



CASE STUDY 25:

A Competent National Authority registers a risk assessment and decision to import herbicide tolerant cotton

Objective:

- To learn how to register a risk assessment and a decision in the Biosafety Clearing House.

References:

- BCH Training Site (Go to <http://bch.cbd.int> > Help (or Resources) > Training Site of the BCH)
- Training Manual 06: Registering Information in the BCH Central Portal
- Training Manual 07: Registering National Information in the BCH Central Portal

Scenario:

You are working for a Competent National Authority and you have recently approved the attached decision to import herbicide tolerant cotton. Your task is to register this LMO, risk assessment and regulatory decision with the BCH.

Important note:

Please be sure that you are using the *BCH Training Site* for this exercise!

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**Release Document CNA2007-17:
Safety Assessment of Roundup Ready® Flex Cotton**

Determination by Authority of the CNA for the Environment

I. Identification of the novel organism

<i>Designation:</i>	Cotton line MON 88913, UID MON-88913-8
<i>Applicant:</i>	Monsanto Inc
<i>Parent species:</i>	Cotton (<i>Gossypium hirsutum</i> L.)
<i>Introduced trait:</i>	Herbicide tolerance (glyphosate)
<i>Proposed use:</i>	Production of cotton for fibre, cottonseed and cottonseed meal for livestock feed, and cottonseed oil for human consumption. These materials will be grown in the US and cottonseed and cottonseed meal will be imported for livestock feed use only.

II. Background information

Monsanto Inc. has developed a cotton line, designated MON 88913, which has enhanced tolerance to glyphosate, the active ingredient in the herbicide Roundup®. The enhanced herbicide tolerant trait in cotton line MON 88913 will enable the over-the-top application of Roundup® agricultural herbicides at later stages of development than is possible with cotton lines MON 1445 and MON 1698. This is intended to provide greater flexibility and convenience in weed control options during crop production.

Cotton line MON 88913 was developed using *Agrobacterium*-mediated gene transfer technology, resulting in the introduction of two copies of the *epsps* (5-enolpyruvyl shikimate-3-phosphate synthase) gene from *Agrobacterium* sp. strain CP4. The CP4 version of the EPSPS protein imparts reduced glyphosate sensitivity to the modified plant. The use of two different promoters to control the expression of the *cp4 epsps* genes confers the enhanced tolerance to glyphosate. The *cp4 epsps* coding region is fused to a chloroplast transit peptide sequence, which directs the translated protein to the chloroplast, the site of amino acid biosynthesis.

The CP4 EPSPS protein from MON 88913 was shown to be equivalent to the protein produced in an *Escherichia coli* expression system developed to produce the protein. The *E. coli* produced CP4 EPSPS was used to generate sufficient quantities of pure protein for safety studies. References to relevant scientific publications have been reviewed by the Authority.

Phenotypic data for cotton line MON 88913 were collected from fourteen field locations under confined research field trial conditions. Compositional and expression data were collected from four of these locations. Agronomic characteristics of cotton line MON 88913 such as plant morphology, disease susceptibility, agronomic performance and reproductive fitness were compared to those of unmodified cotton counterparts. Nutritional components of cotton line MON 88913 such as proximates, amino acids and fatty acids were compared with unmodified cotton counterparts.

The following assessment criteria were considered when determining the safety and efficacy as livestock feed and the environmental safety of this novel feed:

- potential impact of cotton line MON 88913 on livestock nutrition,
- potential impact of cotton line MON 88913 on livestock and workers,

- potential of cotton line MON 88913 to become a weed of agriculture or be invasive of natural habitats,
- potential for gene flow from cotton line MON 88913 to wild relatives whose hybrid offspring may become more weedy or more invasive,
- potential of cotton line MON 88913 to become a plant pest,
- potential impact of cotton line MON 88913 or their gene products on non-target species, including humans, and
- potential impact of cotton line MON 88913 on biodiversity.

III. Description of the Novel Traits

Development Method: Cotton line MON 88913 was created through the insertion of a fragment of DNA containing two copies of a gene derived from the *Agrobacterium* sp. strain CP4 (*cp4 epsps*), which imparts field level tolerance to glyphosate, the active ingredient in Roundup® herbicides. Cotton line Coker 312 was transformed with a plasmid vector carrying the synthetic *cp4 epsps* genes, both of which had been fused with a plant-derived coding sequence expressing an optimized chloroplast transit peptide.

Glyphosate Tolerance: EPSPS is an enzyme involved in the shikimic acid metabolic pathway which is essential for the production of the aromatic amino acids. The native cotton EPSPS enzyme is sensitive to glyphosate. The herbicide disrupts the shikimic acid pathway, leading to growth suppression or death of the plant. The CP4 EPSPS version of this enzyme is expressed in cotton line MON 88913 and confers glyphosate tolerance since it continues to catalyze the production of aromatic amino acids in the presence of glyphosate due to a reduction in the binding of glyphosate to the CP4 EPSPS in comparison to the native EPSPS.

The expression of the novel enzyme in the plant is driven by constitutively active promoters and was quantified by enzyme-linked immunosorbent assay (ELISA). The CP4 EPSPS protein levels were assessed in young leaf, overseason leaf (OSL), root, seed and pollen tissues collected from field trials performed in 2002. ELISA assays demonstrated that the mean CP4 EPSPS protein levels across four sites for young leaf, OSL1, OSL2, OSL3, root and seed tissues were 970, 1400, 690, 630, 99 and 340 µg/g dry weight respectively. The mean CP4 EPSPS protein level in pollen across the four sites was 4.0 µg/g fresh weight.

Unlike typical allergens, the CP4 EPSPS protein is present at low levels in cotton line MON 88913 (less than 0.12% of total protein in seed), is not glycosylated and was shown to be labile to digestion. Following incubation in simulated gastric fluid it was shown, through western blotting, that more than 95% of the CP4 EPSPS protein was digested within 15 seconds. Also, unlike known allergens, it was shown that EPSPS activity was reduced by more than 90% within 15 seconds incubation time in simulated gastric fluid.

The amino acid sequence of the CP4 EPSPS protein was compared to several protein sequence databases and was shown to share no significant structural similarity with any known toxic, allergenic or pharmacologically relevant proteins. Further analysis also showed that the CP4 EPSPS protein lacks immunologically relevant sequences. An acute mouse study reported no deleterious side effects when animals were administered CP4 EPSPS protein by oral gavage at doses up to 475 mg/kg.

Due to the low levels of CP4 EPSPS protein expressed in cotton line MON 88913 it was necessary to produce CP4 EPSPS by bacterial fermentation to obtain sufficient quantities to conduct some of the safety studies (acute oral mouse toxicity study, simulated gastric fluid digestion study). The bacterial produced protein was compared to the plant produced protein and shown to be of similar molecular weight, immunological reactivity and to have similar functional activity as the plant produced protein.

Southern blot analysis showed stability of the introduced DNA in the first five generations removed from the original transformant. Data presented also demonstrated that the genes segregated according to Mendelian inheritance in these generations.

The Authority has been provided with a method for detection and identification of MON 88913.

IV. Criteria for the Environmental Assessment

1. Potential of Cotton line MON 88913 to Become a Weed of Agriculture or be Invasive of Natural Habitats

Cotton (*Gossypium hirsutum*) is a perennial member of the *Malvaceae* (Mallow family), but it is cultivated as an annual crop. Neither plants nor seeds of *Gossypium* can survive freezing temperatures. The genus *Gossypium* shows no particular weedy aggressive tendencies. Cotton has been grown for centuries and has never been reported as a serious weed problem.

Dormancy and germination characteristics of line MON 88913, the negative segregant control (which shares similar background genetics to MON 88913), and several reference varieties were measured in temperature-controlled growth chambers under seven different temperature regimes. All values for MON 88913 were either within the range of reference values or incrementally outside of the range provided. No significant differences were detected in percent viable hard seed (a potential measure of dormancy).

Measurements on height, yield, and seed/boll characteristics of line MON 88913 and the control were recorded at harvest. When pooled across all locations, there were no significant differences between height, number of nodes, total number of bolls, number of vegetative bolls, number of abnormal bolls, total seed per boll, mature seed per boll, or immature seed per boll. A significant difference of 0.3 grams was recorded for seed index (grams per 100 fuzzy seed), but this difference likely has little biological impact on weediness.

Line MON 88913 is therefore considered to be substantially equivalent to lines 1445 and 1698 with regards to invasiveness and potential to become a weed of agriculture. CFIA concludes that cotton line MON 88913 is unlikely to become a weed of agriculture or invasive of natural habitats.

2. Potential for Gene Flow to Wild Relatives and Potential for Hybrid Offspring to Become More Weedier or More Invasive

Cotton pollen remains viable for about 12 hours but the grains are relatively large and heavy and not easily dispersed by wind. *G. hirsutum* is generally self-pollinating but can exhibit outcrossing in the presence of suitable insect pollinators (such as bumble bees (*Bombus* spp.) and honey bees (*Apis mellifera*)). Outcrossing

frequency decreases with increasing distance from the pollen source. Wild species of *Gossypium* are generally restricted to arid tropical or subtropical regions.

The Authority has concluded that gene flow from glyphosate-tolerant cotton lines to wild *Gossypium* relatives is very unlikely in managed or unmanaged ecosystems in this country.

3. Altered Plant Pest Potential

Cotton is not a plant pest and the intended effect of the novel trait is unrelated to plant pest potential. Susceptibility of line MON 88913 to insect, disease, and abiotic stressors were evaluated in field experiments at 14 locations. Susceptibilities to aphids, beet armyworm, cotton bollworm, lygus, pink bollworm, stink bugs, tarnished plant bugs, thrips, tobacco budworm, whiteflies, boll rot, Pythium, Rhizoctonia, Verticillium, cold, drought, and heat were similar in line MON 88913 to the susceptibilities of the control plants to these stressors. No differences between line MON 88913 and the control were observed that would contribute to increased plant pest potential.

The Authority has therefore determined that Roundup Ready® Flex cotton line MON 88913 does not display any altered plant pest potential.

4. Potential Impact of Non-Target Organisms

The source of the coding sequence for the CP4 EPSPS protein produced in line MON 88913 is a common soil bacterium which is not a known human or animal pathogen and has not been previously reported as an allergen. The protein is rapidly digested in simulated gastric fluid (more than 95% of the protein was digested within 15 seconds) and there were no immunologically relevant homologous sequences to any known allergenic proteins, indicating a lack of allergenic potential. Additionally, no documented cases of allergy or adverse effects from the consumption of this protein in foods or feeds derived from other Roundup Ready® crops have been reported since their introduction in 1996.

EPSPS proteins naturally occur in plant and microbially-based foods that have a history of safe consumption by humans and animals. CP4 EPSPS has been consumed directly or as processed products of Roundup Ready® crops since their commercialization in 1996 with no reported cases of toxicity. A compositional and nutritional assessment compared cottonseed, oil, and meal from line MON 88913 to cottonseed, oil, and meal from a negative control cotton line with similar background genetics. This assessment found no component values in cotton line MON 88913 that were outside of the range of those found in the unmodified counterparts.

Cotton is well-known for naturally occurring toxicants and antinutrients (cyclopropenoid fatty acids and gossypol). Aflatoxins are toxic by-products of several varieties of fungi that can grow in cotton. Line MON 88913 was tested for four aflatoxins (B1, B2, G1, and G2) but, because 50% of all the samples taken were below the LOQ, further statistical analyses were not conducted. Cyclopropanoid fatty acids (malvalic acid, dihydrosterculic acid, and sterculic acid) levels in line MON 88913 were assessed and were found to be within the range of values observed in conventional cotton counterparts. Levels of gossypol were assessed and were also found to be within the range of values observed in conventional cotton counterparts.

An acute oral toxicity study using *E. coli*-produced CP4 EPSPS protein was administered to mice. No adverse effects were observed at 475 mg of CP4 EPSPS

protein/kg mouse body weight, and there were no differences in body weight, cumulative body weight, or food consumption between mice fed CP4 EPSPS protein and mice fed with a control protein (bovine serum albumin) at that level.

The Authority therefore concludes from the above data that the use of line MON 88913 will not have a greater impact on non-target organisms (including humans) than the use of conventional cotton.

5. Potential Impact on Biodiversity

No varieties of cotton, nor any wild relatives that can readily interbreed with cotton, can grow in the local environment. Roundup Ready® Flex cotton line MON 88913 has no observed or expected modifications that would allow it to survive in the local environment better than unmodified cotton. The Authority therefore concludes that line MON 88913 will have no impact on biodiversity in this country.

V. Criteria for the Environmental Assessment

1. Potential Impact on Livestock Nutrition

The composition of cottonseed, cottonseed meal and cottonseed oil from line MON 88913 was compared with a negative-segregant control line. Acid-delinted whole cottonseed was analyzed in two trials and analysis included proximates, minerals, amino acids, fatty acids, cyclopropenoid fatty acids, gossypol, and vitamin E. Cottonseed meal and cottonseed oil were analysed in one of the trials. Cottonseed meal analysis included proximates, minerals, amino acids, cyclopropenoid fatty acids, and gossypol, while cottonseed oil analysis included fatty acids, cyclopropenoid fatty acids, gossypol, and vitamin E. Oleic acid, when expressed as a percentage of total fatty acids, was significantly altered but this difference was due to elevated values in the control line. Phenylalanine, when expressed as a percentage of total amino acids, was significantly higher in line MON 88913 than its control in both trials. However, both line MON 88913 and its control line fell within the range of the commercial reference varieties and the range of literature values for this analyte. There was no difference in phenylalanine when expressed as a percentage of total amino acids in cottonseed meal derived from whole cottonseed used in this trial. Several other analytes (tryptophan, linoleic acid and manganese) were found to be significantly different, but all of these values fell within the tolerance interval for the commercial reference varieties.

Malvalic and sterculic acids, when expressed as a percentage of total fatty acids, were slightly, but significantly, different at one location in one of the trials. The means for these fatty acids fell within the range of the commercial reference varieties. The evidence provided by Monsanto Inc. supports the conclusion that the nutritional composition of Roundup Ready® Flex cotton line MON 88913 is substantially equivalent to conventional cotton varieties.

2. Potential Impact on Livestock and Workers

EPSPS is an enzyme present in many foods with a long history of safe use, and therefore would not be expected to be toxic or allergenic. The CP4 EPSPS enzyme is from *Agrobacterium* strain CP4, a soil bacterium, which is not a known human or animal pathogen. The amino acid sequence of the CP4 EPSPS protein found in line MON 88913 is identical to the CP4 EPSPS protein in Roundup Ready® crops previously approved for use as feed. CP4 EPSPS shares no biologically relevant significant homology with known toxins or allergens, it is present in small amounts in

the feed, it is heat labile and it is rapidly degraded under the conditions present in the gastrointestinal tract. Additionally, a mouse acute oral toxicity study indicated no adverse effects at 475 mg/kg body weight CP4 EPSPS. Based on the information provided by Monsanto Inc, CP4 EPSPS is unlikely to be a novel toxin or allergen.

Cotton is not known for the production of endogenous allergens and the transformation event which produced cotton line MON 88913 would not be expected to induce their synthesis. Based on the predicted exposure levels and the results of the above tests, no significant risk to livestock and workers is expected from exposure to the CP4 EPSPS protein.

VI. New Information Requirements

If at any time, Monsanto Inc. becomes aware of any information regarding risk to the environment, or risk to human or animal health that could result from release of these materials, Monsanto Inc. will immediately provide such information to the Authority. On the basis of such new information, the Authority will re-evaluate the potential impact of the proposed use and will re-evaluate its decision with respect to the livestock feed authorization of cotton line MON 88913.

VII. Regulatory Decision

Based on the review of data and information submitted by Monsanto Inc., including comparisons of cotton line MON 88913 with the unmodified parental counterparts, the Authority has concluded that the novel gene and its corresponding trait does not confer to the plants any characteristic that would raise any concerns regarding the safety or nutritional composition of cotton line MON 88913. Cotton line MON 88913 has been assessed and found to be as safe and as nutritious as traditional cotton varieties. Cotton line MON 88913 and its products are considered to meet the present ingredient definitions and are approved for use as livestock feed ingredients. Cotton line MON 88913 will not be grown locally nor can the seed overwinter, therefore the release of the feed into the environment would result in neither intended nor unintended environmental effects.

Livestock feed use of cotton line MON 88913 is therefore authorized as of November 23, 2007. Cotton line MON 88913 and any other cotton lines derived from it may be imported and/or released, provided no inter-specific crosses are performed, provided the intended uses are similar, and provided it is known, based on characterization that these plants do not display any additional novel traits and are substantially equivalent to currently grown cotton, in terms of their specific use and safety for the environment and for human and animal health.

Cotton line MON 88913 is subject to the same phytosanitary import requirements as its unmodified counterparts.

VIII. Regulatory Contact

Competent National Authority for the Environment
123 Authority Street
National Capital 9999

Note: This release document is also available on the Internet at www.decisions.com/CNA2007-17.pdf



CASE STUDY 25:

A Competent National Authority registers a risk assessment and decision to import herbicide tolerant cotton

TRAINER NOTES

Training Objective:

To learn how to use the BCH Management Centre to register a risk assessment and the decision to import herbicide tolerant cotton.

Requirements:

BCH account and access to the BCH.

Notes:

- Users can work singly or in small groups for this exercise.
- Participants need to have their own BCH account ready. They will login the **BCH Training Site** using their own BCH account. Refer to MO06 Training Manual for details on how to create a BCH account.
- It is recommended for you to setup your own Training Space for your workshop before you start this exercise. Otherwise, the participants can select the “Public BCH Training” training space.
- For this exercise, participants should use common format for national records “Risk assessment generated by regulatory process”
- At the end of the exercise, the participant that is logged in with the role “BCH-NFP” will need to validate completed records so that participants can access the records they entered through the “Finding Information” section of the BCH Training Site.