Agriculture and Agri-Food Canada
Food Production and Inspection Branch
Plant Products Division

Agriculture et Agroalimentaire Canada
Direction générale, Production et inspection des aliments
Division des produits végétaux

Decision Document DD97-18

Determination of the Safety of Pioneer Hi-Bred International Inc.'s European Corn Borer (ECB) Resistant Corn (*Zea mays* L.) Line MON809

This Decision Document has been prepared to explain the regulatory decision reached under the guidelines Dir94-08 *Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits* and its companion document Dir94-11 *The Biology of* Zea mays *L. (Corn/Maize)* and the guidelines Dir95-03 *Guidelines for the Assessment of Livestock Feed from Plants with Novel Traits.*

Agriculture and Agri-Food Canada (AAFC), specifically the Plant Biotechnology Office and the Feed Section of the Plant Products Division, with input from the Plant Health Risk Assessment Unit, has evaluated information submitted by Pioneer Hi-Bred International Inc. This information is in regard to the European Corn Borer (ECB) resistant corn line MON809. AAFC has determined that this plant with novel traits does not present altered environmental interactions or pose concerns for the safety of livestock consuming feed derived from the PNT, when compared to currently commercialized corn varieties in Canada.

Unconfined release into the environment and use as livestock feed of the corn line MON809 is therefore authorized. Any other *Zea mays* lines and intraspecific hybrids resulting from the same transformation event and all their descendants, are also approved, provided no inter-specific crosses are performed, provided the intended use is similar, provided it is known following thorough characterization that these plants do not display any additional novel traits and that the resulting lines are substantially equivalent to currently grown corn, in terms of their potential environmental impact and livestock feed safety and provided that pest resistance management requirements described in the present document are applied.

Please note that, while determining the environmental safety of plants with novel traits is a critical step in the commercialization of these plant types, other requirements still need to be addressed, such as the evaluation of food safety (Health Canada) and Variety Registration (AAFC).

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I. Brief Identification of Plant with Novel Traits (PNT)

Designation(s) of the PNT: MON809

Applicant: Pioneer Hi-Bred International Inc.

Plant Species: *Zea mays* L.

Novel Traits: Resistance to European Corn Borer (*Ostrinia nubilalis*); tolerance to the herbicide glyphosate.

Trait Introduction Method: Microprojectile bombardment of plant cells.

Proposed Use of PNT's: Production of *Z. mays* for seed oil for human consumption and seed oil, meal and silage for livestock feed. These materials will not be grown outside the normal production area for corn in Canada.

II. Background Information

Pioneer Hi-Bred International Inc. in co-operation with Monsanto Company, has developed a corn line resistant to the European Corn Borer (ECB) larvae, a periodic pest of corn in Canada. This corn line, referred to in this document as MON809, was developed to provide a method to control yield losses from insect feeding damage caused by the larval stages of ECB, without the use of conventional pesticides.

MON809 was developed using recombinant DNA technology, resulting in the introduction of bacterial genes conferring ECB resistance and glyphosate herbicide tolerance, into elite hybrid lines. Herbicide tolerance was used to select modified plants, but is not expressed at sufficient levels to confer field tolerance.

Pioneer Hi-Bred International Inc. has provided data on the identity of line MON809, a detailed description of the transformation method, data and information on the gene insertion sites, copy number and levels of expression in the plant, the role of the inserted genes and regulatory sequences in donor organisms and full nucleotide sequences. The novel proteins were identified, characterized and compared to the original bacterial proteins, including an evaluation of their potential toxicity to livestock and non-target organisms. Relevant scientific publications were supplied.

These materials have been field tested in Canada under confined conditions in Saskatchewan (1994-96), Alberta (1996), Manitoba (1995, 96) and Ontario (1996).

Agronomic characteristics of corn hybrids derived from MON809 such as seed dormancy, seedling vigour, early stand establishment, plant vigour, time to maturity, flowering

period, susceptibilities to various *Z. mays* pests and pathogens, and seed production were compared to those of unmodified *Z. mays* counterparts.

The Plant Biotechnology Office of the Plant Products Division, AAFC, has reviewed the above information, in light of the assessment criteria for determining environmental safety of plants with novel traits, as described in the regulatory directive Dir94-08:

- potential of the PNT's to become weeds of agriculture or be invasive of natural habitats,
- potential for gene flow to wild relatives whose hybrid offspring may become more weedy or more invasive,
- potential for the PNT's to become plant pests,
- potential impact of the PNT's or their gene products on non-target species, including humans, and
- potential impact on biodiversity.

AAFC has consulted with the Pest Management Regulatory Agency of Health Canada on issues related to potential development of ECB populations resistant to the insecticidal protein produced by the PNT's.

The Feed section of the Plant Products Division AAFC, has also reviewed the above information with respect to the assessment criteria for determining the safety and efficacy of livestock feed, as described in Dir95-03:

- potential impact to livestock and
- potential impact on livestock nutrition.

III. Description of the Novel Traits

1. Resistance to the European Corn Borer (ECB)

- *Bacillus thuringiensis* var. *kurstaki* HD-1(B.t.k.) is a common gram-positive soil-borne bacterium. In its spore forming stage, it produces several insecticidal protein crystals, including the δ-endotoxin Cry1A(b), which is active against certain lepidopteran insects, such as ECB. This protein has been shown to be non-toxic to humans, other vertebrates and beneficial insects. B.t.k. based foliar insecticides have been registered for over 30 years in Canada and have a long history of safe use.

- A synthetic *cryIA(b)* gene was developed for maximum expression in corn, and introduced into line MON809. The gene codes for a protein similar to the *Bacillus thuringiensis* var. *kurstaki* HD-1 insecticidal crystal protein. The protein is insecticidal to lepidopteran larvae after cleavage to a bio-active, trypsin resistant core. Insecticidal activity is believed to depend on the binding of the active fragment to specific receptors present in susceptible insects on midgut epithelial cells, forming

pores which disrupt osmotic balance and eventually results in cell lysis. Specific Lepidopteran pests of corn sensitive to the protein are ECB and corn earworm.

- The *cry1A(b)* gene is linked to a strong constitutive promoter. Average protein expression evaluated at six locations was 1.63 µg/g (f.w.) in leaves, 0.55µg/g (f.w.) in seeds and 1.23 µg/g (f.w.) in the whole plant. Cry 1A(b) protein was not detected in pollen. Protein expression ranged from 0.88-2.37 µg/g (f.w.) in leaves, from 0.28-0.73 µg/g (f.w.) in grain and from 0.73-1.73 µg/g (f.w.) in the whole plant. Protein expression declined over the growing season as indicated by the Cry1A(b) levels present in leaves assayed over the growing season.

- The Cry1A(b) protein was shown to degrade readily in the environment. The plant expressed protein had DT_{50} and DT_{90} values (time to degrade to 50% and 90 % of the original bioactivity) of 2 and 15 days respectively.

- Protein allergens are normally resistant to digestion unlike the Cry1A(b) protein which was shown to degrade readily in simulated gastric fluid. Unlike many known allergens the insecticidal protein is not glycosylated. A search for amino acid sequence similarity between the Cry1A(b) protein and known allergens, using a database assembled from the public domain databases GenBank, EMBL, Pir and SwissProt, revealed no significant amino acid sequence homologies. A search of a similarly constructed database of known toxins indicated no amino acid sequence homologies between known toxins and the Cry1A(b) protein, with the exception of homologies to other B.t. insecticidal proteins.

- Corn products are an important alternative to wheat flour for individuals afflicted with coeliac disease, an immune mediated food intolerance for which wheat gliadins have been implicated as the causal agent. In light of the importance of corn products to these individuals, a sequence similarity search was conducted and no amino acid sequence homologies between the Cry1A(b) protein and gliadins were detected.

- The full nucleotide sequence and corresponding amino acid sequence were provided.

- The 131 kDa protein expressed in plants and the resulting proteolytic fragments were compared to the bacterial proteins and shown to be of similar molecular weight, amino acid sequence, immunological reactivity and trypsin resistance. The protein was not glycosylated and showed similar bioactivity and host range specificity to the native protein.

2. Glyphosate Tolerance

- Two genes have been introduced into MON809 to provide tolerance to glyphosate, the active ingredient in Roundup® herbicide**.** The tolerance, although not sufficient to provide field tolerance to the herbicide, was used to select transformed plants.

- The CP4 EPSPS gene expresses a bacterial version from *Agrobacterium tumefaciens* strain CP4, of 5-enolpyruvlshikimate-3-phosphate synthase**,** a plant enzyme involved in the shikimate biochemical pathway for the production of aromatic amino acids. Glyphosate inhibits the native plant EPSPS, thus blocking the shikimate pathway and halting the production of these necessary amino acids. The CP4-EPSPS is a glyphosate tolerant version of the enzyme with high catalytic activity. Under glyphosate selection, the addition of the bacterial, glyphosate tolerant enzyme will permit the normal production of aromatic amino acids.

- The second gene, *gox*, is derived from *Achromobacter* sp., a ubiquitous soil bacteria species which expresses an enzyme that degrades glyphosate by conversion to aminomethylphosphonic acid (AMPA) and glyoxylate. In line MON809 only a partial copy of the gene is inserted and no gene expression was detected.

- A plant-derived coding sequence expressing a chloroplast transit peptide was co-introduced with each of the glyphosate resistance genes. The chloroplast transit peptides facilitates the import of the protein products from the glyphosate resistance genes into the chloroplast, which is the location of the shikimate pathway and the site of action for glyphosate.

- Expression of the CP4 EPSPS gene averaged 21.68 µg/g (f.w.) in leaves, 9.41 µg/g (f.w.) in grain and 1.6 µg/g (f.w.) in the whole plant.

- The plant expressed enzyme showed no significant homology with any known toxins or allergens. It is common in nature and no toxic or allergenic effects would be expected to occur. Data was presented which showed that the CP4 EPSPS enzyme is rapidly inactivated by heat and by digestion in simulated mammalian gastric fluid.

3. Development Method

- MON809 was co-transformed with two vectors, one carrying a synthetic *cry1A(b)* gene and the second bearing the two herbicide resistance genes. The two plasmid vectors were introduced by microprojectile bombardment into cultured plant cells. Glyphosate tolerant transformed cells were selected, then cultured in tissue culture medium for plant regeneration.

4. Stable Integration into the Plant's Genome

Southern analysis indicated one integrated DNA segment which included a complete and a partial copy of the *cryIA(b)* gene, two complete copies of the CP4 EPSPS genes and a partial *gox* gene.

Segregation data indicate a stable insertion, with the trait segregating according to Mendelian genetics, through five generations of crossing.

IV. Assessment Criteria for Environmental Safety

1. Potential of the PNT to Become a Weed of Agriculture or be Invasive of Natural Habitats

The biology of corn (*Zea mays*), described in Dir94-11, shows that unmodified plants of this species are not invasive of unmanaged habitats in Canada. Corn does not possess the potential to become weedy due to traits such as lack of seed dormancy, the non-shattering aspect of corn cobs, and poor competitive ability of seedlings. According to the information provided by Pioneer Hi-Bred, MON809 and derived corn hybrids were determined not to be different from their counterparts in this respect.

AAFC evaluated data submitted by Pioneer Hi-Bred on the reproductive and survival biology of corn hybrids derived from MON809, and determined that early stand establishment, vegetative vigour, time to maturity and seed production were within the normal range of expression of these traits currently displayed by commercial corn hybrids. No genes were inserted for cold tolerance or winter survival.

No competitive advantage was conferred to these plants, other than that conferred by resistance to European Corn Borer. Resistance to ECB will not, in itself, render corn weedy or invasive of natural habitats since none of the reproductive or growth characteristics were modified. Glyphosate tolerance of MON809 is not sufficiently high for effective weed control in the field. Also, tolerance to glyphosate would not render corn weedy, since this herbicide is not presently used in crop rotation cycles involving corn. Glyphosate is commonly used for chemical fallow production, nonetheless the emergence of resistant corn volunteer plants should not be problematic since glyphosate tolerant corn volunteer plants can be easily managed by either mechanical means or by the use of other available herbicides.

NOTE: A longer term concern, if there is general adoption of several different crop and specific herbicide weed management systems, is the development of crop volunteers with novel tolerances to specific herbicides. This could result in the loss of the use of these herbicides in some crop rotation cycles. Some canola (*Brassica napus*) varieties modified to express glyphosate tolerance (see DD95-02) were

recently registered in Canada. Should glyphosate tolerant corn and canola be grown in rotation, volunteers would not be controlled with this herbicide. Agricultural extension personnel, in both the private an public sectors, should therefore promote careful management practices for growers who use any of these herbicide tolerant crops.

The above considerations, together with the fact that the novel traits have no intended effects on weediness or Invasiveness, led AAFC to conclude that MON809 has no altered weed or Invasiveness potential compared to currently commercialized corn.

2. Potential for Gene Flow to Wild Relatives Whose Hybrid Offspring May Become More Weedy or More Invasive

The biology of corn, as described in Dir94-11, indicates that there are no wild relatives in Canada that can hybridize with *Zea mays*.

AAFC therefore concludes that gene flow from MON809 to corn relatives is not possible in Canada.

3. Altered Plant Pest Potential

The intended effects of both novel traits are unrelated to plant pest potential, and corn is not a plant pest in Canada (Dir94-11). In addition, agronomic characteristics of the modified corn hybrids were shown to be within the range of values displayed by currently commercialized corn hybrids, and indicate that the growing habit of corn was not inadvertently altered. Field observations did not indicate modifications of disease and pest susceptibilities, other than to ECB.

AAFC has therefore determined that MON809 does not display any altered pest potential.

4. Potential Impact on Non-Target Organisms

The enzymes responsible for glyphosate tolerance have very specific enzymatic activities, do not possess proteolytic or heat stability, and do not affect plant metabolism. EPSPS are common enzymes in bacteria, plants and animals. A search of the public domain databases GenBank, EMBL, Pir and SwissProt showed no significant amino acid sequence homology with any known allergens.

The history of use and literature suggest that the bacterial B.t.k. δ-endotoxin is not toxic to humans, other vertebrates, and beneficial insects and the B.t.k. protein produced in corn was shown to be equivalent to the original microbial protein. This protein is active only against specific lepidopteran insects; no lepidopteran species are listed as threatened or endangered species in Canada.

In addition, Pioneer Hi-Bred has submitted data from dietary toxicity studies on the effect of the microbial B.t.k. protein on non-target insects, including pollinators (honeybees), predaceous insects (green lacewing larvae, ladybird beetles) and parasitic Hymenoptera. There was no discernible effect at approximately 10 times the usual LC_{50} dose for a target insect.

Based on the above, AAFC has determined that the unconfined release of MON809, when compared with currently commercialized corn, will not result in altered impacts on interacting organisms, including humans, with the exception of specific lepidopteran insect species.

5. Potential Impact on Biodiversity

MON809 has no novel phenotypic characteristics which would extend its use beyond the current geographic range of corn production in Canada. Since corn does not outcross to wild relatives in Canada, there will be no transfer of novel traits to unmanaged environments.

At present, the use of chemical insecticides to control ECB is not recommended for grain corn produced in Canada, as they are only effective when the larvae first emerge and before these begin to move to the whorl of the plant and into the stalk. It is not considered cost-effective to use insecticides to control ECB in grain corn, but treatment is recommended in sweet corn given the higher cash value of the crop and lower consumer tolerance for insect-damaged cobs. Current recommendations by provincial extension services for the control of ECB in grain corn are: to grow corn hybrids which have been traditionally bred for natural resistance to ECB, to avoid planting at densities that are too high for the chosen hybrid, to plant early to reduce infestations by the second generation of ECB, to harvest early in order to decrease losses from dropped ears and broken stalks, and to practice crop rotation. Despite the promotion of no-till practices in corn for soil conservation, it is still recommended to plow-down corn residues in the fall, in order to kill over-wintering larvae. Thus, the cultivation of MON809 will not result in any overall change in the use of chemical insecticides.

AAFC has therefore concluded that the potential impact on biodiversity of MON809 will not be altered.

6. Potential for Development of ECB Resistance to the PNT

The potential for lepidopteran insects to develop resistance to conventional chemical insecticides is well documented. A B.t.k. foliar insecticide is currently registered for control of ECB on hybrid seed corn production. Another B.t.k. insecticide is registered to control cabbage looper (but not ECB) on pepper, a crop also visited and attacked by ECB. Resistance may develop as a result of increased use of these B.t.k.

foliar sprays; resistance to the B.t.k. proteins could also develop following continued exposure to ECB-resistant hybrid corn.

The development of such a resistance would result in the loss of valuable B.t.k. tools for the control of ECB infestations in corn, and to a lesser extent, pepper.

MON809 steadily produces high levels of B.t.k. in leaves resulting in mortality of ECB feeding on this plant. Target insects will thus be exposed to significantly higher levels of B.t.k. than through the current foliar spray treatments, leading to high selection pressures for resistant ECB individuals. It is currently accepted that ECB has one or more generations a year in Canada. The number of ECB generations that will develop in any one season will be influenced by the environmental conditions in a given area, particularly temperature and day length. The potential for development of resistant ECB populations may also increase in areas with multi-generations.

A component of a possible resistance management strategy linked to the use of MON809 is the presence of non-selecting refugia (unmodified corn) in close proximity, where susceptible insect populations are maintained. Should resistant insects occur, they would then be able to mate with susceptible insects to produce heterozygotes, which are expected to be susceptible to the ECB-resistant corn hybrids. The behaviour of ECB during mating is such that individuals migrate to grassy areas adjacent to corn fields to mate, hence increasing the likelihood that any resistant ECB individuals mate with susceptible ones. Initially there will exist sufficient unstructured non-Bt corn refugia and this may delay the development of resistance. Should the acreage of ECB-resistant corn become greater than the non-Bt hybrids, careful management resulting in the maintenance of non-Bt corn (structured refugia) may be necessary to provide the required non-Bt refugia. Even though the majority of the scientific community agrees that this approach sounds effective in theory, it is very difficult at this point to predict the extent and rapidity of resistance development without field validation of the proposed strategy. These corn plants should therefore be responsibly managed and ECB populations monitored for development of resistant individuals in a regular and consistent manner.

Consideration must be given to the possibility that ECB populations developing resistance to the corn produced B.t.k. protein could also develop cross-resistance to other B.t. δ-endotoxins, resulting in the loss of other B.t. protein types that may be used for the control of ECB infestations.

The development of resistance to the Cry1Ab protein by non-target insect pests, that may then cause further problems in other crops, is another consideration. Armyworm (Lepidoptera: *Pseudaletia unipuncta*) is a sporadic pest in Canada. It feeds on corn and other crops such as forage grasses (e.g. timothy), wheat, oats and barley. Corn earworm (Lepidoptera: *Helicoverpa zea*) feeds on the silks and developing ears (pre-dough stage of kernels) of corn. Presently, B.t.k. foliar sprays are not registered for control of these insects in Canada, so even should resistance

occur, control of these insects would not be compromised. AAFC has therefore concluded that development of resistance in non-target insect pests is unlikely to have an impact on the conventional control of these pests.

AAFC believes that sound management practices can reduce and delay the development of resistant ECB populations, and that ECB populations must be monitored for the development of resistance in a regular and consistent manner. AAFC understands that Pioneer Hi-Bred has developed and will implement a pest resistance management plan that includes the following key components:

- The early detection of ECB populations resistant to the corn-expressed insecticidal protein is extremely important. Close monitoring for the presence of such populations, in ECB-resistant corn fields and surrounding areas, is therefore warranted. Monitoring includes the development of appropriate detection tools such as visual field observations and laboratory bioassays, education of growers, reporting schedules, and enforcement procedures in case of resistance development.

- Education tools will be developed and provided to all growers, district managers and field managers. These will include information on product performance, resistance management, monitoring procedures and timetables, detection protocols for resistant ECB individuals, instructions to contact Pioneer Hi-Bred and strategies to be followed if unexpected levels of ECB damage occur.

- Pioneer Hi-Bred will have procedures in place for responding to these reported instances of unexpected ECB damage. These procedures will include, where warranted, the collection of plant tissue and ECB and use of appropriate bioassays to evaluate suspected Cry1Ab resistant individuals, and a protocol for immediate action to control resistant individuals.

- Detection of confirmed resistant ECB populations and following action plan will immediately be reported to AAFC.

- Integrated Pest Management practices will be promoted, such as prediction of infestation problems from previous years and crop rotation.

- The strategy for resistance management of ECB when using plants that continually produce high concentrations of a B.t.k. δ-endotoxin and refugia has not been previously tested in the field on a large scale. Continued research in this area using sound science will be conducted.

- The developed plans, information and data from the above are available to AAFC.

AAFC also strongly encourages Pioneer Hi-Bred to develop novel ECB control systems with different modes of action that would offer additional or alternative management practices to growers.

If at any time, Pioneer Hi-Bred becomes aware of any information regarding risk to the environment, including risk to agriculture such as development of ECB resistance, or risk to animal or human health, that could result from release of these materials in Canada, or elsewhere, Pioneer Hi-Bred will immediately provide such information to AAFC. On the basis of such new information, AAFC will re-evaluate the potential impact of the proposed release, and will re-evaluate its decision with respect to the unconfined release of these corn hybrids.

V. Nutritional Assessment Criteria for Use as Livestock Feed

1. Nutritional Composition of the PNT

Comparisons of protein, fat and fibre of corn grain and whole plant material from each PNT line and its respective parent line were made. In both the grain and the whole plant, there were occasional significant differences between the PNT and its respective control but there was no consistent pattern of differences from the control for any nutrient for either grain or the whole plant.

Protein, fat and fibre concentration were within the published range for corn, in both the grain and the whole plant in the PNT. The observed variations in nutritional composition was judged to arise from normal variability rather than as a result of the inserted novel traits. AAFC has determined that line MON809 is substantially equivalent to traditional corn varieties.

2. Anti-Nutritional Factors

The parent plant *Zea mays* is not known for the production of anti-nutritional factors and the transformation event which produced MON809, would not be expected to induce their synthesis.

VI. Regulatory Decision

Based on the review of data and information submitted by Pioneer Hi-Bred, and through comparisons of corn hybrids derived from MON809 with unmodified corn counterparts, the Plant Biotechnology Office of the Plant Products Division, AAFC, has concluded that the novel genes and their corresponding traits do not confer to these plants any characteristic that would result in intended or unintended environmental effects following unconfined release. Pioneer Hi-Bred has developed and will implement a resistance management plan.

Based on the review of submitted data and information, the Feed Section of the Plant Products Division has concluded that the novel traits do not in themselves raise any concerns regarding the safety or nutritional composition of MON809. Grain corn, its byproducts and corn oil are currently listed in Schedule IV of the Feeds Regulations and are, therefore approved for use in livestock feeds in Canada. The ECB resistant corn hybrids have been assessed and found to be substantially equivalent to traditional corn varieties, MON809 and its byproducts are considered to meet present ingredient definitions and are approved for use as livestock feed ingredients in Canada.

Unconfined release into the environment and use as livestock feed of the corn line MON809 is therefore authorized. Any other *Zea mays* lines and intraspecific hybrids resulting from the same transformation event, and all their descendants, are also approved, provided no inter-specific crosses are performed, provided the intended use is similar, provided it is known following thorough characterization that these plants do not display any additional novel traits and that the resulting lines are substantially equivalent to currently grown corn, in terms of their potential environmental impact and livestock feed safety and provided that pest resistance management requirements described in the present document are applied.

Please note that, while determining the environmental safety of plants with novel traits is a critical step in the commercialization of these plant types, other requirements still need to be addressed, such as the evaluation of food safety (Health Canada) and Variety Registration (AAFC).