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français	Contact Us	Help	Search	Canada Site
Table of Contents	What's New	Acts and Regulations	Site Map	
Food Safety	Animal Health	Plant Protection	Corporate Affairs	

Quick Pick By Commodity / Key Topic

- [Main Page - Plant Biosafety Office](#)
- [Main Page - Plant Health and Production Division](#)
- [The Regulation of Plants With Novel Traits in Canada](#)
- [Guidelines for the Environmental Release of Plants with Novel Traits within Confined Field Trials in Canada \(Dir2000-07\)](#)
- [Decision Documents](#)
- [Contacts](#)

Plant Health and Production Division,  
Plant Biosafety Office

## Decision Document DD96-12: Determination of Environmental Safety of Northrup King Seeds' European Corn Borer (ECB) Resistant Corn (*Zea mays* L.)

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, specifically the Plant Biotechnology Office (PBO) and the Feed Section of the Plant Health and Production Division, with input from the Plant Health Risk Assessment Unit, AAFC, and advice from the Pest Management Regulatory Agency, Health Canada, has evaluated information submitted by Northrup King Seeds Ltd. regarding two corn hybrids referred to as X4334CBR and X4734CBR in the present document. These plants were transformed with genes conferring resistance to the European Corn Borer (ECB) and tolerance to glufosinate ammonium. AAFC has determined that these plants with novel traits should not pose concern to environmental safety. Northrup King Seeds has developed and will implement a ECB resistance management plan.

**Unconfined release into the environment, including feed use, of the corn hybrids X4334CBR, X4734CBR, and other *Z. mays* lines derived from them, but without the introduction of any other novel trait, is therefore authorized.**

Please note that, while determining the environmental and feed 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 for Variety Registration (AAFC) and for the evaluation of food safety (Health Canada).

### Table of Contents

- I. [Brief Identification of the Plants with Novel Traits \(PNT's\)](#)
- II. [Background Information](#)
- III. [Description of the Novel Traits](#)
  1. [Resistance to the European Corn Borer \(ECB\)](#)
  2. [Glufosinate Ammonium Tolerance](#)
  3. [Development Method](#)
  4. [Stable Integration into the Plants' Genomes](#)
- IV. [Assessment Criteria for Environmental Safety](#)

1. [Potential of the PNT's to Become Weeds of Agriculture or Be Invasive of Natural Habitats](#)
  2. [Potential for Gene Flow to Wild Relatives Whose Hybrid Offspring May Become More Weedy or More Invasive](#)
  3. [Altered Plant Pest Potential](#)
  4. [Potential Impact on Non-Target Organisms](#)
  5. [Potential Impact on Biodiversity](#)
  6. [Potential for Development of ECB Resistance to the PNT's](#)
- V. [Nutritional Assessment Criteria as Livestock Feed](#)
1. [Anti-Nutritional Factors](#)
  2. [Nutritional Composition of the PNT's](#)
- VI. [Regulatory Decision](#)
- 

## I. Brief Identification of the Plants with Novel Traits (PNT's)

<i>Designations of the PNT's:</i>	Corn hybrids X4334CBR and X4734CBR
<i>Applicant:</i>	Northrup King Seeds Ltd., the Canadian subsidiary of Northrup King Co. (USA)
<i>Plant Species:</i>	Corn ( <i>Zea mays</i> L.)
<i>Novel Traits:</i>	Resistance to European Corn Borer ( <i>Ostrinia nubilalis</i> ); tolerance to glufosinate ammonium
<i>Trait Introduction Method:</i>	Direct DNA transfer system
<i>Proposed Use of PNT's:</i>	Production of field corn for livestock feed and human consumption. These materials will not be grown outside the usual production area for corn in Canada.

## II. Background Information

Northrup King has developed two corn hybrids resistant to the European Corn Borer (ECB) larvae, a periodic pest of corn in Canada. These corn hybrids, referred to as X4334CBR and X4734CBR in the present document, will provide an alternative for control of larval stages of ECB, thus resulting in potential yield increases following reduction of insect damage in infested areas.

The development of these ECB resistant corn hybrids was based on recombinant DNA technology, by the introduction of two bacterial genes into an elite inbred line. A gene conferring resistance to ECB was inserted, coding for an insecticidal protein active against specific lepidopteran species. Another gene, conferring tolerance to glufosinate ammonium, was also inserted; this gene may be of agronomic interest but was primarily introduced to select modified plants from those that remained unmodified at the development stage.

These lines have been tested in Canada under confined conditions since 1993 in Ontario.

Northrup King Seeds Ltd. has provided data on the identity of X4334CBR and X4734CBR corn hybrids, a detailed description of the cloning method and intermediate vector

construction, full description of the final plasmid used for DNA transfer, data and information on the gene insertion sites, copy numbers 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 their potential toxicity to livestock and non-target organisms with particular attention given to beneficial arthropods. Full amino acid sequences and relevant scientific publications were also supplied.

Agronomic characteristics such as vegetative vigour, male and female fertility, time to maturity, seed yield, kernel size, weight and density, were compared to those of unmodified corn counterparts.

Data to support the suitability of these corn hybrids as livestock feed were provided. Proximate analyses to include crude protein, crude fat, crude fibre and starch, were supplied.

Both hybrids have received non-regulated status from the United States Department of Agriculture (USDA). They were recommended by the Ontario Corn Registration Committee for one year interim variety registration.

The potential development of ECB resistance to the insecticidal protein was discussed, and an insect resistance management plan will be implemented by Northrup King Seeds in Canada.

The Plant Biotechnology Office of the Plant Health and Production Division, with input from the Plant Health Risk Assessment Unit on behalf of the Plant Protection Division, AAFC, has reviewed the above information, in light of the following 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 to 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 Health and Production Division, AAFC, has also reviewed the above information in light of the assessment criteria for determining 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 subsp. kurstaki* (B.t.k.) is a common gram-positive soil-borne

- bacterium. In its spore forming stage, it produces several insecticidal protein crystals, including Cry1Ab that is active against lepidopteran insects such as ECB, corn earworm, spruce budworm, tent caterpillar, gypsy moth, diamondback moth, cabbage looper, tobacco budworm, and cabbage worm. The 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 and have a history of safe use.
- The ECB resistance gene engineered into the corn hybrids X4334CBR and X4734CBR codes for a truncated version of Cry1Ab. Insecticidal activity results from the Cry1Ab protein binding to specific insect gut epithelium receptors, pore formation, loss of cations, and disruption of digestive processes following an osmotic pressure imbalance. Current knowledge indicates that only sensitive insects possess such receptors.
  - The *cry1Ab* gene, truncated and modified for enhanced plant expression, is linked to a strong constitutive promoter. Protein production (referred to as B.t.k. protein in the present document) was quantified in roots, leaves, stalk epidermis, stalk pith, tassel, pollen, silk, ear shank, husk, cob, brace root and kernel. It was found throughout the plant with highest levels detected in leaf tissues and at younger stages of development, and lowest levels detected in pollen grains. Expression was found to average 28.87 µg/g (f.w.) of young leaf tissue, 15.23 µg/g (f.w.) of mature leaf tissue and 4.76 µg/g (f.w.) of homozygous seed tissue.
  - The resulting B.t.k. proteins include a 69 kDa protein, a major proteolytic fragment of 65 kDa and two minor fragments of 40 and 15 kDa. These fragments result from trypsin digestion and are also released following tryptic digestion of the original bacterial B.t.k. Cry1Ab protein purified from *Escherichia coli*.
  - The expressed 69 kDa protein and the proteolytic fragments were compared to the bacterial proteins, and shown to be of similar molecular weight, primary amino acid sequences, immunological reactivity, trypsin resistance and potential glycosylation sites, indicating that there were no insertional or post-transcriptional modifications.
  - The biological activity of the trypsinized Cry1Ab protein from the modified corn was the same as that of the native B.t.k. Cry1Ab on ECB, with a LC50 of 0.46-0.51 µg/ml (f.w.) and an EC50 of 0.060-0.067 µg/g (f.w.). Studies have shown that B.t.k. proteins were rapidly inactivated when subjected to simulated mammalian gastric fluids. After one week incubation in soil, 99% of the leaf B.t.k. proteins' bioactivity was lost due to aerobic degradation.
  - The gene nucleotide sequence and the enzyme amino acid sequence were provided.

## 2. Glufosinate Ammonium Tolerance

- Phosphinothricin (PPT), the active ingredient of glufosinate ammonium, inhibits glutamine synthetase, which results in the accumulation of lethal levels of ammonia in susceptible plants within hours of application.
- The phosphinothricin tolerance gene engineered into hybrids X4334CBR and 4734CBR was isolated from a common aerobic soil actinomycete and codes for PPT-acetyltransferase (PAT). This enzyme detoxifies phosphinothricin by acetylation into an inactive compound. It has extremely high substrate specificity; experimental data clearly showed that neither L-PPT's analog L-glutamic acid, D-PPT, nor any protein amino acid can be acetylated by the PAT enzyme.
- The gene, modified for enhanced gene expression, is linked to a constitutive promoter. Expression was estimated to average 0.049 µg/g (f.w.) of leaf tissue, 0.027 µg/g (f.w.) of tassel tissue and 0.005 µg/g (f.w.) in silks. It was not detected in roots, kernels nor pollen.
- The expressed PAT enzyme was compared to the bacterial protein: molecular weights were similar, indicating that the protein had not been glycosylated nor had it undergone post transcriptional modifications.
- Studies showed that the enzyme was inactivated within one minute when subjected to typical mammalian gastric conditions and is thus digested as a conventional dietary protein.
- The gene nucleotide sequence and the enzyme amino acid sequence were provided. The nucleotide sequence showed no significant homology with the toxins or allergens

entered in the GENE BANK DNA database.

### 3. Development Method

- The original transformations were made through the use of a direct transfer system which did not involve the use of a DNA carrier. These transformations were conducted under contract for Northrup King by a third party and the exact method is considered to be confidential business information.

### 4. Stable Integration into the Plants' Genomes

- The data provided showed that only one copy of the gene was integrated at one insertion site. The single insertion site was mapped to the long arm of chromosome 8 using Restriction Fragment Length Polymorphism (RFLP) markers.
- Mendelian inheritance of the insect resistance and herbicide tolerance genes was shown over two generations.

## IV. Assessment Criteria for Environmental Safety

### 1. Potential of the PNT's to Become Weeds 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 Northrup King Seeds, hybrids X4334CBR and X4734CBR were determined not to be different from their counterparts in this respect.

AAFC evaluated data submitted by Northrup King Seeds on the reproductive and survival biology of X4334CBR and X4734CBR corn hybrids, and determined that vegetative vigour, overwintering capacity, time to maturity, male and female fertility, and seed yield, were within the normal range of expression currently displayed by commercial corn hybrids. No genes were inserted for cold tolerance or winter survival.

No competitive advantage was conferred to these corn hybrids, 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. Tolerance to glufosinate ammonium will not render corn weedy, since this herbicide is not presently used in crop rotation cycles involving corn. Any glufosinate 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 glufosinate ammonium tolerance (see [DD95-01](#)) were recently registered in Canada. Should glufosinate ammonium tolerant corn and canola be grown in rotation, volunteers would not be controlled with this herbicide. Agricultural extension personnel, in both the private and 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 X4334CBR and X4734CBR corn hybrids have 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 hybridized with *Zea mays*.

AAFC therefore concludes that gene flow from X4334CBR and X4734CBR hybrids 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 such as: plant vigour, growth, male and female fertility, time to maturity, flowering period, and seed yield, 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.

AAFC has therefore determined that X4334CBR and X4734CBR corn hybrids do not display any altered pest potential.

## **4. Potential Impact on Non-Target Organisms**

The PAT enzyme responsible for glufosinate ammonium tolerance has very specific enzymatic activity, does not possess proteolytic or heat stability, and does not affect plant metabolism. Acetyltransferases are common enzymes in bacteria, plants and animals. A search of the GENE BANK DNA sequence database revealed no significant homology with the toxins or allergens entered in that database.

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

In addition, Northrup King Seeds submitted data from dietary toxicity studies using the B.t.k. microbial protein on honeybee larvae and adults, and on parasitic wasps. No negative effect was observed.

Based on the above, AAFC has determined that the unconfined release of these corn hybrids, 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**

Hybrids X4334CBR and X4734CBR have no novel phenotypic characteristics which would extend their 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 hybrid chosen, to plant early to reduce infestations by the second generation, 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 corn hybrids X4334CBR and X4734CBR, as hybrid corn, will not result in any overall change in the use of chemical insecticides.

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

## **6. Potential for Development of ECB Resistance to the PNT's**

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 on pepper, a crop also visited and attacked by ECB. To date, resistance of ECB to B.t.k. has not been observed under field conditions. 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 these valuable B.t.k. tool for the control of ECB infestations in corn and pepper.

The hybrids X4334CBR and X4734CBR steadily produce high levels of B.t.k. in leaves resulting in mortality of ECB feeding on these hybrids. 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 daylength. The potential for development of resistant ECB populations may also increase in areas with multi-generations.

A component of the resistance management strategy linked to the use of these corn hybrids 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.  $\delta$ -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 Northrup King Seeds 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, reporting schedules, education of growers, 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 contract Northrup King Seeds and strategies to be followed if unexpected levels of ECB damage occur.
- Northrup King Seeds 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.  $\delta$ -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 plans, information and data from the above are available to AAFC.

AAFC has also strongly encouraged Northrup King Seeds 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, Northrup King Seeds becomes aware of any new 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, Northrup King Seeds 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 as Livestock Feed**

## 1. Anti-Nutritional Factors

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

## 2. Nutritional Composition of the PNT's

The nutritional composition of the ECB resistant corn hybrids was analysed at six locations for grain and silage. For the grain analysis, protein, oil, starch and fibre content of the ECB resistant corn lines were shown to be substantially equivalent to the untransformed controls. Silage samples from the ECB resistant corn hybrids had significantly higher levels of ADF and NDF than samples from the control lines, however the values were well within the normal range for corn silage. All other nutrients tested (protein, calcium, magnesium, phosphorus and potassium) were shown to equivalent to their respective controls.

Proximate analysis (protein, fat, fibre) was performed using Near Infrared Reflectance (NIR) technology. This method has been submitted to the Association of Official Analytical Chemists (AOAC) for accreditation and official approval is expected shortly. The method was conducted with appropriate calibration and statistical analysis, to show equivalence between the NIR technique and traditional chemistry. Sample falling outside the normal range using NIR were analysed using AOAC approved, traditional chemical methods.

The proximate analysis of the ECB resistant corn hybrids gave values well within the published range for traditional corn cultivars. Accordingly these hybrids have been determined to be substantially equivalent to traditional corn varieties.

## VI. Regulatory Decision

Based on the review of data and information submitted by Northrup King Seeds, and through comparisons of corn hybrids X4334CBR and X4734CBR with unmodified corn counterparts, the Plant Biotechnology Office of the Plant Health and Production Division, AAFC, has concluded that the novel genes and their corresponding traits do not confer to these corn hybrids any characteristic that would result in intended or unintended environmental effects following unconfined release.

Based on the review of submitted data and information, the Feed Section of the Plant Products Division has concluded that the novel genes and their corresponding traits do not in themselves raise any concerns regarding the safety or nutritional composition of X4334CBR and X4734CBR. Grain corn and its various fractions, and corn oil, are currently listed in Schedule IV of the Feeds Regulations and are, therefore, approved for use in livestock feeds in Canada. As X4334CBR and X4734CBR have been assessed and found to be substantially equivalent to traditional corn, these hybrids and their byproducts are considered to meet present ingredient definitions and are approved for use as livestock feed ingredients in Canada.

**Unconfined release into the environment, including feed use, of the corn hybrids X4334CBR, X4734CBR, and other *Z. mays* lines derived from them, but without the introduction of any other novel trait, is therefore authorized.**

Please note that, while determining the environmental and feed 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 for Variety Registration (AAFC) and for the evaluation of food safety (Health Canada).

This bulletin is published by the Plant Health and Production Division, Canadian Food Inspection Agency. For further information, please contact the Plant Biosafety Office or the Feed Section at:

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[Top of Page](#)

[Important Notices](#)