtually eliminates corn borer-induced tissue damage in corn products which produce Cry1Ab protein throughout the plant, the fungal spores are less able to germinate and reproduce. Munkvold *et al.* (1997, 1999) showed that *Fusarium* ear rot levels and the resulting levels of fumonisin mycotoxin were dramatically reduced in *Bt*-protected corn compared to non-*Bt* corn over several years of observations (Fig. 3). Research from Iowa State University and the U.S. Department of Agriculture showed up to a 96% reduction in *Fusarium* ear rot levels in insect-damaged ears with *Bt* corn hybrids compared to non-*Bt* corn hybrids. The same research in 1997, a year with high corn borer pressure, showed a 90 to 93% reduction in fumonisin levels (Munkvold *et al.*, 1997, 1999). From their research, Munkvold *et al.* (1997) concluded "Genetic engineering of maize for insect resistance may enhance its safety for animal and human consumption. The magnitude of the differences in fumonisin concentrations between transgenic and non-transgenic hybrids was sufficient to impact the toxicity of these maize kernels to horses and to human cell cultures." Similar reductions of approximately 90% in fumonisin levels have been observed in *Bt* corn hybrids grown in Italy (Masoero *et al.*, 1999). The levels of fumonisin reduction will depend on environmental and varietal differences. Less information has been developed on the impact of *Bt* corn on other mycotoxins, like aflatoxin. Aflatoxin levels appear to be much more variable with no consistent correlation to the presence of *Bt*.

SAFETY CONSIDERATIONS FOR *Bt*-PROTECTED CROPS

Bt microbial products are the most widely used biopesticide in the world, comprising 1 to 2% of the global insecticide market in the 1990s (Baum *et al.*, 1999). Cry proteins are highly specific to their target insect pest. Cry proteins are highly specific to their target insect pest. Cry proteins have little or no effect on other organisms. In almost 40 years of widespread use, microbial *Bt* products have caused no adverse human health or environmental effects (EPA, 1998a; McClintock *et al.*, 1995). Having been registered in the United States since 1961, there are currently at least 180 registered microbial *Bt* products (EPA, 1998b) and over 120 microbial products in the European Union. These products have been used continuously since then for an expanding number of applications in agriculture, disease vector control, and forestry.

The U.S. EPA has determined that the numerous toxicology studies conducted with *Bt* microbial products show no adverse effects and has concluded that these products are not toxic or pathogenic to humans (McClintock *et al.*, 1995; EPA, 1998a). EPA, in its 1998 reregistration eligibility decision, concluded that microbial *Bt* products pose no unreasonable adverse effects to humans or the environment and that all uses of those products are eligible for reregistration (EPA,

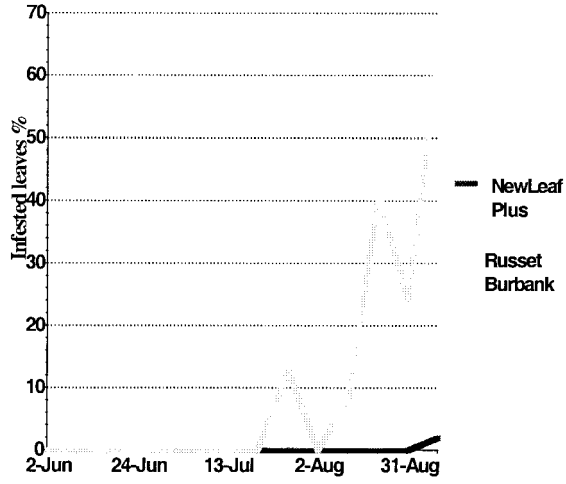

FIG. 2. Spider mite infestation of NewLeaf Plus and nongenetically modified Russet Burbank potatoes, Ephrata, WA, 1998. Mite infestations were found to be lower in untreated NewLeaf Plus than comparison Russet fields treated with insecticides and miticide (Reibe, unpublished).

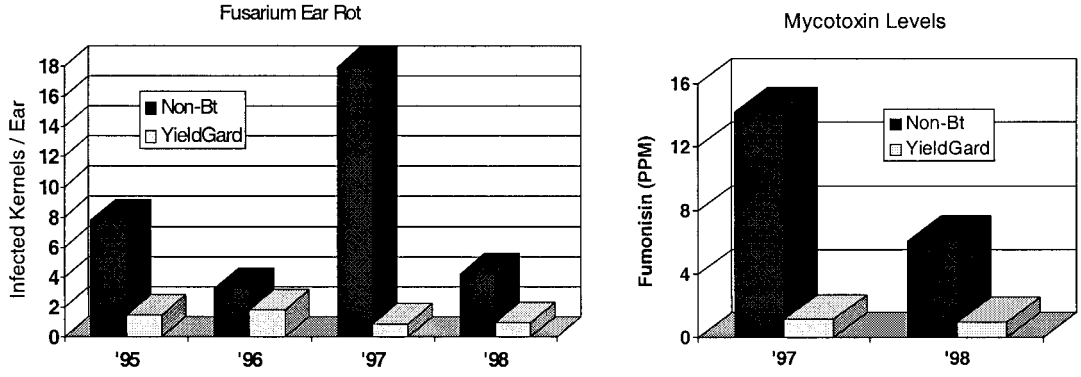

FIG. 3. Reduced ear rots and mycotoxins. (*Source:* 1995–1998 Iowa State University Research, natural European corn borer infestations.)

1998a). The World Health Organization's (WHO) International Program on Chemical Safety report on environmental health criteria for *Bt* concluded that: "*Bt* has not been documented to cause any adverse effects on human health when present in drinking water or food" (IPCS, 2000).

Microbial *Bt* formulations are used commercially in the United States, Canada, Mexico, and numerous South American countries, as well as in virtually all of the countries comprising the European Union. These products are also commonly used in numerous other countries around the world including Russia, China, Australia, and Eastern European countries. The WHO recently reviewed the extensive safety database on *Bt* microbial formulations and concluded that: "Owing to their specific mode of action, *Bt* products are unlikely to pose any hazard to humans or other vertebrates or to the great majority of non-target vertebrates provided they are free from non-*Bt* microorganisms and biologically active products other than *ICPs* (insect control proteins)" (IPCS, 2000).

The following data and scientific reasoning support an affirmative human health and environmental safety assessment for Cry proteins:

- Results of extensive acute oral or dietary studies representing numerous commercial *Bt* microbial pesticide products containing different combinations of Cry proteins establish no mammalian toxicity.
- Studies on representative proteins from three classes of Cry proteins (Cry1, Cry2, and Cry3) confirm that these materials are not toxic to mammals when administered orally at high doses. All the proteins from these classes of Cry proteins degrade rapidly in simulated gastric fluid.
- Genetically modified Cry proteins (Cry proteins with changes introduced by molecular methods), *a priori*, pose no unique human health concerns. The data on naturally occurring Cry proteins are applicable to the native and genetically modified Cry proteins produced in insect-protected plants.
- Cry proteins have a complex, highly specific mode of action. In addition, there are specific binding sites which are present in the target invertebrates and required for Cry protein to exert the insecticidal activity. Immunocytochemical analyses of Cry1A have revealed no comparable binding sites in mammals or unaffected insects.
- *Bt* microbial products have a long history (approximately 40 years) of safe use. There have only been two reports of potential adverse effects in humans from the use of microbial *Bt* products, neither of which was attributable to exposure to Cry proteins (EPA, 1988a; McClintock *et al.*, 1995).

Human Health Implications

Bt microbial pesticides are nontoxic to mammals. Numerous animal safety studies conducted over the past 40 years have demonstrated that *Bt* microbial insecticide mixtures containing Cry proteins are nontoxic when fed to mammals. "Toxicology studies submitted to the U.S. Environmental Protection Agency to support the registration of *B. thuringiensis* subspecies have failed to show any significant adverse effects in body weight gain, clinical observations or upon necropsy" (McClintock *et al.*, 1995). Collectively, these studies demonstrate the absence of acute, subchronic, and chronic oral toxicity associated with *Bt* microbial pesticides (Table 6). These findings are relevant to the safety assessment of *Bt*-protected plants because the microbial preparations contain the same classes of Cry proteins (Cry1, Cry2, and Cry3) that have been introduced into insect-protected plants (Table 7).

Acute oral toxicity studies conducted in rats and rabbits revealed no mortalities at the highest doses tested, which ranged up to thousands of milligrams of *Bt* microbial product per kilogram of body weight (Table 6). In the studies listed in Table 6, there were no deleterious effects observed in animals based on the absence of mortality, changes in body weight and food consumption, and gross pathology findings at necropsy (McClintock *et al.*, 1995). Subchronic toxicity studies in rats demonstrated "no-effect levels" (NOELs) of up to

TABLE 6
Mammalian Toxicity Assessment of *Bacillus thuringiensis*—Microbial Pesticides (Oral Exposure)[a]

Bt Microbial	Cry gene content	Test substance	Type of study	Results (NOEL)[b]	Toxicity findings	Reference
Kurstaki (Crymax)	Cry1Ac Cry2A Cry1C	Technical	Acute oral toxicity/ pathogenicity (rat)	>2.5–2.8 × 10^8 CFUs/rat	No evidence of toxicity	Carter and Liggett (1994) and EPA Fact Sheet (1996a) (Ecogen)
Kurstaki (Lepinox)	Cry1Aa Cry1Ac Cry3Ba	Technical	Acute oral toxicity/ pathogenicity (rat)	>1.19 × 10^8 CFUs/rat	No evidence of toxicity	Barbera (1995)
Kurstaki (Raven)	Cry1Ac Cry3Aa Cry3Ba	Technical	Acute oral toxicity/ pathogenicity (rat)	>4 × 10^8 CFUs/rat	No evidence of toxicity	Carter *et al.* (1993)
Kurstaki (Cutlass)	Cry1Aa Cry1Ab Cry1Ac Cry2A Cry2Ab	Technical	Acute oral toxicity/ pathogenicity (rat)	>10^8 CFUs/ml, dosing rate is 1 ml/rat	No evidence of toxicity	David (1988)
Tenebrionis (San Diego)	Cry3Aa	Technical	Acute oral toxicity (rat)	>5050 mg/kg	No evidence of toxicity	EPA Fact Sheet (1991) (Mycogen)
Kurstaki (Dipel)	Cry1Aa Cry1Ab Cry1Ac Cry2Aa	Technical	Acute oral (rat)	≥4.7 × 10^{11} spores/kg	No evidence of toxicity	EPA Fact Sheet (1986) (Abbott) and McClintock *et al.* (1995)
Kurstaki (Dipel)	Cry1Aa Cry1Ab Cry1Ac Cry2Aa	Technical	13-week oral—(gavage) (rat)	>1.3 × 10^9 spores/kg	No evidence of toxicity	McClintock *et al.* (1995)
Kurstaki (Dipel)	Cry1Aa Cry1Ab Cry1Ac Cry2Aa	Technical	13-week oral—(feed) (rat)	>8400 mg/kg/ day	No evidence of toxicity	McClintock *et al.* (1995)
Kurstaki (Dipel)	Cry1Aa Cry1Ab Cry1Ac Cry2A	Technical	2-year chronic—rat (feed)	8400 mg/kg/ day	Statistically significantly decreased body weight gain in females from week 10 to week 104 (not considered related to Cry proteins); no infectivity/ pathogenicity was found.	McClintock *et al.* (1995)
Kurstaki	Cry1Aa Cry1Ab Cry1Ac Cry2Aa	Technical	Human—oral	1000 mg/adult or 1 × 10^{10} spores daily for 3 days	No toxicity/infectivity; all blood cultures were negative; 5 of 10 patients showed viable *Bt* microbes in stool samples 30 days postfeeding.	EPA Fact Sheet (1986) (Abbott) and McClintock *et al.* (1995)
Berliner	Cry1Ab Cry1B	Technical	5-day human oral exposure	1000 mg/adult or 3 × 10^9 spores in capsules daily for 5 days[h]	All subjects remained well during the course of the experiment (~5 weeks) and all laboratory findings were negative (subjects were evaluated before treatment, after the 5-day treatment period, and 4 to 5 weeks posttreatment).	Fisher and Rosner (1959)
Israelensis (Teknar)	Cry4A Cry4B Cry10A Cry11A Cyt1Aa	Technical	Acute oral toxicity/ infectivity (rat)	>1.2 × 10^{11} spores/kg	No evidence of toxicity	McClintock *et al.* (1995)
Israelensis (h-14)	Cry4A Cry4B Cry10A Cry11A Cyt1Aa	Technical	13-week oral (feed) rat	>4000 mg/kg/ day	No evidence of toxicity	McClintock *et al.* (1995)

[a] Doses are expressed in various units for *Bt* microbial technical-grade materials, e.g., mg technical ingredient/kg body wt, or more commonly CFUs or spores/animal or kg body wt. For purposes of comparison with Table 8, it would have been desirable to convert all doses into mg/kg units. Unfortunately, this is not possible since the colony forming units (CFUs) or spore count can range from approximately 10^8 to 10^{11} per gram of technical-grade *Bt* microbial material (McClintock *et al.*, 1995). Second, the Cry protein content in different *Bt* microbial preparations may vary depending on the microorganism and fermentation conditions. It is possible to conclude from Table 7 that the Cry2 protein dosages administered to animals in the referenced studies ranges from milligrams to grams/kg body wt.

[b] Highest dose in the toxicity study that produced no adverse effects. In all referenced studies, the highest tested dose produced no test article related adverse effects.

TABLE 7
Mammalian Toxicity of *Bacillus thuringiensis* Cry Proteins[a] Expressed in Crops: Calculated Dietary Exposure Margins (NOEL Animal Study/Human Exposure Levels)

Cry protein	Type of study	Results (NOEL)[b] mg/kg/day	Toxicity findings	Dietary exposure margin[c]	Reference
Cry1Ab	Acute oral toxicity (mouse)	>4000	No evidence of toxicity	>22,000,000 (corn)	EPA Fact Sheet (1996b) (Monsanto)
Cry1Ab	Acute oral toxicity (mouse)	>3280	No evidence of toxicity	>3,000,000,000 (corn)	EPA Fact Sheet (1995a) (Ciba Seeds)
Cry1Ab	28-day mouse drinking water study	>0.45 via drinking water	No evidence of toxicity, no evidence of immunological responses	>20,000 (tomato)	Noteborn *et al.* (1994)
Cry1Ab	31-day rabbit drinking water study	>0.06 via drinking water	No evidence of toxicity	>2600 (tomato)	Noteborn *et al.* (1994)
Cry1Ac	Acute oral toxicity (mouse)	>4200	No evidence of toxicity	>22,000,000 (cottonseed oil) >16,000,000 (tomato)	EPA Fact Sheet (1995c) (Monsanto)
Cry1Ac	Acute oral toxicity (mouse)	>5000	No evidence of toxicity	>560,000,000 (corn)	Spencer *et al.* (1996) (Dekalb)
Cry2Aa	Acute oral toxicity (mouse)	>4011	No evidence of toxicity	>1,000,000,000 (cottonseed oil)	Monsanto, unpublished
Cry2Ab	Acute oral toxicity (mouse)	>1450	No evidence of toxicity	2,800,000 (corn)	Monsanto, unpublished
Cry3A	Acute oral toxicity (mouse)	>5220	No evidence of toxicity	>652,500 (potato)	EPA Fact Sheet (1995b) (Monsanto)
Cry3Bb	Acute oral toxicity (mouse)	>3780	No evidence of toxicity	>291,000 (corn)	Monsanto, unpublished

[a] In contrast to Table 6, individual Cry proteins rather than microbial mixtures were tested in animals.
[b] Highest dose in the toxicity study that produced no adverse effects. In all referenced studies, the highest tested dose produced no adverse effects.
[c] Exposure margin calculation:

$$\text{Exposure margin} = \frac{\text{Toxicity Study NOEL } (\mu\text{g/kg body wt/day})}{\text{Human Cry Protein Consumption } (\mu\text{g/kg body wt/day})}$$

$$\text{Human Cry Protein Consumption } (\mu\text{g/kg body wt/day}) = \frac{\text{Human Consumption of Food Item (g/day)} \times \text{Maximum Cry Protein Concentration } (\mu\text{g/g})}{\text{Average Human Body Weight (60 kg)}}.$$

Consumption calculations assume that there has been no loss of the Cry protein during processing of food. Human food consumption values were obtained from the USDA TAS database (USDA, 1993) and the GEMS/Food Regional Diets (WHO, 1998). The crop in parentheses refers to the crop for which the respective Cry protein was produced and published or submitted for approval to the EPA.

8400 mg *Bt* microbial product/kg body wt/day. In the 2-year chronic rat feeding study, there were observations of decreased weight gain in females dosed with 8400 mg/kg/day. However, in the absence of other adverse findings, this effect was not considered of toxicological concern and the 8400 mg/kg dose was considered the NOEL (McClintock *et al.*, 1995). In two separate studies, human volunteers have been fed 1000 mg of *Bt* microbial preparations per day for up to 5 days and exhibited no symptoms of toxicity or other ill effects (Table 6). The *Bt* preparations used in the human feeding studies contained genes encoding the following Cry protein families: Cry1Aa, Cry1Ac, Cry1Ab, Cry1B, and Cry2A.

EPA guidance documents for reregistration of *Bt* microbial formulations (EPA, 1988a) and other published literature contain additional references to mammalian toxicology studies in which animals have been administered *Bt* microbial preparations via one of several nonoral routes of exposure, such as pulmonary, dermal, ocular, intraperitoneal, subcutaneous, intravenous, or intracerebral injection. These studies were done to assess the potential pathogenicity/infectivity of the *B. thuringiensis* organisms in the microbial formulations. These studies were also performed as quality control measures to confirm the absence of non-Cry protein toxins (e.g., exotoxins) which can be produced in certain *Bt* microbial strains. When large doses (10^8 CFUs) of *Bt* microorganisms were administered by injection to rodents, there were occasional reports of mortality in test animals. Mortality was also observed in rodents injected with similar large doses of related

nonpathogenic bacteria, e.g., *Bacillus subtilis*. Since mortality can occur following injection of large doses of nonpathogenic microorganisms, the mortality observed in rodents given large doses of *Bt* microbes was not attributed to the Cry proteins present in *Bt* microbial formulations (EPA, 1998a; McClintock *et al.*, 1995). The results of injection and irritation studies are not summarized here because they are not relevant to assessing potential health risks from dietary exposure to Cry proteins produced *in planta*.

The safety testing requirements for registration of *Bt* microbial products has evolved over the years based on EPA review of completed toxicity/pathogenicity studies in 1982, in 1989, and again in 1998 (EPA, 1998a,b). While subchronic and chronic safety studies were conducted with the first *Bt* microbial products that were developed, the EPA has subsequently decided that acute hazard assessment is sufficient to assess the safety of new *Bt* microbial products. This decision is based on the fact that Cry proteins in *Bt* microbial products act through acute mechanisms to control insect pests, and these mechanisms are not functional in man. "A battery of acute toxicity/pathogenicity studies is considered sufficient by the Agency to perform a risk assessment for microbial pesticides. Furthermore, the *Bacillus thuringiensis* delta-endotoxins affect insects via a well known mechanism in which they bind to unique receptor sites on the cell membrane of the insect gut, thereby forming pores and disrupting the osmotic balance. There are no known equivalent receptor sites in mammalian species which could be affected, regardless of the age of the individual. Thus, there is a reasonable certainty that no harm will result to infants and children from dietary exposures to residues of *Bacillus thuringiensis*" (EPA, 1998a).

Cry proteins produced in Bt-protected plants are nontoxic to mammals. For safety assessment of Cry proteins expressed *in planta*, acute toxicity testing along with digestive fate testing *in vitro* is considered appropriate and sufficient to assess health risks from dietary exposure to Cry proteins (Sjoblad *et al.*, 1992). Pathogenicity and infectivity testing, which has been conducted with viable *Bt* microbial technical-grade material would be inappropriate for Cry proteins. Dermal, ocular, and inhalation exposure testing is generally not appropriate since farm worker exposure to Cry proteins expressed in plants is anticipated to be negligible. In plants, Cry proteins are expressed at low levels (ppm) and contained within the cells of the plants.

All of the mammalian toxicity testing of individual Cry proteins expressed *Bt*-protected plants has demonstrated an absence of toxicity. No treatment-related adverse effects have been observed in any of the acute oral mammalian toxicity studies conducted with individual representatives of the Cry1, Cry2, and Cry3 family of proteins (Table 7). The NOELs for these Cry proteins range up to 5220 mg/kg. These exposure levels which produced no toxicity are thousands to millions of times higher than potential dietary exposures to these proteins (Table 7). For example, the expression level of Cry1Ab in corn grain is approximately 1 ppm. A 60-kg person would have to eat 120,000 kg/day of corn grain to achieve the same acute high dose of 4000 mg/kg Cry1Ab protein which produced no adverse effects when fed to mice (Table 7). Based on the lack of toxic effects and the large margins of safety for both dietary exposures, it is concluded that these Cry proteins pose no foreseeable risks to human or animal health.

Cry proteins are highly specific. Mammals and most other species are not susceptible to Cry proteins. This is explained, in part, by the fact that conditions required for the complex steps in the mode of action described by English and Slatin (1992) do not exist in mammals or most invertebrates. Cry proteins must first be solubilized. The Cry1 class of Cry proteins require alkaline pH's to be soluble, with pH values of 10 or above required for effective solubility. At the pH 1.2 of the gastrointestinal tract of humans, the Cry proteins have extremely limited solubility (English and Slatin, 1992). Some of the Cry proteins must then be proteolytically digested to the insecticidally active form. Cry proteins must remain active rather than being further degraded. Data in the next section will show that Cry proteins are rapidly degraded under conditions which simulate the gastrointestinal conditions of the mammalian system. Therefore, these Cry proteins will be rapidly degraded and inactivated upon consumption. Finally, receptor-mediated binding to the brush-border membrane in midgut epithelium cells leads to membrane-bound forms of the Cry protein. This is believed to take place in three steps: binding to midgut receptor proteins, partitioning into the brush-border membrane, and, finally, forming channels and pores.

Binding to these receptors is required for a Cry protein to exert any activity (English and Stalin, 1992). If receptor binding does not occur, the Cry protein will have no effect on that organism. Noteborn *et al.* (1993) detected no specific binding of Cry1Ab protein to mouse and rat gastrointestinal tract tissue *in vivo*. These researchers also adapted an *in vitro* immunocytochemical assay (for detecting Cry protein binding in insect cells) to evaluate binding of Cry1Ab protein to mammalian gut tissue sections. Their analysis of mouse, rat, monkey, and human tissue sections did not reveal any Cry1Ab-binding sites in these tissues. These results are consistent with those of Hofmann *et al.* (1988) who did not detect specific binding of Cry protein to rat intestinal cell membrane preparations. These findings further support the dietary safety of Cry proteins for humans and animals due to: (1) the lack of appropriate conditions to solubilize the Cry proteins; (2) the rapid

TABLE 8
***In Vitro* Digestibility of *Bacillus thuringiensis* Cry Proteins in Simulated Gastric Fluid**

Cry protein	Results	Findings	Reference
Cry1Ab	Degraded within 30 s	No longer detectable after incubation for 30 s in gastric fluid; in intestinal fluid, converted to trypsin-resistant core, as expected.	EPA Fact Sheet (1996b) (Monsanto)
Cry1Ab	Degraded within 1 min	Rapidly degraded in the presence of pepsin.	EPA Fact Sheet (1995a) (Ciba Seeds)
Cry1Ab	Substantially degraded	Study of the activity of Cry1Ab under simulated GI tract conditions and applying multienzymatic methods, the Cry1Ab protein was substantially degraded via digestion.	Noteborn *et al.* (1994)
Cry1Ac	Degraded within 30 s	No longer detectable after incubation for 30 s in gastric fluid; in intestinal fluid, converted to trypsin-resistant core, as expected.	EPA Fact Sheet (1995c) (Monsanto)
Cry1Ac	Degraded within 30 s	The protein was found to rapidly degrade in full strength and diluted simulated gastric fluid; degraded to below detection limits after a few seconds in full strength simulated gastric fluid; in simulated gastric fluid in which the pepsin concentration had been reduced 110-fold, Cry1Ac degraded to below detection in 5 min.	Spencer *et al.* (1996) (Dekalb)
Cry2Aa	Degraded within 30 s	No longer detectable after incubation for less than 30 s in gastric fluid; in intestinal fluid, converted to trypsin-resistant core, as expected.	Monsanto, unpublished
Cry3A	Degraded within 30 s	No longer detectable after incubation for less than 30 s in gastric fluid; in intestinal fluid, converted to trypsin-resistant core, as expected.	EPA Fact Sheet (1995b) (Monsanto)

degradation of the Cry proteins upon consumption; and (3) the lack of Cry-specific receptors, which are required for Cry activity.

Cry proteins are rapidly digested. Cry1, Cry2, and Cry3 classes of proteins have been shown to be rapidly degraded *in vitro* using simulated gastric fluids (Table 8). Results of seven *in vitro* assays conducted with representative Cry1, Cry2, and Cry3 proteins establish that the proteins are rapidly degraded, usually within 30 s in *in vitro* simulated digestion models (Table 8). Cry proteins range in size from approximately 60 to 130 kDa in size. These proteins are degraded in the simulated digestion models to polypeptides of less than 2 kDa, which translates to less than 10 amino acids in length, the lower limit of separation on Western blot analyses. These *in vitro* models are significantly less robust than the gastrointestinal systems of humans and animals, which suggests that the Cry proteins will be rapidly and extensively degraded upon consumption.

The demonstrated rapid degradation of Cry1, Cry2, and Cry3 classes of proteins following ingestion minimizes the potential for the protein to induce an allergic reaction since the potential for absorption is greatly reduced. Food allergens generally persist in the gastrointestinal model, whereas common food proteins with no allergenic history degrade rapidly in simulated gastric fluid (Metcalfe *et al.*, 1996; Astwood *et al.*, 1996). In addition, the very low level of these Cry proteins in the food greatly decreases the extent of exposure and hence the likelihood of absorption. To further investigate the potential for allergenicity, searches of allergen sequence databases have been conducted. These searches have shown no significant matches with the Cry1, Cry2, and Cry3 classes of proteins to known allergens (Metcalfe *et al.*, 1996; Astwood *et al.*, 1996; EPA Fact Sheets, 1995a,b,c, 1996b, 1997), establishing a lack of structural similarity to known allergenic proteins. In addition, in the almost 40-year history of their commercial use, there have been no confirmed cases of allergenic reactions to the microbial *Bt* products (61 FR 40340, August 2, 1996).

Modified Cry proteins pose no unique concerns. The Cry proteins in *Bt*-protected plants typically differ slightly, if at all, from their naturally occurring Cry counterparts. Some of the plant-expressed proteins are truncated, resembling the naturally occurring proteins after they are cleaved in the insect gut, while others may vary from the natural proteins by a few amino acids. There is no reason to expect that these genetically modified proteins pose any unique human health concerns compared to their naturally occurring counterparts. In fact, there is evidence that modified Cry proteins have already been generated in nature. The Cry1Ab δ-endotoxin appears to have arisen from a recombination event between ancestral *cry1Aa*- and *cry1Ac*-like toxin genes (Geiser *et al.*, 1986).

Similarly, amino acid sequence alignments of Cry1Ca, Cry1Cb, Cry1Ea, and Cry1Eb provide evidence that *cry1Ea* and *cry1Cb* could have arisen from a recombination event between ancestral *cry1Ca* and *cry1Eb* toxin genes (Thompson *et al.*, 1995). Multiple alignments of the Cry1Ca, Cry1Cb, Cry1Ea, and Cry1Eb amino acid sequences highlight the probable recombination site near amino acid 450. Analyses such as this suggest that recombination between related *cry* genes is a normal process in *cry* gene evolution.

TABLE 9

Comparison of the Biochemical Characteristics of Cry and Selectable Marker Proteins and Known Allergenic Proteins

Characteristic	Allergens	Cry or marker proteins
Prevalent protein in food	Yes	No
Stable to digestion	Yes	No
Stable to processing	Yes	No

Source: Fuchs *et al.* (1993b); Sanders *et al.* (1998); Lavrik *et al.* (1995); Berberich *et al.* (1996).

Food safety of Bt-protected crops is established. The safety of *Bt*-protected crops currently in the market as human food or animal feed has been established. In addition to assessing the safety of the Cry proteins, the edible portions of the crops and the accompanying selectable marker gene proteins have been examined. *Bt*-protected corn, cotton, and potato crops have been shown to be substantially equivalent (comparable in composition) to their non-*Bt* counterparts. No biologically significant differences in the composition of components including grain, seed, tuber, oil, silage, or other crop by-products have been observed between *Bt*-protected crops and their non-*Bt* counterparts (Sanders *et al.*, 1998; Lavrik *et al.*, 1995; Berberich *et al.*, 1996). The only difference is that *Bt*-protected crops provide protection against certain pests by virtue of the expression of the specific Cry protein, which, along with the marker protein, has been shown to be safe for human consumption.

Analysis of the agronomic and morphological characteristics of *Bt*-protected crops confirm the efficacy and stability of the introduced traits and the lack of significant unintended effects that may be attributable to the genetic modification process. *Bt*-protected crops meet the stringent product performance standards established for new plant varieties. Evaluations consisting of plant vigor, growth habit characteristics, yield, crop quality, and insect and disease susceptibility have shown *Bt*-protected crops to be morphologically and ergonomically equivalent to their parental plants.

Detailed molecular analyses have been performed on each *Bt*-protected crop to characterize and confirm that the intended genetic material has been introduced. Further analyses confirm that the Cry and marker proteins are produced as predicted from the molecular characterization. Cry proteins have a long history of safe use in microbial *Bt* products. To confirm the safety of Cry proteins in *Bt*-protected crops, additional mammalian toxicology studies have been conducted as described earlier.

The neomycin phosphotransferase II (NPTII)-selectable marker protein has been used in *Bt*-protected cotton and potato products to enable selection of the rare cells which have acquired the *Bt* gene. The *nptII* gene and encoded protein has a history of safe use based on the ubiquitous presence of the *nptII* gene and hence the encoded protein in gut and soil microbes. The safety of the NPTII protein has been evaluated in mouse acute oral and digestive fate studies comparable to those studies described above for Cry proteins (Fuchs *et al.*, 1993a,b). No adverse effects were observed in the acute oral studies with up to a 5 million-fold higher level than that from the projected consumption. The marker proteins were rapidly degraded, e.g., half-life less than 30 s, in simulated digestive fate studies.

The Cry- and NPTII-selectable marker proteins have been shown to pose no significant allergic concerns. Commonly allergenic proteins are typically prevalent in food, stable to the acidic and proteolytic conditions of the digestive system and stable to food processing and are glycosylated (Taylor and Lehrer, 1996). None of the three classes Cry proteins (Cry1, Cry2, or Cry3 classes) nor the NPTII-selectable marker protein share any of these characteristics (Table 9). Although none of these biochemical criteria alone enable prediction of the allergenic potential of proteins, the combination of the characteristics provides a strong basis to conclude that these proteins do not pose a significant allergic concern. The lack of any reports of sensitization to the commercial microbial formulations also supports the lack of allergic concerns with the Cry proteins (McClintock *et al.*, 1995).

The important nutrients and antinutrients have been assessed in detail for each of the *Bt*-protected crops and compared to their parental lines and to the literature published on crop varieties. A list of the individual components analyzed for these crops is

TABLE 10

Recommended List of Key Nutrients and Antinutrients to Establish Substantial Equivalence in Representative Crops[a]

Component	Corn	Cotton	Potato
Protein	√	√	√
Fat	√	√	
Starch (carbohydrates)[b]	√		√
Specific gravity (solids)			√
Crude fiber		√	√
Acid detergent fiber	√		
Neutral detergent fiber	√		
Individual amino acids	√	√	
Major fatty acids	√	√	
Gossypol		√	
Minerals	√		√
Vitamins	√	√	√
Glycoalkaloids			√
Cyclopropenoid fatty acids		√	
Mycotoxins	√	√	

[a] In seed, grain, tuber, or fruit of the pertinent crop.
[b] Determined by calculation.

TABLE 11
Toxicity of Cry Proteins to Nontarget Organisms

Nontarget organism	Test results and findings		
	Cry3A (potato)[a]	Cry1Ac (cotton, corn)[b]	Cry1Ab (corn)[c]
Lady bird beetle	Practically nontoxic	Practically nontoxic: fed at 1,700× and 10,000× level in cotton pollen and nectar	Practically nontoxic NOEC > 20 ppm
Collembola	NOEC > 200 ppm[d]	NOEC > 200 ppm[d]	NOEC > 200 ppm[d]
Honey bee	Practically nontoxic to larvae	Practically nontoxic: fed at 1,700× and 10,000× level in cotton pollen and nectar	Practically nontoxic to larvae NOEC > 20 ppm (larvae)
Earthworm	—	—	Practically nontoxic NOEC > 20 ppm
Parasitic wasp	Practically nontoxic	Practically nontoxic: fed at 1,700× and 10,000× level in cotton pollen and nectar	Practically nontoxic NOEC > 20 ppm
Green lacewing	Practically nontoxic	Practically nontoxic: fed at 1,700× and 10,000× level in cotton pollen and nectar	Practically nontoxic NOEC > 16.7 ppm
Bobwhite quail	Practically nontoxic LC_{50} > 50,000 ppm (potato tubers)	Practically nontoxic	NOEC > 100,000 ppm corn grain containing the Cry1Ab protein
Daphnia	NA[e]	NA	Practically nontoxic NOEC > 100 ppm of corn pollen containing Cry1Ab[f]
Fish	NA	—	No effect on channel catfish fed ground corn grain containing Cry1Ab protein (35%)

Note. NOEC refers to the no observed effect concentration.
[a] *Source:* EPA Fact Sheet (1995b) (Monsanto Cry3A).
[b] *Source:* EPA Fact Sheet (1995c) (Monsanto Cry1Ac).
[c] *Source:* EPA Fact Sheet (1996b) (Monsanto Cry1Ab).
[d] Sims and Martin (1997).
[e] Not applicable.
[f] Graves and Swigert (1997).

shown in Table 10. The data derived from these analyses establish that the commercially introduced *Bt*-protected crops (corn, cotton, potato) are substantially equivalent in composition to their parental lines (Sanders *et al.*, 1998; Lavrik *et al.*, 1995; Berberich *et al.*, 1996). In summary, the data generated for the safety of Cry and marker proteins and the compositional analyses confirm the safety of the food and feed derived from *Bt*-protected crops which are fully approved for marketing.

Environmental Implications

Environmental impact of Cry proteins. The U.S. EPA has concluded "that toxicity and infectivity risks due to δ-endotoxin effects to nontarget avian, freshwater fish, freshwater aquatic invertebrates, estuarine and marine animals, arthropod predators/parasites, honey bees, annelids, and mammalian wildlife will be minimal to nonexistent at the label use rates of registered *B. thuringiensis* active ingredients" (EPA, 1998a). This provides strong evidence that Cry proteins in produced *Bt*-protected plants approved for marketing will pose low risk to nontarget organisms. The level of exposure to Cry protein in *Bt* plants would be significantly lower than that resulting from the application of *Bt* microbial products at label rates. However, because microbial Cry proteins degrade rapidly and Cry proteins are typically produced continuously in *Bt* plants, the duration of exposure in the environment would likely be longer for *Bt*-protected crops compared to microbial *Bt* products.

The Cry proteins expressed in fully approved *Bt* products in the United States have been shown to have little or no effect on birds, fish, aquatic invertebrates, and a wide range of beneficial insects (Table 11). Studies administering the respective purified Cry protein produced in microbial systems were conducted to characterize the toxicological properties of these proteins to nontarget organisms. Grain, pollen, or leaf tissue from the various *Bt*-protected pesticidal plant products was also fed to animals to more closely simulate actual exposure to the protein. In general, no mortality or

behavioral effects were observed in nontarget animals fed Cry protein in amounts that exceed actual exposure by at least 10-fold and usually more than 100-fold. In no instances were adverse effects observed at Cry protein levels approaching those that would occur under actual use conditions. Overall, Cry proteins are characterized as being practically nontoxic to nontarget organisms (EPA Fact Sheet, 1996c).

The lack of activity of Cry proteins against nontarget organisms is not surprising in view of their high degree of target specificity. Even the beneficial predatory ladybird beetle (related to the Colorado potato beetle) is unaffected by Cry3Aa, a protein that is highly effective against an important pest, the Colorado potato beetle (Dogan *et al.*, 1996). Because many of the Cry proteins are effective against lepidopteran pests, activity against nonpest lepidopterans (butterflies and moths) might be anticipated; however, *Bt*-protected plants generally pose very low risk to these nontarget species for several reasons. First, like other nontarget organisms, nonpest lepidopterans may not be susceptible to the Cry protein. If nontarget organisms are sensitive, typically only the first few instars are sensitive. For example, adult insects, even for target insects, show greatly reduced susceptibility. Halcomb *et al.* (1996) showed that the fifth instar of tobacco budworm and pink bollworm are much less sensitive to Cry1Ac than earlier instars of these insects. Second, the potential for exposure to Cry protein in *Bt*-protected plants is extremely limited unless the insect feeds directly on the plant or plant parts. Caterpillars that feed directly on *Bt*-protected crops will be exposed, but they are generally considered to be pests. Adult butterflies and moths may visit flowering *Bt*-protected crops to feed on nectar, but little or no Cry protein is present in nectar and, in any case, the adult life stages of lepidopterans are not sensitive to Cry protein.

Inadvertent feeding by caterpillar larvae on windblown pollen appears to be a potential opportunity for exposure of Cry protein to nontarget lepidopterans (i.e., monarch butterfly). This situation poses a potential hazard only if all of the following spatial, temporal, and biological conditions are met: (1) the pollen must contain some level of Cry protein; (2) sufficient quantities of the Cry pollen must be dispersed onto, and remain on, plants fed upon by nontarget lepidopterans; (3) the lepidopteran must be sensitive to Cry protein; (4) pollen shed must occur when the larvae are in the Cry-sensitive early instar stages of development; and (5) the larvae must ingest Cry pollen in sufficient quantities to alter normal larval development. Occasionally, all of these circumstances may coincide for a limited portion of the insect population. However, there is no indication that such conditions occur with such frequency as to pose any significant hazard to populations of monarch butterflies (Sears *et al.*, 1999; Sears and Stanley-Horn, 2000).

Following the publication of a controversial laboratory experiment (Losey *et al.*, 1999), several studies were initiated to quantify the concentration of corn pollen on leaves of milkweed plants (the primary source of exposure to corn pollen for monarch butterflies) during the peak period of pollen shed. Dively *et al.* (2000) examined pollen deposition within and at the edges of corn fields at 81 sites in Delaware and five sites in Nebraska. They found mean pollen deposition in Delaware ranged from 56.8 grains/cm^2 of leaf area inside and at the field edge, to 21.8 grains/cm^2 within the first 5 m from the field edge, to 12.7 grains/cm^2 within 6 to 10 m from the field edge, and after 10 m, pollen deposition averaged 7.0 grains/cm^2 or less. In Nebraska, the pollen deposition values ranged from 6.0 grains/cm^2 at the field edge to less than 1.0 grains/cm^2 beyond 10 m from the field edge. Observed differences between these sites was likely due to differences in rainfall and average wind speed between the two sites. Pleasants *et al.* (1999) reported that heavy rainfall reduced the amount of pollen initially deposited on leaves by approximately 90%.

Dively *et al.* (2000) calculated the percentage of milkweed leaves within and at various distances from the field edge with pollen densities exceeding 150 grains/cm^2 from the empirical data. The level of 150 grains/cm^2 was selected since Hellmich *et al.* (2000) reported that survival and growth of young monarch larvae were not impacted when larvae consumed milkweed leaf tissue treated with 150 grains/cm^2 of *Bt* corn pollen. Dively *et al.* (2000) found that only 8% of the leaves within and at the edge of the corn field and only 2% of the leaves from 1 to 5 m away from the field had deposits of pollen exceeding 150 grains/cm^2. Similarly, Pleasants *et al.* (1999) found that higher pollen densities on milkweed leaves (150 grains/cm^2) are not present beyond 2 m from the field edge. When the pollen deposition and toxicity data are considered together, the probability that monarch larvae would be exposed to harmful concentrations of *Bt* corn pollen (i.e., *Bt* pollen deposition that exceeds 150 grains/cm^2) is low even within the corn field where the majority of corn pollen is deposited. The conclusions reached for monarch butterflies reiterate a basic tenant for risk assessment; that risk is the combination of the potential to cause harm and exposure. Exposure must be factored into the assessment to reach scientifically valid conclusions.

Environmental impact of Bt-protected plants. Because Cry proteins have little or no effect on nontarget vertebrates and invertebrates, including a wide range of beneficial insects, nontarget populations are expected to increase as the introduction of *Bt* crops leads to reductions in broad-spectrum insecticide use. These population increases will result in overall increases in biodiversity within agricultural systems and reduced

disruption in a number of key ecological processes. As discussed in earlier sections, nontarget insect populations are expected to increase as broad-spectrum insecticide use decreases, and this has been confirmed in the case of *Bt* cotton and *Bt* potatoes. These population increases allow biological control to play a greater role in the population regulation of primary and secondary pest species. Impacts on biological processes occurring in the soil also will be reduced as *Bt* crops, with their absence of effects on important soil-dwelling invertebrates like Collembola and earthworms, replace conventional insecticides that negatively affect these same species. At higher trophic levels, there have been anecdotal reports within the United States of increasing populations of vertebrates, particularly birds, associated with reductions in the use of various insecticides. These reports have ranged from observations of hummingbirds in *Bt* cotton fields to quail around *Bt* corn fields.

Cry proteins can enter the soil through incorporation of plant material in the soil. Data from a number of studies show that the Cry proteins are rapidly degraded in soil, at rates comparable to the rate of degradation of Cry proteins in microbial *Bt* products (Palm *et al.*, 1993, 1994; 1996; Ream *et al.*, 1992; Sims and Holden, 1996). Data have been generated for the Cry proteins produced in each *Bt*-protected crop for regulatory submissions (Ream *et al.*, 1992; Sims and Holden, 1996). In addition, EPA scientists have reported data on the Cry3Aa protein in *Bt* potato and the Cry1Ab and Cry1Ac proteins which were produced in *Bt* cotton lines, which show that these Cry proteins are rapidly degraded in the soil (Palm *et al.*, 1993, 1994, 1996). Recently, questions have been raised in a letter to *Nature* regarding the rate of degradation of the Cry proteins contained in *Bt* crops after the crop has been harvested and the plant residue incorporated into the soil (Saxena *et al.*, 1999). Based on the information presented above, the stringent specificity of these Cry proteins and their rapid degradation in the soil provide strong evidence that the Cry proteins in crop residues from current *Bt* crops do not pose a significant risk to the environment.

The widespread planting of *Bt*-protected crops also raises questions of the potential for the flow or spread of *cry* genes to wild plant species and the development of pest resistance to Cry proteins. These issues have been thoroughly examined and were addressed prior to deployment of *Bt*-protected potato, corn, and cotton in the United States, Canada, Mexico, Argentina, and other countries in which these products have been approved or are considering approval.

With respect to gene flow, the taxonomy, genetics, mode of reproduction, and outcrossing potential for cotton, corn, and potato establish either: (1) the inability of *cry* genes introduced into commercial varieties of these crops to outcross to wild species or (2) that if gene flow occurs, the potential impact is assessed. There is no outcrossing issue for corn in the United States because the crop has no wild relatives in the United States, whereas outcrossing to wild species in Mexico is being assessed in detail due to the presence of compatible wild species.

Currently, Russet Burbank is the most common commercialized *Bt*-protected potato cultivar. Because this cultivar is male sterile, gene flow is precluded. In addition, potato is not sexually compatible with any related species in North America, so outcrossing is not a concern.

In the case of cotton, there are only two wild relatives that occur in the United States—*Gossypium thurberi* in Arizona and *G. tomentosum* in Hawaii—that could possibly outcross with commercial varieties of cotton (Fryxell, 1979; Stephens, 1964). Cultivated cotton is an allotetraploid, whereas *G. thurberi* is a diploid, so these are incompatible and would not produce fertile offspring. *G. tomentosum* is morphologically and temporally incompatible with commercial cotton varieties. There is, therefore, no reasonable mechanism for outcrossing of the genes introduced into wild cotton relatives in the United States (Fuchs *et al.*, 1993c).

As additional *Bt*-protected crops are developed, the potential for and potential impact of gene flow from these crops to wild species will have to be determined. If outcrossing can occur, questions such as the potential to confer increased fitness to the recipient plant and the potential exposure of nontarget organisms to Cry protein will need to be assessed on a case-by-case basis.

Pest populations exposed to *Bt*-protected crops have the potential to develop resistance to Cry proteins. Resistance is not unique to *Bt*-protected crops. Resistance has arisen repeatedly with conventional chemical insecticides. In fact, one reason for the wide acceptance of *Bt* cotton has been the reduced efficacy of synthetic pyrethroid compounds due to the onset of pest resistance (Smith, 1999).

In the United States, companies in partnership with the regulatory authorities have implemented aggressive resistance management plans to ensure the prolonged efficacy of *Bt*-protected crops. A key element of these plans is to require that growers plant sufficient acreage of non-*Bt* crops to serve as a refuge for *Bt*-sensitive pests. The refuge strategy is designed to ensure that *Bt*-sensitive pests will be available to mate with *Bt*-resistant pests, should they arise. The offspring of these matings will be *Bt* sensitive, thus mitigating the spread of resistance in the population (Gould, 1998). Management plans also call for monitoring and reporting of any incidents of resistance so that remedial actions can be taken. Looking to the future, additional *cry* genes and/or other insecticidal genes will be combined in crops to increase the durability of the Cry proteins for insect control. By simul-

taneously presenting pest populations with multiple modes of action, the onset of resistance is expected to be slowed dramatically.

CONCLUSIONS

Advantages

Bt-protected crops, particularly corn and cotton, have demonstrated significant benefits since their introduction beginning in 1995/1996. These products provide a level of insect protection that is generally superior to that of conventional chemical pesticides. As a result, *Bt*-protected crops require fewer applications of externally applied pesticides, thus significantly reducing the overall use of chemical pest control products and preserving the population of beneficial insects. *Bt*-protected cotton and corn provide higher crop yields and economic value to growers and *Bt* corn results in reduced levels of the fungal toxin (fumonisin) in the harvested corn crop.

Safety

Bt-protected plants are thoroughly studied before they are introduced into commercial agriculture. These studies establish that:

- Cry and marker proteins are not toxic to humans and pose no significant concern for allergenicity;
- *Bt*-protected plants are substantially equivalent to their non-*Bt* counterparts, except for the presence of the Cry and marker proteins;
- Based on the previous two points, food and feed derived from *Bt*-protected crops are safe to consume;
- Cry proteins are virtually nontoxic to nontarget organisms, except for certain insects that are closely related to the target pest; and
- Cry and marker proteins and the *Bt*-protected plants themselves pose no foreseeable risks to the environment.

Numerous regulatory authorities around the world have evaluated the data on *Bt*-protected crops. Consistent with their regulatory mandates, they have concluded that these products are safe and fully suitable for introduction into commercial agriculture. These affirmations are further supported by the almost 40-year history of safe use of Cry proteins in *Bt* microbial products around the world.

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