Attachment III: Environmental Risk Assessment of Plant Incorporated Protectant (PIP) Inert Ingredients

This environmental risk assessment of PIP inert ingredients is divided into three sections which cover herbicide tolerance markers, antibiotic resistance markers, and other markers.

HERBICIDE TOLERANCE MARKERS

Phosphinothricin acetyltransferase (PAT)

SUMMARY

The Agency has conducted an environmental risk assessment of the plant-incorporated protectant (PIP) inert ingredient phosphinothricin acetyltransferase (PAT), and the genetic material necessary for its production. Topics covered in this assessment include mode of action, ecological effects, endangered species considerations, and gene flow from a modified plant to wild or weedy relatives. Data cited in this assessment were submitted to the Agency in support of Dekalb's DBT 418 and Ciba Seed's Event 176 Bt corn registrations, and Syngenta's COT 102 Bt cotton registration. Ecological data and published information on the biology of this protein indicate that this PIP inert ingredient is not a known toxin and/or pathogen of plant or animal species. Due to the low human health risks associated with this protein, the Agency has granted an exemption from the requirement of a tolerance for this PIP inert ingredient (40 CFR 180.1151; 62 FR 17719, Aug. 11, 1997).

MODE OF ACTION

The PAT enzyme is an acetyltransferase that catalyzes the acetylation the active ingredients L-phosphinothricin (L-PPT) and demethylphosphinothricin (DMPT), conferring tolerance to glufosinate ammonium herbicides. L-PPT and DMPT inhibit glutamine synthetase, resulting in the accumulation of toxic ammonium ions and a decrease in the amount of glutamine, an essential amino acid used in many anabolic processes. In the presence of acetyl-CoA, the PAT protein catalyses the acetylation of the free amino group of L-PPT to N-acetyl-L-PPT, a compound that does not inhibit glutamine synthetase (OECD 2002). The PAT enzyme is highly specific for L-PPT and does not acetylate other L-amino acids, such as glutamate (Thompson *et al.*, 1987).

ENVIRONMENTAL EFFECTS

The Agency assessed the toxicity of PAT protein to representative non-target organisms that could be exposed to this PIP inert ingredient. Toxicity evaluations included the following Tier I studies[1]: Mammalian (mouse), Avian (northern bobwhite quail), Freshwater Fish (channel

[1] Tier I testing consists of maximum dose single species hazard assessments that are used to evaluate the potential for toxicity, infectivity, and pathogenicity of a pesticidal agent to nontarget organisms.

catfish), Aquatic Invertebrate [cladoceran (*Daphnia magna*)], and Non-target Arthropod (honey bee larvae, lady beetle, green lacewing, collembola, and earthworm). Tier IV[2] field studies of non-target abundance are also discussed below.

The wild mammal hazard assessment was performed on the basis of rodent acute oral toxicity data prepared for human health risk assessment purposes which utilized pure PAT protein. Submitted data indicate no significant adverse effects among mice dosed with up to 2500 mg/kg body weight PAT protein (DBT 418 MRID 439995-02).

The remaining single species studies were maximum hazard dose evaluations, where PAT protein toxicity was evaluated by feeding select non-target organisms corn pollen, grain, or leaf tissue, which has been shown to contain the PIP inert ingredient PAT and the active ingredient Cry1Ab or VIP3A. Note: Studies submitted in support of the COT102 registration utilized corn plant material in place of cotton plant material.

An avian study, where northern bobwhite quail were dosed with lyophilized DBT 418 corn leaf tissue, reported no adverse effects to the test species at field exposure levels (MRID 439995-10). The freshwater fish corn grain feeding study indicated that channel catfish diets may contain up to 50% PAT-containing corn grain with no abnormalities or adverse effects on fish growth (final report not submitted as of 9-5-03). In three Event 176 and four COT102 corn pollen dietary toxicity studies, no adverse effects to *Daphnia magna*, a representative of aquatic invertebrate species, honey bee larvae, lady beetle, or green lacewing were seen at field level exposures (Event 176 MRIDs 433236-10, 433396-02, 434157-03; COT102 MRIDs 457921-01, 457665-09, and 458358-08). Results of DBT 418 and COT102 corn leaf tissue feeding studies indicate that PAT does not adversely affect reproduction of Collembola, a representative plant tissue decomposer (DBT 418 MRID 439995-12; COT102 MRID 458358-10). Finally, no adverse effects were seen among earthworms exposed for 14 days to soil containing lyophilized DBT 418 corn leaf tissue (MRID 439995-13).

A semi-field study of honeybee colonies showed that the overall ability of colonies to produce brood, manage stores of food and recruit new bees was not affected by exposure to the VIP3A corn containing the PAT protein (MRID 458358-13). Field studies that evaluated arthropod abundance in plots of stacked VIP3A x Cry1Ab Bt corn were also conducted. To date, results show that beneficial arthropods were significantly more abundant in plots containing Bt plants than in plots treated with conventional chemical insecticides (interim report MRID 458358-07).

The reviewed data showed no toxicity to non-target test species. As a result of these findings and knowledge of the mode of action of PAT, the Agency concludes that no unreasonable adverse effects on non-target organisms are expected from exposure to PAT protein.

ENDANGERED SPECIES CONSIDERATIONS

[2] Tier IV testing refers to field testing under simulated or actual field conditions. These studies are designed on a case-by-case basis to evaluate specific problems that cannot be resolved by lower tier testing.

Because this PIP inert ingredient is neither a known toxin and/or pathogen of plant or animal species, EPA does not expect that exposure to the PAT protein will result in a "may affect" finding for any endangered or threatened species.

GENE FLOW CONSIDERATIONS

Gene flow may occur when the PAT protein is expressed in a plant that can form viable hybrids in nature with wild or weedy relatives of that species. With the assistance of the October 2004 SAP, EPA has identified the following plants as not having wild or weedy relatives in the United States, its possessions, or territories, with which they can produce viable hybrids in nature: almond (*Prunus communis*), apricot (*Prunus armeniaca*), asparagus (*Asparagus officinale*) avocado (*Persea americana*), banana (*Musa acuminata*), barley (*Hordeum vulgare*), bean (*Phaseolus vulgaris*), black-eyed pea (*Vigna unguiculata*), cacao (*Theobroma cacao*), celery (*Apium graveolens*), chickpea (*Cicer arietinum*), citrus (*Citrus* spp.), coffee (*Coffea arabicua*), corn (*Zea maize*), cucumber (*Cucumis sativus*), eggplant (*Solanum melongena*), guava (*Psidium guajava*), kiwi (*Actinidia* spp.), mango (*Mangifera indica*), nectarine (*Prunus persica*), okra (*Abelmoschus esculentus*), olive (*Olea europaea*), papaya (*Carica papaya*), parsley (*Petroselinum crispum*), pea (*Pisum sativum*), peach (*Prunus persica*), peanut (*Arachis hypogaea*), pineapple (*Ananas comosus*), pistachio (*Pistacia vera*), plum (*Prunus domestica*), potato (*Solanum tuberosum*), soybean (*Glycine max*), spinach (*Spinacia oleracea*), starfruit (*Averrhoa carambola*), taro (*Colocasia esculenta*), tomato (*Lycopersicon lycopersicum*), or watermelon (*Citrullus lanatus*). Since hybridization with wild or weedy relatives is not known to occur among plants included on this list, EPA has concluded that, when introduced into these species, PAT presents a low probability of risk to human health and the environment.

CP4 enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS)

SUMMARY

The Agency has conducted an environmental risk assessment of the plant-incorporated protectant (PIP) inert ingredient CP4 enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS), and the genetic material necessary for its production. Topics covered in this assessment include mode of action, ecological effects, endangered species considerations, and gene flow from a modified crop to wild or weedy relatives. Data cited in this assessment was submitted to the Agency in support of Monsanto's MON 810 Bt Corn registration. Ecological data and published information on the biology of this protein indicate that this PIP inert ingredient is not a known toxin and/or pathogen of plant or animal species. Due to the low human health risks associated with this protein, the Agency has granted an exemption from the requirement of a tolerance for this PIP inert ingredient (40 CFR 180.1174; 61 FR 40340, Aug. 2, 1996).

MODE OF ACTION

When conventional plants are treated with glyphosate they cannot produce the aromatic amino acids needed to grow. Glyphosate blocks biosynthesis of aromatic amino acids by binding to 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in plants (Steinrucken and Amrhein, 1980).

The CP4 EPSPS enzyme, isolated from the *Agrobacterium tumefaciens* strain CP4, functions in the presence of glyphosate and thus confers tolerance to glyphosate herbicides when expressed in plants. The EPSPS enzyme is naturally present in plants, bacteria, and fungi (Levin and Sprinson, 1964).

ENVIRONMENTAL EFFECTS

The Agency assessed the toxicity of CP4 EPSPS protein to representative non-target organisms that could be exposed to the PIP inert ingredient. Toxicity evaluations included the following Tier I studies: Mammalian (mouse), Avian (juvenile northern bobwhite quail), and Freshwater Fish (channel catfish).

The wild mammal hazard assessment was performed on the basis of rodent acute oral toxicity data prepared for human health risk assessment purposes which utilized pure CP4 EPSPS protein. Submitted data indicate no significant adverse effects among mice dosed with up to 572 mg/kg body weight for CP4 EPSPS protein (MRID 436433-03).

The avian study was a maximum hazard dose evaluation, where CP4 EPSPS protein toxicity was evaluated by feeding northern bobwhite quail MON 801 corn grain. Results of this feeding study showed no mortality among northern bobwhite quail fed diets containing MON 801 corn grain (MRID 435332-05).

Results of a freshwater fish corn grain feeding study reported that no adverse effects on fish growth and survival were seen among channel catfish fed MON 801 corn (MRID No. 438879-01).

The reviewed data showed no toxicity to non-target mammalian and avian test species. As a result of these findings, knowledge of the mode of action of CP4 EPSPS, and the presence of a similar enzyme, EPSPS, in all plants, the Agency concludes that no unreasonable adverse effects on non-target organisms are expected from exposure to the CP4 EPSPS protein.

ENDANGERED SPECIES CONSIDERATIONS

Because this PIP inert ingredient is neither a known toxin and/or pathogen of plant or animal species, EPA does not expect that exposure to the CP4 EPSPS protein will result in a “may affect” finding for any endangered or threatened species.

GENE FLOW CONSIDERATIONS

Gene flow may occur when the CP4 EPSPS protein is expressed in a plant that can form viable hybrids in nature with wild or weedy relatives of that species. With the assistance of the October 2004 SAP, EPA has identified the following plants as not having wild or weedy relatives in the United States, its possessions, or territories, with which they can produce viable hybrids in nature: almond (*Prunus communis*), apricot (*Prunus armeniaca*), asparagus (*Asparagus officinale*) avocado (*Persea americana*), banana (*Musa acuminata*), barley (*Hordeum vulgare*), bean (*Phaseolus vulgaris*), black-eyed pea (*Vigna unguiculata*), cacao (*Theobroma cacao*), celery (*Apium graveolens*), chickpea (*Cicer arietinum*), citrus (*Citrus* spp.), coffee (*Coffea*

arabicua), corn (*Zea maize*), cucumber (*Cucumis sativus*), eggplant (*Solanum melongena*), guava (*Psidium guajava*), kiwi (*Actinidia* spp.), mango (*Mangifera indica*), nectarine (*Prunus persica*), okra (*Abelmoschus esculentus*), olive (*Olea europaea*), papaya (*Carica papaya*), parsley (*Petroselinum crispum*), pea (*Pisum sativum*), peach (*Prunus persica*), peanut (*Arachis hypogaea*), pineapple (*Ananas comosus*), pistachio (*Pistacia vera*), plum (*Prunus domestica*), potato (*Solanum tuberosum*), soybean (*Glycine max*), spinach (*Spinacia oleracea*), starfruit (*Averrhoa carambola*), taro (*Colocasia esculenta*), tomato (*Lycopersicon lycopersicum*), or watermelon (*Citrullus lanatus*). Since hybridization with wild or weedy relatives is not known to occur among plants included on this list, EPA has concluded that, when introduced into these species, CP4 EPSPS presents a low probability of risk to human health and the environment.

Glyphosate oxidoreductase (GOX)

SUMMARY

The Agency has conducted an environmental risk assessment of the plant-incorporated protectant (PIP) inert ingredient glyphosate oxidoreductase (GOX), and the genetic material necessary for its production. Topics covered in this assessment include mode of action, ecological effects, endangered species considerations, and gene flow from a modified crop to wild or weedy relatives. Data cited in this assessment was submitted to the Agency in support of Monsanto's MON 810 Bt Corn registration. Ecological data and published information on the biology of this protein indicate that this PIP inert ingredient is not a known toxin and/or pathogen of plant or animal species. Due to the low human health risks associated with this protein, the Agency has granted an exemption from the requirement of a tolerance for this PIP inert ingredient (40 CFR 180.1190; 62 FR 52509, Oct. 8, 1997).

MODE OF ACTION

GOX catalyzes the conversion of glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate; by increasing the speed of AMPA degradation, GOX confers glyphosate tolerance to plants. The GOX proteins that EPA evaluated for registration of MON 810 Bt Corn and for the GOX tolerance exemption were derived from a gene originally isolated from *Achromobacter* sp. strain LBAA. Two modified GOX proteins are specified in the tolerance exemption. They are designated GOX and GOXv247. The GOX protein has the same amino acid sequence as the native protein, with an additional four amino acid sequence on the N-terminus (remnants of an added signal sequence). In GOXv247, in addition to the four amino acids at the N-terminus from the signal sequence, the gene sequence was altered resulting in changes to three amino acids in the protein compared to the native protein. These protein variants are similar to the native GOX protein in terms of molecular weight, immunoreactivity, amino acid sequence, and enzymatic activity.

ENVIRONMENTAL EFFECTS

The Agency assessed the toxicity of GOX protein to mice, a representative non-target mammalian species that could be exposed to the PIP inert ingredient in the animal's natural

environment. The Tier 1 hazard assessment was performed on the basis of rodent acute oral toxicity data prepared for human health risk assessment purposes which utilized pure GOX protein. Submitted data indicate no significant adverse effects among mice dosed with up to 91.3 and 104 mg/kg body weight for GOX and GOXv247 proteins, respectively (MRID 439037-07 and 439037-08).

The reviewed data showed no toxicity to the non-target mammalian test species. As a result of this finding, and knowledge of the mode of action of the enzyme, the Agency concludes that no unreasonable adverse effects on non-target organisms are expected from exposure to GOX protein.

ENDANGERED SPECIES CONSIDERATIONS

Because this PIP inert ingredient is neither a known toxin and/or pathogen of plant or animal species, EPA does not expect that exposure to the GOX protein will result in a "may affect" finding for any endangered or threatened species.

GENE FLOW CONSIDERATIONS

Gene flow may occur when the GOX protein is expressed in a plant that can form viable hybrids in nature with wild or weedy relatives of that species. With the assistance of the October 2004 SAP, EPA has identified the following plants as not having wild or weedy relatives in the United States, its possessions, or territories, with which they can produce viable hybrids in nature: almond (*Prunus communis*), apricot (*Prunus armeniaca*), asparagus (*Asparagus officinale*) avocado (*Persea americana*), banana (*Musa acuminata*), barley (*Hordeum vulgare*), bean (*Phaseolus vulgaris*), black-eyed pea (*Vigna unguiculata*), cacao (*Theobroma cacao*), celery (*Apium graveolens*), chickpea (*Cicer arietinum*), citrus (*Citrus* spp.), coffee (*Coffea arabicua*), corn (*Zea maize*), cucumber (*Cucumis sativus*), eggplant (*Solanum melongena*), guava (*Psidium guajava*), kiwi (*Actinidia* spp.), mango (*Mangifera indica*), nectarine (*Prunus persica*), okra (*Abelmoschus esculentus*), olive (*Olea europaea*), papaya (*Carica papaya*), parsley (*Petroselinum crispum*), pea (*Pisum sativum*), peach (*Prunus persica*), peanut (*Arachis hypogaea*), pineapple (*Ananas comosus*), pistachio (*Pistacia vera*), plum (*Prunus domestica*), potato (*Solanum tuberosum*), soybean (*Glycine max*), spinach (*Spinacia oleracea*), starfruit (*Averrhoa carambola*), taro (*Colocasia esculenta*), tomato (*Lycopersicon lycopersicum*), or watermelon (*Citrullus lanatus*). Since hybridization with wild or weedy relatives is not known to occur among plants included on this list, EPA has concluded that, when introduced into these species, GOX presents a low probability of risk to human health and the environment.

ANTIBIOTIC RESISTANCE MARKER

Neomycin phosphotransferase II (NPTII)

SUMMARY

The Agency has conducted an environmental risk assessment of the plant-incorporated protectant (PIP) inert ingredient neomycin phosphotransferase II (NPTII), and the genetic material required for its production. Topics covered in this assessment include mode of action, ecological effects, endangered species considerations, and gene flow from a modified crop to wild or weedy relatives. Data cited in this assessment was submitted to the Agency in support of Monsanto's NewLeaf Potato and YieldGard Plus Corn registrations and is discussed in more detail in the *Bacillus thuringiensis* Plant-Incorporated Protectant and MON863 Biopesticide Registration Action Documents. Ecological data and published information on the biology of this protein indicate that this PIP inert ingredient is not a known toxin and/or pathogen of plant or animal species. Due to the low human health risks associated with this protein, the Agency has granted an exemption from the requirement of a tolerance for this PIP inert ingredient (40 CFR 180.1134; 59 FR 49125, Sep. 28, 1994).

MODE OF ACTION

The NPTII enzyme inactivates, by phosphorylation, a range of aminoglycoside antibiotics, including neomycin and kanamycin. NPTII occurs naturally in bacteria, is not known to be toxic, and degrades rapidly under simulated gastric conditions. Microbially-produced and plant-produced NPTII enzymes have similar molecular weights and terminal amino acids, indicating that glycosylation and post-transcription modifications do not occur with plant-expressed NPTII.

ENVIRONMENTAL EFFECTS

The Agency assessed the toxicity of NPTII protein to representative non-target organisms that could be exposed to the PIP inert ingredient. Toxicity evaluations included the following Tier I studies: Mammalian (mouse), Avian (juvenile northern bobwhite quail), Freshwater Fish (channel catfish), Aquatic Invertebrate [cladoceran (*Daphnia magna*)], and Non-target Arthropod (collembola, lady beetle, and monarch butterfly). A Tier IV field study of non-target abundance is also discussed below.

The wild mammal hazard assessment was performed on the basis of rodent acute oral toxicity data prepared for human health risk assessment purposes which utilized pure NPTII protein. Submitted data indicate no significant adverse effects among mice dosed with up to 5000 mg/kg body weight NPTII protein (MRID 430547-01).

The remaining single species studies were maximum hazard dose evaluations, where NPTII protein toxicity was evaluated by feeding select non-target organisms NewLeaf or YieldGard Plus plant material, which has been shown to contain the PIP inert ingredient NPTII and the active ingredient Cry3A (NewLeaf) or Cry3Bb1 (YieldGard Plus). The avian studies, where northern bobwhite quail were fed potato tuber tissue for the NewLeaf and corn grain for the YieldGard Plus evaluations, reported no adverse effects to the test species at field exposure levels (NewLeaf MRIDs 429322-14, 429322-15; MON863 MRID 449043-15). The freshwater fish corn grain feeding study indicated that channel catfish diets may contain up to 35% NPTII protein-containing corn grain meal with no adverse effects on fish growth, feed conversion efficiency, survival, behavior, or body composition (MON863 MRID 449043-19). In a corn pollen feeding study, no adverse effects to *Daphnia magna*, a representative of aquatic invertebrate species, were seen at field level exposure (MON863 MRID 449043-18). In separate

corn pollen dietary toxicity studies, no adverse effects to adult and larval lady beetles were seen at field level exposure (diet composed of 50% pollen) (MON863 MRIDs 455382-04, 453613-02). A dietary toxicity study with the monarch butterfly also showed no adverse effects to larvae when exposed to field levels of corn pollen (MON863 MRID 455382-05). Finally, results of a corn leaf tissue feeding study indicated that the NPTII protein does not adversely affect reproduction of Collembola, a representative plant tissue decomposer (MON863 MRID 449043-17).

A field study that evaluated arthropod abundance in YieldGard Plus fields was also conducted. Results showed that 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 *Bt* plants than in plots treated with conventional chemical insecticides (MRID 456530-03).

The reviewed data showed no toxicity to non-target test species. As a result of these findings, and knowledge of the mode of action of the enzyme, the Agency concluded that no unreasonable adverse effects on non-target organisms are expected from exposure to NPTII protein.

ENDANGERED SPECIES CONSIDERATIONS

Because this PIP inert ingredient is neither a known toxin and/or pathogen of plant or animal species, EPA does not expect that exposure to the NPTII protein will result in a “may affect” finding for any endangered or threatened species.

GENE FLOW CONSIDERATIONS

EPA anticipates that gene flow may occur if NPTII is expressed in plants that have wild or weedy relatives in the United States, its possessions, or territories. However, no unreasonable adverse effects to the environment are expected from movement of this inert into wild plant populations, because the trait is unlikely to confer selective advantage to recipient plants.

EPA considers the frequency of horizontal gene transfer (HGT) from NPTII-expressing plants to microbes to be very low. Studies conducted under a range of test conditions could not demonstrate HGT from plants to microbes, nor is there a clear mechanism for such transfer (Nap *et al.*, 1992; Redenbaugh *et al.* 1994; Schlüter *et al.*, 1995; SAP Report, 2001). Nonetheless, if HGT was to occur it would not have a significant affect on existing populations of neomycin and kanamycin resistant bacteria, because resistance to these groups of antibiotics is widespread in naturally occurring microbes in humans and the environment.

Due to the low probability that gene flow from NPTII-containing plants would confer a selective advantage on wild or weedy relatives of that species and the low frequency of HGT from NPTII-expressing plants to microbes, EPA has concluded that this inert marker poses low risk to human health and the environment when used in any plant as part of a PIP.

OTHER MARKERS

Beta-D-glucuronidase (GUS) from *Escherichia coli*

SUMMARY

The Agency has conducted an environmental risk assessment of the *Escherichia coli*-derived plant-incorporated protectant (PIP) *beta*-D-glucuronidase (GUS), and the genetic material necessary for its production. Topics covered in this assessment include mode of action, ecological effects, endangered species considerations, and gene flow wild or weedy relatives. Data cited in this assessment were submitted to the Agency in support of Monsanto's Bollgard II Bt cotton registration and are discussed in the Bollgard II BRAD. Ecological data and published information on the biology of this protein indicate that this PIP inert ingredient is not a known toxin and/or pathogen of plant or animal species. Due to the low human health risks associated with this protein, the Agency has granted an exemption from the requirement of a tolerance for this PIP inert ingredient (40 CFR 180.1216; 66 FR 42961, Aug. 16, 2001).

MODE OF ACTION

E.coli β-glucuronidase (GUS), an enzyme that catalyzes the hydrolysis of glucuronides, is introduced into plants to serve as a visual marker of transformation (Guivarc'h et al., 1996). GUS from *E.coli* has a pH optimum near 7.0 and maintains enzymatic activity for approximately 2 hours at 55º C, but is rapidly inactivated at 60º C. This enzyme is ubiquitous in the digestive system of humans and other vertebrates. Other types of GUS enzyme are present in the liver, spleen, kidneys, salivary glands, breast milk and a variety of other tissues in humans, other vertebrates and a number of invertebrates, as well as in the fruit, seed coat, and endosperm of various plants.

ENVIRONMENTAL EFFECTS

The Agency assessed the toxicity of GUS protein to representative non-target organisms that could be exposed to the PIP inert ingredient. Toxicity evaluations included the following Tier I studies: Mammalian (mouse), Avian (juvenile northern bobwhite quail), Freshwater Fish (channel catfish), and Non-target Arthropod (collembola).

The wild mammal hazard assessment was performed on the basis of rodent acute oral toxicity data prepared for human health risk assessment purposes which utilized pure GUS protein. Submitted data indicate no significant adverse effects among mice dosed with up to 100.0 mg/kg body weight GUS protein (MRID 449888-00).

The remaining single species studies were maximum hazard dose evaluations, where GUS protein toxicity was evaluated by feeding select non-target organisms Bollgard II plant tissue, which has been shown to contain the PIP inert ingredient GUS and the active ingredients Cry1Ac and Cry2Ab in cotton leaf and seed material. The avian cottonseed meal feeding study reported no adverse effects to northern bobwhite quail at field exposure levels (MRID 450863-16). The freshwater fish cottonseed meal feeding study indicated that channel catfish diets may contain up to 20% GUS protein-containing cotton seed meal with no adverse effects on fish growth, feed conversion efficiency, survival, behavior, or body composition (MRIDs 450863-18 and 453371-03). Finally, results of a cotton tissue feeding study indicated that the GUS protein is non-toxic to

Collembola, a representative plant tissue decomposer, and did not adversely affect the rate of Collembola reproduction (MRID 450863-14).

The reviewed data showed no toxicity to non-target test species. As a result of these findings, and the natural occurrence of this protein in a variety of species, and knowledge of the mode of action of the enzyme, the Agency concluded that no unreasonable adverse effects on non-target organisms are expected from exposure to GUS protein.

ENDANGERED SPECIES CONSIDERATIONS

Because this PIP inert ingredient is neither a known toxin and/or pathogen of plant or animal species, EPA does not expect that exposure to the GUS protein will result in a "may affect" finding for any endangered or threatened species.

GENE FLOW CONSIDERATIONS

EPA anticipates that gene flow may occur if GUS is expressed in plants that have wild or weedy relatives in the United States, its possessions, or territories. However, due to the low probability that gene flow from a GUS-containing plant would confer a selective advantage on wild or weedy relatives of that species, EPA has concluded that this inert marker poses low risk to human health and the environment when used in any plant as part of a PIP.

Phosphomannose isomerase (PMI)

SUMMARY

The Agency has conducted an environmental risk assessment of the *Escherichia coli*-derived plant-incorporated protectant (PIP) phosphomannose isomerase (PMI), and the genetic material required for its production. Topics covered in this assessment include mode of action, ecological effects, endangered species considerations, and gene flow from a modified crop to wild or weedy relatives. Data cited in this assessment were submitted to the Agency in support of Syngenta's MIR604 Bt corn registration. Ecological data and published information on the biology of this protein indicate that this PIP inert ingredient is not a known toxin and/or pathogen of plant or animal species. Due to the low human health risks associated with this protein, the Agency has granted an exemption from the requirement of a tolerance for this PIP inert ingredient (40 CFR 180.1252; 69 FR 26770, May 14, 2004).

MODE OF ACTION

The PMI protein is a ubiquitous enzyme involved in carbohydrate metabolism. Plant cells take up mannose and convert it to mannose-6-phosphate, an inhibitor of glycolysis. PMI activity converts mannose-6-phosphate to fructose-6-phosphate, an intermediate of glycolysis, which positively supports growth of transformed cells (Todd and Tague, 2001). PMI, or a highly homologous enzymatic protein, is expressed in many species including enteric bacteria, fungi, insects, some species of nematodes, and mammals including monkeys, mice and man.

ENVIRONMENTAL EFFECTS

The Agency assessed the toxicity of PMI protein to representative non-target organisms that could be exposed to the PIP inert ingredient. Toxicity evaluations included the following Tier I studies: Mammalian (mouse) and Freshwater Fish (rainbow trout).

The wild mammal hazard assessment was performed on the basis of rodent acute oral toxicity data prepared for human health risk assessment purposes which utilized pure PMI protein. Submitted data indicate no significant adverse effects among mice dosed with up to 5,050 mg/kg of dosing solution or 3,080 mg/kg of PMI protein (MRID 459344-07).

The freshwater fish study was a maximum hazard dose evaluation, where PMI protein toxicity was evaluated by feeding juvenile rainbow trout Event MIR604 corn grain, which has been shown to contain the PIP inert ingredient PMI and the active ingredient Cry1Ab. Results of the feeding study indicate that rainbow trout diets may contain up to 50% PMI protein-containing corn seed meal with no adverse effects on fish weight or length (MRID 461556-17).

The reviewed data showed no toxicity to non-target mammalian and freshwater fish test species used in the evaluations. As a result of these findings, and the natural occurrence of this protein in a variety of microbe, insect, plant and mammalian species, the Agency has concludes that no unreasonable adverse effects on non-target organisms are expected from exposure to PMI protein.

ENDANGERED SPECIES CONSIDERATIONS

Because this PIP inert ingredient is neither a known toxin and/or pathogen of plant or animal species, EPA does not expect that exposure to the PMI protein will result in a “may affect” finding for any endangered or threatened species.

GENE FLOW CONSIDERATIONS

EPA anticipates that gene flow may occur if PMI is expressed in plants that have wild or weedy relatives in the United States, its possessions, or territories. However, due to the low probability that gene flow from a GUS-containing plant would confer a selective advantage on wild or weedy relatives of that species, EPA has concluded that this inert marker poses low risk to human health and the environment when used in any plant as part of a PIP.

REFERENCES

Guivarc'h, A., Caissard, J.C., Azmi, A., Elmayan, T., Chriqui, D., and M. Tepfer (1996). In situ detection of expression of the *gus* reporter gene in transgenic plants: ten years of blue genes. *Transgenic Res.* 5:281–288.

Levin, J.G. and Sprinson, D.B. (1964). The enzymatic formation and isolation of 5-enolpyruvylshikimate 3-phosphate. J. Biol. Chem. 239:1142-1150.

Nap, J.P., Bijvoet, J. and Strikema, W.J. (1992). Biosafety of kanamycin-resistant transgenic plants: an overview. Transgenic Crops 1:239-249.

OECD (2002). Module II: Herbicide biochemistry, herbicide metabolism, and the residues in glufosinate-ammonium (Phosphinothricin)- tolerant transgenic plants. Series on Harmonization of Regulatory Oversight in Biotechnology. OECD Environmental Health and Safety Publications 25:1-19.

Redenbaugh, K., Hiatt, W., Martineau, B., Lindemann, J. and Emlay, D. (1994). Aminoglycoside 3'-phosphotransferase II: review of its safety and use in the production of genetically engineered plants. Food Biotechnology 8:137-165.

SAP Report No. 2000-17 (2001). Bt Plant-Pesticides Risk and Benefit Assessments.

Schlüter, K., Futterer, J. and Potrykus, I. (1995). "Horizontal" gene transfer from a transgenic potato line to a bacterial pathogen (*Erwinia chryanthemi)* occurs - if at all - at an extremely low frequency. Bio/Technology 13:1094-1098.

Steinruken, H.C., Amrhein, N. (1980). The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl shikimic acid-3-phosphate synthase. Biochem. Biophys. Res. Commun. 94:1207-1212.

Thompson, C.J., N.R.Movva, R. Tizard, R. Crameri, J.E. Davies, M. Lauwereys, J. Botterman. (1987). Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J. 6:2519-23.

Todd, R. and B.W. Tague (2001). Phosphomannose Isomerase: A versatile selectable marker for *Arabidopsis thaliana* germ-line transformation. Plant Molecular Biology Reporter 19: 307-319. pp 758-5016.

UNPUBLISHED STUDIES (identified by MRID Numbers)

429322-14 Cambell, S.; Beavers, J.; Jaber, M. (1993) A Dietary Toxicity Study with Russet Burbank Potatoes in the Northern Bobwhite: Lab Project Number: 139-356: WL-93-11: 93-081E. Unpublished study prepared by Wildlife International Ltd. 87 p.

429322-15 Campbell, S.; Beavers, J.; Jaber, M. (1993) A Dietary Toxicity Study with Russet Burbank Potatoes in the Northern Bobwhite: Lab Project Number: 139-357: WL-93-12. Unpublished study prepared by Wildlife International Ltd. 50 p.

430547-01 Naylor, M. (1992) Acute Oral Toxicity Study of (inert ingredient) in Albino Mice: Lab Project Number: 91177: ML-91-409: EHL 91177. Unpublished study prepared by Monsanto Co. Environmental Health Lab. 161 p.

433236-10 Collins, M. (1994) Bt Maize Pollen (PHO176-0194): Acute Toxicity to Daphnids (*Daphnia magna*) under Static-Renewal Conditions: Lab Project Number: 94/3/5217: 1781/0394/6419/110. Unpublished study prepared by Springborn Laboratories, Inc. 59 p.

433396-02 Obrycki, J. (1994) Feeding, Development, and Survival of the Lady Beetle Predator *Coleomegilla maculata* (Coleoptera:coccinellidae) on Transgenic "Bt" Maize (Corn) Pollen: Final Report: Lab Project Number: PHO176/0194 ISU. Unpublished study prepared by Iowa State University, Department of Entomology. 62 p.

434157-03 Maggi, V. (1994) Evaluation of the Dietary Effect(s) of Transgenic Bt Maize (Corn) Pollen (Sample PH0176-0294) on Honeybee Development: Lab Project Number: CAR 176-94. Unpublished study prepared by California Agricultural Research, Inc. 57 p.

435332-05 Campbell, S.; Beavers, J. (1994) A Dietary Toxicity Study with MON 80187 Meal in the Northern Bobwhite: Lab Project Number: WL 94-150: 139-387. Unpublished study prepared by Wildlife International Ltd. 32 p.

436433-03 Naylor, M. (1993) Acute Oral Toxicity Study of (inert ingredient) in Albino Mice: Lab Project Number: 92223. Unpublished study prepared by Monsanto Co. 179 p.

438879-01 Jackson, L.; Robinson, E.; Nida, D. et al. (1995) Evaluation of the European Corn Borer Resistant Corn Line MON 801 as a Feed Ingredient for Catfish: Lab Project Number: 94-01-39-16: 14066: 95-459-720. Unpublished study prepared by Mississippi State University Delta Research and Extension Center. 39 p.

439037-07 Nickson, T.; Bailey, M.; Harrison, L. et al. (1994) Preparation and Confirmation of Doses for Acute Oral Toxicity Study in Mice with (Inert Ingredient): Lab Project Number: 93-02-30-03: 12967: 93-447-703. Unpublished study prepared by Monsanto Co. 42 p.

439037-08 Naylor, M. (1994) Acute Oral Toxicity Study of (Inert Ingredient) in Albino Mice: Lab Project Number: ML-93-206: 93079. Unpublished study prepared by Monsanto Environmental Health Lab. 204 p.

439995-02 Merriman, T. (1996) An Acute Oral Toxicity Study in Mice with (inert ingredient): Lab Project Number: DGC-95-A18: 3406.2: 3406.1. Unpublished study prepared by DEKALB Genetics Corp. and Springborn Laboratories (SLI). 50 p.

439995-10 Palmer, S.; Beavers, J. (1996) Lyophilized DBT418 Leaf Tissue: A Dietary Toxicity Study with the Northern Bobwhite: Lab Project Number: DGC-95-A13: 438-101: 438/102495/QLC-TRAN/CHP106. Unpublished study prepared by DEKALB Genetics Corp. and Wildlife Int'l. Ltd. 51 p.

439995-12 Collins, M. (1996) Transgenic Maize Leaf Tissue and Microbially Produced CryIA(c) Protein: Chronic Toxicity To Collembola (*Folsomia candida*), Under Static Conditions: Lab Project Number: DGC-95-A16: 13601.1195.6101.123: 96-3-6408. Unpublished study prepared by DEKALB Genetics Corp. and Springborn Laboratories, Inc. (SLI). 141 p.

439995-13 Garvey, N. (1996) Lyophilized DBT418 Maize Leaf Tissue and Microbially Produced CryIA(c) Protein: Acute (14-Day) Toxicity To Earthworms (*Eisenia foetida*): Lab Project Number: DGC-95-A15: 13601.1095.6100.630: 96-2-6377. Unpublished study prepared by DEKALB Genetics Corp. and Springborn Laboratories, Inc. (SLI). 81 p.

449043-15 Gallagher, S.; Grimes, J.; Beavers, J. (1999) Bacillus thuringiensis Protein 11231 in Corn Grain: A Dietary Toxicity Study with the Northern Bobwhite: Lab Project Number: WL-99-014: 16161: 139-44. Unpublished study prepared by Monsanto Company and Wildlife International Ltd. 43 p.

449043-17 Teixeira, D. (1999) Assessment of Chronic Toxicity of Corn Tissue Containing the Bacillus thuringiensis Protein 11098 to Collembola (*Folsomia candida*): Lab Project Number: SB-98-296: 15988: 252.6149. Unpublished study prepared by Monsanto Company and Springborn Labs. 55 p.

449043-18 Drottar, K.; Krueger, H. (1999) Bacillus thuringiensis Protein 11098 in Corn Pollen: A 48-Hour Static-Renewal Acute Toxicity with the Cladoceran (*Daphnia magna*): Lab Project Number: WL-

98-295: 16163: 139A-236. Unpublished study prepared by Monsanto Company and Wildlife International Ltd. 31 p.

449043-19 Meng, L.; Robinson, E. (1999) Evaluation of Insect Protected Corn Lines MON 853 and MON 859 as a Feed Ingredient for Catfish: Lab Project Number: XX-98-297: 16164. Unpublished study prepared by Monsanto Company and Mississippi State University. 32 p.

449888-00 Monsanto Company (1999) Submission of Toxicity Data in Support of the Petition for Tolerance of (Inert Ingredient) as a Plant Pesticide. Transmittal of 1 Study.

450863-14 Teixiera, D. (2000) Assessment of Chronic Toxicity of Cotton Tissue Containing Insect Protection Protein 2 to Collembola (*Folsomia candida*): Amended Final Report: Lab Project Number: 252.6152: SB-99-063: MSL 16174. Unpublished study prepared by Springborn Laboratories, Inc. 49 p.

450863-16 Gallagher, S.; Grimes, J.; Beavers, J. (2000) Insect Protection Protein 2 In Cottonseed Meal: A Dietary Toxicity Study with the Northern Bobwhite: Lab Project Number: 139-449: WL-99-065: MSL 16178. Unpublished study prepared by Wildlife International, Ltd. 40 p.

450863-18 Li, M.; Robinson, E. (2000) Evaluation of Cottonseed Meal Derived from Insect Protected Cotton Lines 15813 and 15985 as a Feed Ingredient for Catfish: Final Report: Lab Project Number: XX-99-056: MSL 16179: 99-858E. Unpublished study prepared by Thad Cochran National Warmwater Aquaculture Center. 22 p.

453371-03 Li, M.; Robinson, E. (2001) Evaluation of Cottonseed Meal Derived from Insect Protected Cotton Lines 15813 and 15985 as a Feed Ingredient for Catfish: Lab Project Number: XX-99-056: MSL 16179. Unpublished study prepared by Thad Cochran National Warmwater Aquaculture Center 23 p.

453613-02 Bryan, R.; Porch, J.; Krueger, H. (2001) Dietary Effects of Transgenic BT Corn Pollen Expressing a Variant of Cry3Bb1 Protein on Adults of the Ladybird Beetle, *Hippodamia convergens*: Final Report: Lab Project Number: WL-2000-158: MSL-17171: 139-453. Unpublished study prepared by Wildlife International, Ltd. 36 p.

455382-04 Duan, J.; McKee, M.; Nickson, T. (2001) Dietary Effects of Transgenic Bacillus thuringiensis (Bt) Corn Pollen Expressing a Variant of Cry3Bb1 Protein on Larvae of the Ladybird Beetle, *Coleomegilla maculata*: Lab Project Number: MSL-16907: 00-01-39-26. Unpublished study prepared by Monsanto Company. 39 p.

455382-05 Sears, M.; Mattila, H. (2001) Determination of the Toxicity of Corn Pollen Expressing a Cry3Bb1 Variant Protein to First Instar Monarch Butterfly Larvae (*Danaus plexippus*) via Laboratory Bioassay: Lab Project Number: MSL-17235: 01-01-39-26. Unpublished study prepared by University of Guelph. 33 p.

456530-03 Head, G. (2002) Research on the Effects of Corn Rootworm Protected Transgenic Corn Events on Nontarget Organisms: Preliminary Results: Lab Project Number: 00-CR-032E-7. Unpublished study prepared by Monsanto Company. 70 p.

457665-09 Teixeira, D. (2002) Assessment of the Chronic Toxicity of VIP3A and VIP3A/Cry1Ab Maize Pollen to Pink-Spotted Lady Beetle (*Coleomegilla maculata*): Lab Project Number: 1781.6623: PACHA: PACHA-00-05AN. Unpublished study prepared by Springborn Smithers Laboratories. 79 p. {OPPTS 885.4340}

457921-01 Putt, A. (2002) VIP3A Maize (Corn) Pollen--Acute Toxicity to Daphnids (*Daphnia magna*) Under Static-Renewal Conditions: Lab Project Number: 1781.6616: SSB-003-00. Unpublished study prepared by Springborn Smithers Laboratories. 42 p.

458358-07 Valchos, D.; Habig, C. (2002) Environmental Safety Assessment of Bacillus thurnigiensis VIP3A Protein and VIP3A Cotton Event COT102 to Non-Target Organisms: Unpublished study prepared by Syngenta Seeds, Inc. and Exponent. 37 p.

458358-08 Teixeira, D. (2002) VIP3A Inbred Maize (Corn) Pollen: Toxicity to Green Lacewing (*Chrysoperla carnea*): Lab Project Number: 1781.6624. Unpublished study prepared by Springborn Smithers Laboratories. 69 p. {OPPTS 885.4340}

458358-10 Privalle, L. (2002) Impact of VIP3A and Cry1AB Transgenic Maize (Corn) Leaf Tissue (Samples LLPACHA-0100, LLBT11-0100, and LLPACHABT11-0100) On 28-Day Survival and Reproduction of Collembola (*Folsomia candida*): Lab Project Number: PACHA-00-03: SSB-006-01: PACHA. Unpublished study prepared by Syngenta Seeds, Inc. 21 p.

458358-13 Dively, G. (2002) Impact of Transgenic Lepidopeteran-Resistant VIP3A Field Corn (Maize) on Honey Bee Colonies in a Semi-Field Setting. Unpublished study prepared by Wye Research and Education Center, University of Maryland. 34 p.

459344-07 Kuhn, J. (1999) Acute Oral Toxicity Study in Mice: Final Report: (Inert Ingredient): Final Report: Lab Project Number: 4708-98: PMI: PMI-98-01. Unpublished study prepared by Stillmeadow, Inc. 24 p. {OPPTS 870.1100}

461556-17 Hutchings, M.; Caunter, J. (2003) A 28-Day Laboratory Study to Evaluate the Effects of Modified Cry3A Maize Fish Feed (FFMIR604-0103) on the Growth of Juvenile Rainbow Trout (*Oncorhynchus mykiss*). Project Number: 03/0294/A, 2033039. Unpublished study prepared by Astrazeneca Uk Ltd. 19 p.