New Leaf (Cry3A) Potato Case Study as Guidance for SpuntaG2 Cry1Ia1 (Cry V) Potato

Specialty Crop
Regulatory Assistance
Workshop
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http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm

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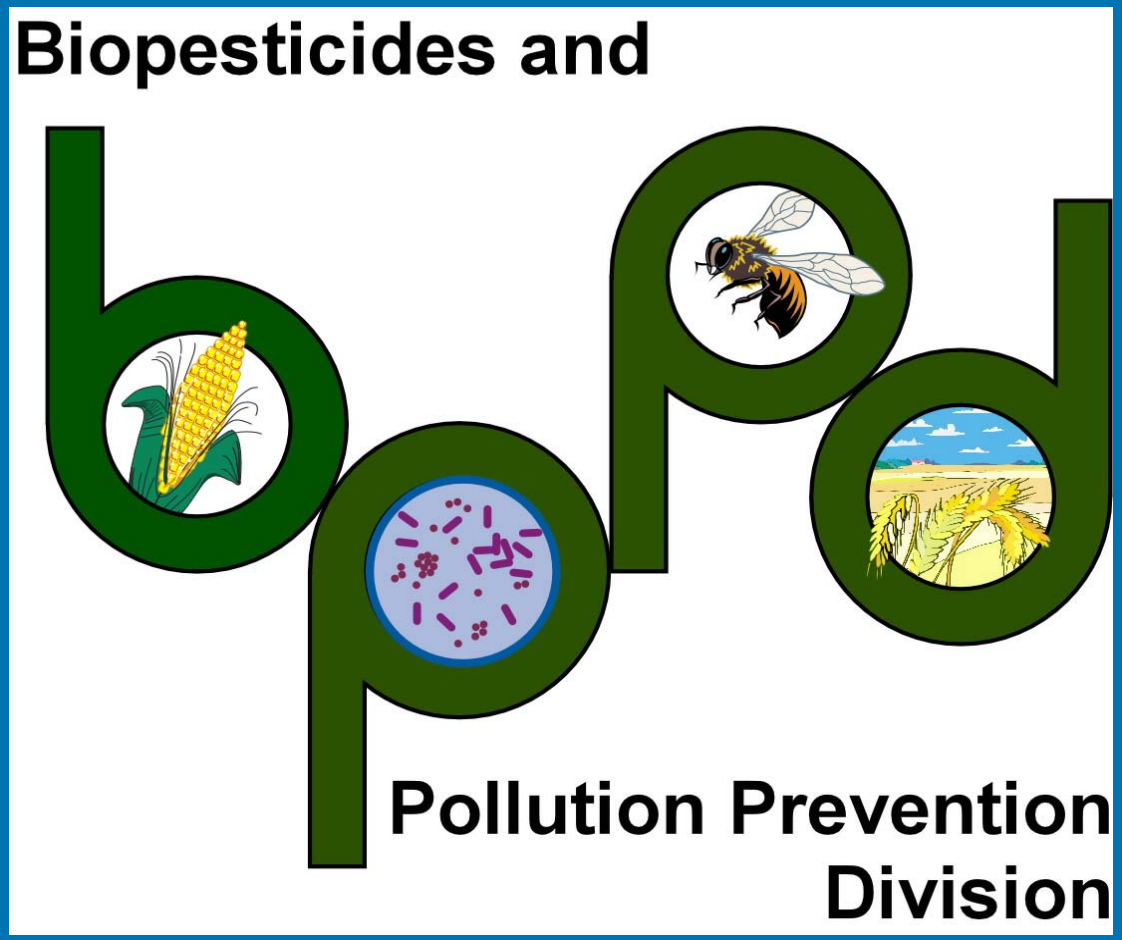
Biopesticides and
Pollution Prevention
Division

Environmental Protection Agency

- Regulates pesticidal substance (e.g. Bt Cry protein) and the genetic material necessary for its production in the plant (e.g. cry gene)

- Plant-Incorporated Protectants (PIPs)

Pesticide Laws

- Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)
- Federal Food, Drug, and Cosmetic Act (FFDCA)
- Food Quality Protection Act (FQPA)

code of federal regulations

40 CFR Part 174

Contains detailed information on PIPs

Product Characterization

- Transformation System
- Characterization of the DNA Inserted in the Plant
- Inheritance and Stability after Transformation
- Protein Characterization and Expression

Study Type	Result	MRID #
Protein Characterization and Expression	Ten insect pest species from 5 families were tested for their sensitivity to *B.t.k.* HD-73 protein. Only in the lepidopteran species was there significant mortality. In one study, the green peach aphid showed marginal effects from treatment with a tryptic digest of the Cry1Ac toxin from *B.t.k.* HD-73 which was not reproducible in a repeat test. The tryptic digest preparation positive control from a *B.t.k* species also showed higher mortality in the tobacco budworm test than that produced in *E. coli.* CLASSIFICATION: ACCEPTABLE.	431452-04

5. Product Characterization of Cry3A Potato (006432)

Monsanto submitted information which adequately described the plant-pesticidal substance, *Bacillus thuringiensis* subsp. *tenebrionis* Cry3A delta endotoxin as produced in potato. Because it would be difficult, or impossible, to extract sufficient biologically-active toxin from the plants to perform toxicology tests, Monsanto used an endotoxin produced in bacteria. Product analysis data were submitted to show that the microbially expressed and purified *Bt* Cry3A delta endotoxin is sufficiently similar to that expressed in the plant to be used for mammalian toxicological purposes.

Study Type	Result	MRID #
Characterization of the DNA Inserted in the Plant	The relative size and number of copies of the DNA inserted into potatoes was demonstrated with endonuclease digested chromosomal DNA from field grown potato plants Southern blotted with the introduced plasmid as the probe. These Southern blots provided information about the number of copies of introduced DNA, the lack of significant amount of DNA introduced outside the border regions and integrity of the introduced DNA near the endonuclease cut site. These results indicate that only the DNA necessary to produce the Cry3A delta endotoxin were introduced into the plant. CLASSIFICATION: ACCEPTABLE	429322-01
Protein Characterization and Expression	Microbially-produced delta endotoxin from the *cry3A* gene as expressed in Escherichia coli and in potato tubers was compared. The data consist of SDS-PAGE co-migration, western blot analysis, staining for carbohydrate residues, N-terminal amino acid sequence analysis and biological equivalence against *Leptinotarsa decemlineata.* These data are adequate to support the equivalence of the microbially- and plant-produced protein for use in the toxicology studies. CLASSIFICATION: ACCEPTABLE	429322-02

Study Type	Result	MRID #
Protein Characterization and Expression	The purity and activity of a 55kD protein released with tryptic digestion of the *Bt* Cry3A delta endotoxin purified from *E. coli* was shown to have a similar size, immunoreactivity and amino acid sequence to the 55kD fragment found in potato tubers. The 55kD protein had somewhat higher bioactivity than the 68kD full-length delta endotoxin from *B.t.t.* These data support the contention that both the 55kD and 68kD forms of the Cry3A delta endotoxin found in the plant were similar to those occurring in *B.t.t.* CLASSIFICATION: ACCEPTABLE	429322-05
Characterization of *E. coli*-Produced Cry3A Protein	The method of preparing by fermentation the delta endotoxin from *B.t.t.* in *E. coli* was presented. The protein was characterized for purity and stability after purification. This data indicates that normal fermentation techniques were used to produce the plant equivalent, microbial Cry3A delta endotoxin. CLASSIFICATION: ACCEPTABLE	429322-04
Protein Characterization and Expression	The Cry3A delta endotoxin as expressed in potato tissue or an *E. coli* alternative gives a similar immunoreactivity and electrophoretic mobility to registered microbial products producing the same delta endotoxin. CLASSIFICATION: ACCEPTABLE	429322-06

B. HUMAN HEALTH ASSESSMENT

1. Background

The basic premise relied on for the toxicology assessment is the fact that all the *Bt* plant-incorporated protectants are proteins. Proteins are commonly found in the diet and, except for a few well described phenomena, present little risk as a mammalian hazard. In addition, for the majority of *Bt* proteins currently registered, the source bacterium has been a registered microbial pesticide which has been approved for use on food crops without specific restrictions. Because of their use as microbial pesticides, a long history of safe use is associated with many *Bt* products.

Several types of data are required for the *Bt* plant-incorporated protectants to provide a reasonable certainty that no harm will result from the aggregate exposure to these proteins. The information is intended to show that the *Bt* protein behaves as would be expected of a dietary protein, is not structurally related to any known food allergen or protein toxin, and does not display any oral toxicity when administered at high doses. These data consist of an *in vitro* digestion assay, amino acid sequence homology comparisons and an acute oral toxicity test. The acute oral toxicity test is done at a maximum hazard dose using purified protein of the plant-incorporated protectant as a test substance. Due to limitations of obtaining sufficient quantities of pure protein test substance from the plant itself, an alternative production source of the protein

Human Health

- *In vitro* Digestibility Assay
- Amino Acid Homology
- Acute Oral Toxicity

Human Health

- ***In vitro* Digestibility Assay**

- A global amino acid sequence comparison for the PIP protein must be conducted. The protein must be compared to other amino acid sequences in available databases to identify any significant global amino acid sequence similarities with known putative toxins, anti-nutrients, and allergens.

Human Health

- ***In vitro* Digestibility Assay**

- Analysis of whether the amino acid sequence of the PIP protein has any significant amino acid sequence similarities to known allergens or allergen epitopes.

material encoding the Cry1Ac delta-endotoxin and (2) its regulatory regions. ``Regulatory regions'' are the genetic material that control the expression of the genetic material encoding the Cry1Ac delta-endotoxin, such as promoters, terminators, and enhancers.

f) Occupational Exposure and Risk Characterization

Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Worker exposure to the Cry protein via seed dust is also expected to be negligible because of the low amount of protein expressed in transformed plants. If such exposure should occur, the Agency concludes that such exposure would not be expected to present any risk due to the lack of toxicity. However, if any unreasonable adverse effects caused by exposure to Cry1Ac are identified, these effects must be reported to the Agency as described in Sec. 6(a)(2) of FIFRA.

BPPD RECOMMENDATION:
There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry1Ac protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because no toxicity to mammals has been observed for the plant-incorporated protectants and anticipated exposures are negligible.

8. Human Health Assessment of Cry3A Potatoes

a. Toxicology Assessment

The delta endotoxin proteins of *B. thuringiensis* have been intensively studied and no indications of mammalian toxicity have been reported. *Bt* microbial pesticides, containing Cry proteins other than Cry3A, have been applied for more than 30 years to food and feed crops consumed by the U.S. population. Furthermore, *B. thuringiensis* products containing Cry3A have been registered and in use for more than a decade, and the Agency has not received any reports of dietary toxicity attributable to their use. The Agency does not anticipate any mammalian toxicity from this protein in plants based on the use history of *B. thuringiensis* products. Therefore, EPA considers that the Cry3A tolerance exemption has been reassessed and meets the 408(c)(2) standard.

The data submitted by Monsanto indicate that this protein would be non-toxic to mammals under the proposed use. Cry3A protein was non-toxic to mice at doses up to 5220 mg/kg bodyweight. This level is >10,000 times the amount found in potato tubers. Adequate information was

submitted to show that the test material derived from microbial cultures was essentially identical to the protein as produced by the potatoes. Production of a microbial Cry 3A delta endotoxin equivalent to plant-produced delta endotoxin was chosen in order to obtain sufficient material for mammalian testing. In addition, the *in vitro* digestibility studies indicate the protein was degraded within 30 seconds in simulated gastric fluid.

Toxicological Endpoints of Cry3A Crops

Study	Result	MRID #
Acute Oral Toxicity of *B.t.* Protein	*Bt* Cry3A delta endotoxin was not toxic by oral gavage when mice were dosed with up to 5220 mg/kg body weight. CLASSIFICATION: ACCEPTABLE These results placed this protein in TOXICITY CATEGORY IV.	429322-17

2) Mutagenicity and Developmental Toxicity, Subchronic Toxicity, and Chronic Exposure and Oncogenicity Assessment

The lack of mammalian toxicity at high levels of exposure demonstrates the safety of the product at levels above possible maximum exposure levels. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. [See 40 CFR Sec. 158.740(b).] For microbial products, further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are only triggered by significant acute effects in studies such as the mouse oral toxicity study.

The acute oral toxicity data submitted support the determination that the Cry3A protein is non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, *et al.*, 1992). Since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry3A delta-endotoxin protein is not considered toxic. Because these proteins break down into their constituent amino acids, there would be no chronic exposure to the protein and therefore no need for chronic toxicity testing. Therefore, the mutagencity, developmental toxicity, subchronic toxicity, chronic exposure and oncogenicity assessment studies are not required.

3) Effects on the Immune System

Since Cry3A is a protein, allergenic potential was considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and proteases, are glycosylated and present at high concentrations in the food. Data has been submitted which demonstrates that the Cry3A delta-endotoxin is degraded within 30 seconds (MRID# 429332-18) by gastric fluid *in vitro* and is non-glycosylated. Studies submitted to EPA done in laboratory animals have not indicated any potential for allergic reactions to *B. thuringiensis* or its components, including the delta-endotoxin in the crystal protein. After decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports of immediate or delayed allergic reactions to the delta-endotoxin itself despite significant oral, dermal and inhalation exposure to the microbial product. Several reports under FIFRA § 6(a)(2) have been made for various *Bacillus thuringiensis* microbial products claiming dermal allergic reactions. However, the Agency determined these reactions were not due to *Bacillus thuringiensis* itself or any of the Cry toxins. The reported reactions were determined to be due to non-Cry proteins produced during fermentation or to added formulation ingredients. Thus, the Cry3A protein is not expected to be a food allergen.

Allergenicity Endpoints of Cry3A Crops

Study Type	Result	MRID #
In-Vitro Digestibility	The 68 kD and 55kD *Bt* Cry3A proteins degraded within 30 seconds in simulated gastric fluid when analyzed by western blot and were not active against Colorado potato beetle after degradation. The 68kD *Bt* Cry3A protein degraded to 55kD within 2 hours of incubation in simulated intestinal fluid. The 55 kD form remained unchanged after 14 hours of incubation and retained its bioactivity and western blot results. These results indicate that, following ingestion by humans, the *Bt* Cry 3A proteins very likely will be degraded like other proteins to amino acids and peptides like other dietary proteins. CLASSIFICATION: ACCEPTABLE	429332-18

4) Effects on the Endocrine System

The pesticidal active ingredients are proteins, derived from sources that are not known to exert an influence on the endocrine system. Therefore, the Agency is not requiring information on the endocrine effects of these plant-pesticides at this time.

5) Dose Response Assessment

No toxicological endpoints are identified so no dose response assessment is required.

6) Dietary Risk Characterization

a) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the Cry3A protein include information on the characterization of the expressed Cry3A delta-endotoxin in cotton, the acute oral toxicity, and *in vitro* digestibility of the delta-endotoxin. The results of these studies were determined to be adequate to evaluate human risk and the validity, completeness, and reliability of the available data from the studies were considered.

Data was submitted to show that the Cry3A test material derived from microbial cultures were biochemically and functionally similar to the proteins produced by the plant-incorporated protectant ingredients. Production of microbially-produced protein was chosen in order to obtain sufficient material for testing.

The acute oral toxicity data submitted supports the determination that the Cry3A protein is non-toxic to humans. Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels (5,000 mg/kg), the Cry3A delta-endotoxin protein is not considered toxic. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. [See 40 CFR Sec. 158.740(b).] Further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are only triggered by significant acute effects in studies such as the mouse oral toxicity study. Because the acute testing showed no toxicity, higher tier studies are not required.

Because Cry3A is a protein and the major exposure is dietary, food allergenic potential was considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and proteases, are glycosylated and present at high concentrations in the food. Data has been submitted which demonstrates that the Cry1Ac delta-endotoxin is degraded within 30 seconds (MRID#429332-18) by gastric fluid *in vitro* and is non-glycosylated. Despite decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports of immediate or delayed allergic reactions to the delta-endotoxin itself despite significant oral, dermal and inhalation exposure to the microbial product. Several reports under FIFRA § 6(a)2 have been made for various *Bacillus thuringiensis* products claiming allergic reactions. However, the Agency determined these reactions were not due to *Bacillus thuringiensis* itself or any of the Cry toxins. Thus, the Cry3A protein is not expected to be a food allergen.

Although Cry3A expression level data was required for an environmental fate and effects assessment, residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity.

Both (1) available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children) and (2) safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food additives, are generally recognized as appropriate for the use of animal experimentation data were not evaluated because the lack of mammalian toxicity at high levels of exposure demonstrate the safety of the product at levels above possible maximum exposure levels.

The genetic material necessary for the production of the plant-incorporated protectants active ingredients are the nucleic acids (DNA) which comprise (1) genetic material encoding these proteins and (2) their regulatory regions. "Regulatory regions" are the genetic material (termed promoters, terminators and enhancers) that control the expression of the DNA encoding proteins. DNA is common to all forms of plant and animal life and the Agency knows of no instance where these nucleic acids have been associated with toxic effects related to their consumption as a component of food. These ubiquitous nucleic acids as they appear in the subject active ingredient have been adequately characterized by the applicant. Therefore, no mammalian toxicity is anticipated from dietary exposure to the genetic material necessary for the production of the subject active plant pesticidal ingredients.

b) Infants and Children Risk Conclusions

FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408 provides that EPA shall apply an additional tenfold margin of exposure (safety) for infants and children in the case of threshold effects to account for pre- and post-natal toxicity and the completeness of the database unless EPA determines that a different margin of exposure (safety) will be safe for infants and children.

In this instance, based on all the available information, the Agency concludes that infants and children will consume minimal residues of this plant-pesticide and that there is a finding of no toxicity.

Thus, there are no threshold effects of concern and, as a result the provision requiring an additional margin of safety does not apply. Further, the provisions of consumption patterns, special susceptibility, and cumulative effects do not apply.

c) Aggregate Exposure (Not Including Occupational Exposure) Risk Conclusions

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectants chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Oral exposure, at very low levels, may occur from ingestion of processed products and drinking water. However, a lack of mammalian toxicity and the digestibility of the plant-incorporated protectants has been demonstrated. The use sites for Cry3A delta endotoxin are all agricultural for control of lepidopteran insects. Therefore, exposure via residential or lawn use to infants and children is not expected. Even if negligible exposure should occur, the Agency concludes that such exposure would present no risk due to the lack of toxicity.

d) Cumulative Effects Risk Conclusions

The Agency has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity to these plant-incorporated protectants, there are no cumulative effects.

e) Tolerance Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry3A protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. Therefore, EPA considers that the Cry3A tolerance exemption has been reassessed and meets the 408(c)(2) standard.

The Agency has arrived at this conclusion because, as discussed above, no toxicity to mammals has been observed for the plant-incorporated protectants. As a result, EPA established an exemption from tolerance requirements pursuant to FFDCA section 408(j)(3) for *Bacillus*

thuringiensis Cry3A delta-endotoxin and the genetic material necessary for its production in all plants.

Bacillus thuringiensis subspecies *tenebrionis* Cry3A delta-endotoxin and the genetic material necessary for its production in all plants are exempt from the requirement of a tolerance when used as plant-incorporated protectants in all plant raw agricultural commodities. ``Genetic material necessary for its production" means the genetic material which comprise (1) genetic material encoding the Cry3A delta-endotoxin and (2) its regulatory regions. ``Regulatory regions" are the genetic material that control the expression of the genetic material encoding the Cry3A delta-endotoxin, such as promoters, terminators, and enhancers.

f) Occupational Exposure and Risk Characterization

Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Worker exposure to the Cry protein via seed dust is also expected to be negligible because of the low amount of protein expressed in transformed plants. If such exposure should occur, the Agency concludes that such exposure would not be expected to present any risk due to the lack of toxicity. However, if any unreasonable adverse effects caused by exposure to Cry3A are identified, these effects must be reported to the Agency as described in Sec. 6(a)(2) of FIFRA.

BPPD RECOMMENDATION:
There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry3A protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because no toxicity to mammals has been observed for the plant-incorporated protectants and anticipated exposures are negligible.

REFERENCES

Bernstein, I.L., J.A. Bernstein, M. Miller, S. Tierzieva, D. I. Bernstein, Z. Lummus, M.K. Selgrade, D.L. Doerfler & Verner L. Seligy (1999) Immune Responses in Farm Workers after Exposure to *Bacillus thuringiensis* Pesticides, Environmental Health Perspectives Volume 107:575-582.

Environmental Assessment

- ***Pesticidal Substance Expression in the Plant***
- ***Environmental Fate***
- ***Non-Target Effects***

Environmental Assessment

- ***Pesticidal Substance Expression in the Plant***

The test substances were cotton lines 531 and 931. Six locations in Mississippi, Louisiana, Texas, Georgia, Arizona, and Alabama were used for field expression studies. Proteins in leaf, seed, whole plant, cottonseed meal and refined cotton seed oil were analyzed. Expression level ranges were identified by validated ELISA procedures. Reported mean *Btk* protein expression levels from field grown plants ranged from 1.10 to 2.04 • g/g of fresh leaf tissue and from 0.49 to 1.62 • g/g per gram fresh seed tissue. Greenhouse studies indicate that *Btk* protein is expressed in pollen (11.5 ng/gram) at a level of detection of 8.0 ng/gram, and is below the level of detection in nectar (<1.6 ng/gram). The Cry protein was reduced to undetectable levels in cottonseed meal after processing. No detectable levels were found in refined oil at a level of detection of 1.3 ppm.

Based upon planting rates of 60,000 plants per acre, a total of 1.44 grams of *Btk* protein would enter the soil per acre due to post harvest incorporation of the plants into the soil.
The submitted data demonstrated a loss, following soil incorporation, in activity of *Btk* endotoxin against a susceptible insect, the tobacco budworm *Heliothis virescens*. Purified endotoxin produced in *E. coli* shows a soil DT_{50} (Degradation Time) of 9.3-20.2 days. Ground, lyophilized Cry1A(c) cotton line 931 tissue has a soil DT_{50} of 41 days.

e. Exposure of Cry3A (006432)

Study	Status, Classification & Comments	MRID #
Expression levels in field grown potatoes	Cry3A delta-endotoxin expression levels for leaf samples: 5.39 to 28.27 micro g/g tissue or 0.03-0.2% of the total foliage protein. Tuber CryIIIA delta-endotoxin expression levels: 0.40 to 2.00 micro g/g or 0.002-0.01% of the total tuber protein. CLASSIFICATION: ACCEPTABLE.	429322-02

Monsanto submitted data for seven advanced CPB resistant Russet Burbank lines and a nontransformed Russet Burbank control grown at seven locations representative of potato growing regions in the United States. Tissues assayed included leaf, whole plant (minus tubers) and tuber, three harvest dates for leaves, two for whole plants and one for tubers were conducted for Cry3A.

Tuber samples were lyophilized before determining protein expression levels. The fresh weight equivalent of each tuber sample was obtained by determining the amount of water removed during the lyophilization process. Leaf and whole plant samples were processed from frozen, but not lyophilized, samples. Percent moisture was not reported for these tissues. Leaf or whole plant samples with low moisture content would be expected to yield higher expression levels than otherwise comparable tissue with relatively higher moisture content.

Cry3A levels expressed as a percentage of total protein were based on the assumption that total protein comprises 1.6 and 2.0% of the fresh weight of foliage and tubers, respectively. These levels are comparable to average values reported in the literature (Burton, 1989). The range expected will vary with genetic and cultural variables. Tuber protein, for example, has been reported to range from 0.7 to 4.6 % of tuber fresh weight (Kadam *et al.*, 1991).

The relatively low expression levels reported for the Cry3A protein in tuber tissue reflect, in part, the high starch concentration in storage parenchyma, cells which comprise the bulk of the tuber. Reported Cry3A delta-endotoxin expression levels for leaf samples ranged from 5.39 to 28.27 micro g/g tissue or 0.03-0.2% of the total foliage protein. Tuber Cry3A delta-endotoxin expression levels ranged from 0.40 to 2.00 micro g/g or 0.002-0.01% of the total tuber protein.

f. Fate of *Bt* Proteins in Soil

Soil organisms may be exposed to • •endotoxins from current transgenic crops by exposure to roots, incorporation of above ground plant tissues into soil after harvest, or by pollen deposited on the soil. Root exposure may occur by feeding on living or dead roots or, theoretically, by ingestion or absorption after secretion of • •endotoxin into the soil. The latter situation is the subject of a recent brief communication in the journal "Nature" by Saxena *et al.* (1999), and is discussed in more detail below. In addition, evidence suggests that some soil components, e.g. clays and humic acids, bind • •endotoxins in a manner that makes them recalcitrant to degradation by soil microorganisms, but without eliminating their insect toxicity. Therefore, exposure to • •endotoxin bound to soil particles may also be a route of exposure for some soil organisms.

Experiments addressing amounts and persistence of • •endotoxins in the soil have been submitted by registrants and reviewed for the current conditional registrations. In addition, a number of publications in the scientific literature have addressed the degradation of Cry proteins in the soil. These experiments consist of the incorporation of purified • •endotoxin or transgenic plant material in soil in a laboratory setting. Cry protein DT_{50} (time to 50% degradation) studies were submitted for registration for corn containing Cry1Ab and Cry1F, and published studies were available for Cry1Ac cotton. Cry1Ab produced an estimated DT_{50} of 1.6 days for Cry protein as expressed in transgenic corn tissue and 8.3 days for purified protein (Sims and Holden 1996). Based on a bioassay with the tobacco budworm (*Heliothis virescens*), a target species, purified Cry1F proteins incorporated into test soils biodegraded with a DT_{50} of approximately 3.13 days. Data produced by Monsanto for Cry1Ac protein and transgenic Cry1Ac in cotton give degradation rates (DT_{50}) of approximately 9-20 days for the purified protein, and 41 days for the protein in cotton tissue (MRID# 43999509). Published data for Cry1Ab or Cry1Ac in cotton tissue or as purified protein produced DT_{50}s of 2.2 to 46 days, where measurable (in 4 of 11 experiments), with DT_{50}s in transgenic tissue shorter than for purified protein in two of three experiments (Palm *et al.* 1994). DT_{50}s of purified Cry1Ac in two different non-sterile soils were

Environmental Assessment

- ***Non-Target Effects***

1) Summary of Non-Target Organism Toxicity Testing of Cry3A (006432)

Study	Status, Classification & Comments	MRID #
Avian Data	Monsanto conducted two dietary avian toxicity studies using the bobwhite quail and seven different potato lines producing the *Bt* Cry3A protein. The studies were both scientifically sound and no treatment mortality, differences in food consumption or behavior was observed between the dosed (50,000 ppm from potato tubers) and control birds. These studies adequately address potential avian toxicity concerns for *Bt* Cry3A protein produced in potato. No additional avian studies should be needed.	429322-14 429322-15
Cry3A Protein Comparison	To ensure that the truncated *Bt* Cry3A protein produced in the potato plants will not have an altered host-range of susceptible insects relative to the native full-length protein, comparative insect host-range studies have been submitted by Monsanto. The data consisted of SDS-PAGE co-migration, Western blot analysis, staining for carbohydrate residues, N-terminal amino acid sequence analysis, and biological equivalence. The results demonstrated that the *Bt* Cry3A protein with respect to the parameters tested was equivalent to the natural protein.	429322-03
Non-Target and Beneficial Insects	Monsanto submitted three standard non-target insect studies (parasitic wasp, ladybird beetle and green lacewing). The results of these studies indicated that the *Bt* Cry3A protein produced in potato plants showed no toxicity to parasitic hymenoptera (*Nasonia vitripennis*), green lacewing (*Chrysopa carnea*) and lady bird beetle (*Hippodamia convergens*).	429322-11 429322-12 429332-13
Honeybee Toxicity Study	The adult and larval honeybees were dosed with *Bt* in a sucrose and honey solution. The testing indicated that there was no significant loss of *Bt* protein bioactivity in honey or sucrose solutions when maintained for up to 7 days at a approximately 28 C. The adult honeybee study was found to be invalid due to excessive mortality in the controls. Since the adult honey bee study was not required, it will not have to be repeated. The larval honeybee study produced useable results and indicated that *Bt* Cry3A protein in potato showed no toxicity to honeybee larvae.	429322-09 429322-10
Evaluation of the Dietary Effects of Purified *Btt* Protein on Honey Bee Larvae	The honey bee study adequately demonstrated that purified *Btt* protein (Cry3A) shows no toxicity to honey bee larvae when exposed to 100 ppm protein. This dose far exceeds the amount expected to be encountered under actual field conditions.	441247-02
Earthworm	The data adequately demonstrated that purified Cry3A protein shows no toxicity to earthworms at levels greater than 100 mg protein/kg soil in a 14 day study.	441247-01

Study	Status, Classification & Comments	MRID #
Collembola	The data adequately demonstrated that Cry3A protein shows no toxicity to two species of Collembola at greater than 200 ppm. No adverse effects expected at field rates.	439416-1

2) Mammals

Monsanto submitted an acute feeding mammalian toxicity study reviewed in the Toxicity Assessment above. The Cry3A protein was found to be nontoxic to mice. EPA has determined that Cry3A is nontoxic to non-target mammalian species.

3) Avian Species

Monsanto conducted two dietary avian toxicity studies using bobwhite quail and 7 different potato lines producing Cry3A protein. The studies were both scientifically sound and no treatment mortality, differences in food consumption or behavior was observed between the dosed (50,000 ppm from potato tubers) and control birds. These studies adequately address potential avian toxicity concerns for Cry3A produced in potato.

4) Aquatic Species

Studies for aquatic species were waived because of expected lack of exposure. Potatoes are not used as fish food. Most *Bt* potato varieties produce a minium amount of pollen and the amount of pollen drops to very low levels within a few meters of the pollen source (Dale et al, 1992) so pollen drift to aquatic sites is minimal to non-existent.

5) Non-target Invertebrates

a) Honeybees

The registrant was required to submit a larval honeybee study. The registrant also submitted an adult honeybee study which was not required for registration. The adult and larval honeybees were dosed with Cry3A protein in a sucrose and honey solution. The registrant wanted to ensure that the Cry3A protein was stable in this type of solution. Testing indicated that there was no significant loss of Cry3A protein bioactivity in honey or sucrose solutions when maintained for up to 7 days at approximately 28°C. The larval honeybee study was scientifically sound and demonstrated that *Btt* in potato has no detectable deleterious effects on honeybee larvae. The adult honeybee study was found to be invalid due to excessive mortality in the controls. Since this study was not required, EPA did not require the study to be repeated. No adverse effects on larval or adult honeybees has been reported since registration in 1995.

b) Predatory, Parasitic and other Non-target Insects

The registrant submitted the three standard non-target insect studies (parasitic wasp, ladybird beetle, and green lacewing). The results of these studies indicated that Cry3A has no observable adverse effects on parasitic Hymenoptera (*Nasonia vitripennis),* green lacewing *(Chrysopa carnea*) and lady beetle (*Hippodamia convergens*).

An additional field study on the comparative impacts of foliar-applied microbial *Btt.*, transgenic potato plants, and conventional insecticides on non-target arthropods was submitted by the registrant. Beneficial arthropods (i.e. lady beetles, damsel bugs, flower flies, soldier beetles, big-eyed bugs, spiders, minute pirate bugs, green lacewings, brown lacewings, stink bugs, and ground beetles) were significantly more abundant in plots containing genetically modified potato plants and foliar-applied microbial *B.t.t.* than in those treated with conventional chemical insecticides. Aphid control was achieved in the plots containing transgenic potatoes solely through predation by natural enemies, while aphid populations rose to high levels in plots where beneficial arthropods were eliminated and no chemical aphid control was applied.

The registrant also submitted a study which tested the sensitivity of selected insect species to the Cry3A protein produced in the potato plants. The tested species were as follows: 3 rootworms; 4 lepidopterans- European corn borer, tobacco hornworm, corn earworm, and tobacco budworm; 1 dipteran-yellow fever mosquito; 1 orthopteran-German cockroach; and 1 hemipteran-green peach aphid. The results demonstrated that no species other than the Colorado potato beetle (*Leptinotarsa decemlineata*) displayed significant mortality. There was a slight reduction in the amount of honeydew produced by the Green peach aphid which was an indication of reduced feeding.

These studies indicate that Cry3A protein produced in potato plants should not adversely affect the non-target insects studied in these tests. Since Cry3A is specific to coleopterans it is not surprising that the non-target coleopteran insects that feed on these potato plants will, in all likelihood, be adversely affected by the Cry3A. Since any coleopteran insect that feeds on these plants would be considered a plant pest, this should not present a risk to non-target, non-pest insects.

c) Soil Invertebrates

Three hundred and five adult earthworms (*Eisenia fetida*) were acclimated for 24 hours to an artificial soil substrate. The worms were rinsed with deionized water and randomly distributed into groups of 10. The worms were not fed during testing. The moisture content of the soil substrate was 33%; the relative humidity of the test chamber was 86%; and the pH of the soil was 6.8/6.9 at day 0, and 7.4 at day 14. The soil was analyzed for actual active ingredient content. Earthworms were exposed to a single test concentration of 100 mg a.i. per kg of soil (approximately 120-fold the amount Cry3A protein estimated to be present in a kg of soil), and observed for mortality and signs of toxicity on day 7 and day 14 of the test. A negative and a

positive control group were maintained concurrently. The no observed effect concentration was determined by visual examination of the mortality, body weight and clinical observation data. The worms exhibited no aversion to the test or control soils. All the worms were normal in appearance and behavior during the course of the study. There were no treatment related effects on body weights. The LC_{50} of Cry3A protein for earthworms (*Eisenia fetida*) as representative beneficial invertebrate soil species is >100 mg a.i./kg dry soil in a 14-day exposure study. The no observed effect concentration is >100 mg a.i./kg dry soil. The 100 mg dose represents a level of exposure 120 times greater than the actual contact the earthworm would have under field conditions.

Two Collembola species, *Folsomia candida* and *Xenylla grisea* were fed a test diet prepared by suspending 1.0 g of Bakers yeast in 3.0 mL of distilled water containing 200 micro g of Cry3A protein. The dose concentration was confirmed by ELISA and/or insect bioassay techniques. Positive control consisted of chlorpyrifos added to yeast to obtain 200, 20, 2, 0.2 and 0.0 (negative control) ppm concentrations. There were ten insects per 5 replicates each for treatment and control groups for *Folsomia candida* and 6 replicates for *Xenylla grisea* in the test system. The test lasted for 21 days with fresh diet being added on days 0, 7 and day 14. The chlorpyrifos response system consisted of 4 replicates per concentration. The Cry3A protein tested did not have a detrimental effect on the survival or reproduction of *F. candida* or *X. grisea*. The NOEC therefore was >200 ppm. Adults and progeny of *X. grisea* were combined for the statistical analysis because of difficulty in discriminating between the initial adults and older progeny. For the chlorpyrifos control the no observed effect (NOEC) for *F. candida* was 2.0 ppm. Progeny production among the survivors at 2.0 ppm was not significantly different from the control. *X. grisea* was considerably less susceptible to chlorpyrifos. The NOEC was >200 ppm. The survival and reproduction appear reduced at 20 and 200 ppm, however these were not statistically significant. The study was scientifically sound and no treatment mortality or behavior change was observed between the dosed and control replicates. No adverse effects were seen to Collembola by chronic exposure to purified Cry3A protein at a maximum hazard dose of 200 ppm. The study showed that at field use rates survival and reproduction of the test insects would not be impaired. The study also shows that *X. grisea* did not exhibit any detrimental effects. This study adequately address potential concerns for Cry3A protein expressed in transgenic potatoes to Collembola (*Folsomia candida*) a representative of beneficial soil insect species. The results of this study demonstrate that Cry3A proteins found in transgenic potatoes pose no hazard to soil inhabiting Collembola species, and by inference to other beneficial soil insects.

6) Threatened and Endangered Species

EPA has determined that Cry3A potatoes will not affect any threatened or endangered species. The known host range for the Cry3A protein is restricted to Coleoptera species. The listed coleopteran threatened/endangered species in potato growing areas are: the American burying beetle, Hungerford's crawling water beetle, Mount Hermon June beetle, Northeastern Beach Tiger beetle, Puritan Tiger beetle and the Valley Elderberry Longhorn beetle. These are not

going to be exposed to Cry3A protein because their habitat does not overlap with potato fields and/or their larvae do not feed on potato tissue and will not be exposed to Cry protein in pollen, or to toxic Cry3A levels in the soil. The amount of pollen that would drift from the potato plants onto plants fed upon by endangered/threatened species would be very small compared to the levels fed to the test species. Submitted data confirm that some coleopteran species tested are not affected, including lady beetles. Generally potatoes do not produce large amounts of pollen which limits exposure. No endangered or threatened avian species feed on potatoes and no aquatic species are known to feed on potato plants.

5. Environmental Reassessment Summary

This reassessment finds no hazard to the environment at the present time from MON810 and *Bt*11 transformation events in corn, Cry1Ac in cotton and Cry3A in potatoes as currently registered. The reassessment considered the following issues.

a) Gene Flow and Weediness

The movement of transgenes from the host plant into weeds and other crops has been considered for each of the *Bt* plant-incorporated protectants currently registered. The Agency has determined that as currently registered there is no significant risk of gene capture and expression of any *Bt* Cry protein by wild or weedy relatives of corn and potatoes in the U.S., its possessions or territories.

There is a possibility for gene transfer in locations where wild or feral cotton relatives exist. If complete isolation and prevention of gene flow for *Bt* cotton is desired, then plantings of *Bt* cotton in Hawaii, Puerto Rico, and the U.S. Virgin Islands may require a minimum 3 mile distance from *Gossypium* spp. with 24 border rows of non-*Bt* cotton surrounding the plots. Monitoring of native populations of established *Gossypium* spp. may be necessary to assess the efficacy of this isolation procedure for *Bt* cotton. This would entail monitoring of wild populations for evidence of gene introgression through PCR or similar sensitive methods. Alternatively, the absolute restriction of planting *Bt* cotton in Puerto Rico, Hawaii, and the U.S. Virgin Islands, would, of course, alleviate any concerns over gene flow.

The current restriction on the planting of *Bt*-cotton in Florida south of Route 60, near Tampa, precludes any chance of outcrossing with feral *Gossypium* spp. due to the very large distance between any commercial plantings and wild populations in the extreme south of the state.

b) Fate in Soils and Indirect Effects on Soil Biota

Most of the Cry protein deposited into soil by *Bt* crops is quickly degraded, although a residual amount may persist in biologically active form for a much longer period of time. It is also reported that the same degree of *Bt* Cry protein persistence takes place in soils that have been

Common Name and Cry Protein	OPP Chemical Code	Study Types
MON810, Cry1Ab *Bt* Corn	6430	1) Cry1Ab expression levels in the root. 2) While much of the data required in the *Bt* corn data call in has been submitted, final published reports and updates from ongoing research is still required for the following categories of data: the distribution of monarch butterflies, milkweed plants and corn; corn pollen release and distribution in the environment; toxicity of *Bt* corn Cry proteins and *Bt* corn pollen to lepidopterans; monarch egg laying and feeding behavior; and monarch population monitoring. 3) Field tests of Cry protein degradation in soil under a range of conditions typical of *Bt* crop cultivation are needed to yield relevant data on persistence and natural variation. 4) Submitted avian toxicity data on Cry1Ab *Bt* corn are not sufficient to make a final hazard assessment from repeated exposure(s) to higher doses of *Bt* corn. A six week study with 60 to 70% corn in the diet is necessary to assess hazards from chronic exposure of wild and domesticated fowl. 5) Yearly insect census estimates from representative fields.
Cry3A Potato	6432	1) Field tests of Cry protein degradation in soil under a range of conditions typical of *Bt* crop cultivation are needed to yield relevant data on persistence and natural variation. 3) Yearly insect census estimates from representative fields.

References:

<u>MRID Numbers</u>

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Insect Resistance Management

- Seven elements that should be addressed in a Bt PIP resistance management plan: 1) knowledge of pest biology and ecology; 2) appropriate dose expression strategy; 3) appropriate refuge; 4) resistance monitoring and a remedial action plan should resistance occur;

Insect Resistance Management

- Seven elements that should be addressed in a Bt PIP resistance management plan cont. ; 5) employment of integrated pest management (IPM); 6) communication and education strategies on use of the product; and 7) development of alternative modes of action.

a. Current Insect Resistance Management (IRM) Plan

The SAP meeting in 1998 on resistance management recommended that the IRM plan for potatoes be mandatory instead of voluntary. Monsanto has made several modifications to its NewLeaf potato IRM plans over the last five years. In 2000, Monsanto amended their registration to make the refuge mandatory. Growers were already signing contracts which included a refuge requirement. In addition, the current plan focuses on placement of the refuge and encompasses the importance of overwintering sites. The Insect Resistance Management Plan includes:

1) Use NewLeaf potatoes in rotation to reduce CPB.

2) Plant and manage "refuges" to maintain susceptible insect populations. Specific grower recommendations are as follows:

1. Do not plant your entire potato acreage to NewLeaf potato varieties, but maintain at least 20% of the total acreage as "refuge".
2. Do not use a foliar *Bt* application for CPB control on refuge acres. You may treat CPB in the refuge with insecticides to prevent damage. It is recommended that you use foliar insecticides only when populations reach damaging levels, according to local IPM recommendations.
3. Plant every NewLeaf potato field within ½ mile or less of the appropriate current year refuge or Plant every NewLeaf potato field within ½ mile of land that was the designated refuge (non-*Bt* potatoes) last year.

3) Use of every method available to reduce CPB populations such as crop rotation, propane flaming, trench trapping, and overwintering habitat destruction.

4) Monitoring for survival of CPB including a toll free number.

5) Grower education plan.

6) Monitoring for resistance development.

7) Remedial action plan.

b. Analysis of the Risks Associated with Current IRM Plans and Alternatives

The 1998 SAP Subpanel concluded that NewLeaf® and NewLeaf Plus® potato hybrids are maintaining a " high dose" expression of Cry3A throughout the growing season to control Colorado potato beetle (CPB). The dose is at least 50 times that necessary to kill first-instar larvae. Experts meeting in December 1999 agreed that a 20% refuge is sufficient to produce the 500:1 susceptible insects to resistant insects needed for an efficient refuge. They also agree that a one-half mile maximum distance restriction for the refuge is a reasonable recommendation. EPA agrees with these experts. Monsanto has developed a discriminating dose assay, a surveillance and remedial action plan, and an extensive grower education communication and training program to

convey appropriate resistance management tactics. IPM and scouting are discussed in the technical material provided by Monsanto/NatureMark. Based on Monsanto's annual grower surveys, grower compliance with the 20% refuge is >99%. In addition, the recent amendments to make the refuge mandatory and the focus on managing insect overwintering habitat have further decreased the likelihood that resistance of CPB to Cry3A will occur from exposure to *Bt* potatoes. The Agency's full risk assessment of insect resistance development and insect resistance management assessment is found in the Agency's memorandum from S. Matten OPP/BPPD to W. Nelson, OPP/BPPD, dated July 5, 2000.

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