

Caso hipotético de maíz resistente a insectos y tolerante a herbicida, para liberación en México



Resumen del caso estudio

Nombre del evento	IR+HT maize
Especie	<i>Zea mays ssp mays</i> L.
Característica insertada	Resistente a Coleopteros, Lepidopteros y tolerante al herbicida glifosato.
Gen, Producto del gen, o fuente y fragmento del gen	<ul style="list-style-type: none"> • <i>Cry1Ab</i> del <i>Bacillus thuringiensis ssp. kurstaki</i>. • <i>Cry3Bb1</i> del <i>Bacillus thuringiensis subsp. kumamotoensis</i>. • <i>Epsps</i> del <i>Agrobacterium</i> strain CP4. • Promotor del virus del mosaico de la coliflor (<i>CaMV</i>) asociado con altos niveles de expresión en las raíces, conteniendo 4 copias tandem de la secuencia de activación 1 (AS1) que corresponde a una secuencia de 21 pb derivada del <i>CaMV</i> 35S promotor (35S) fusionado a una porción adicional del 35S. • La región no codificante 3' de la pequeña subunidad de Rubisco en trigo (<i>Triticum aestivum</i>) intensifica la traducción, termina la transcripción y dirige la poliadenilación (terminación). • La región no codificante 3' de la pequeña subunidad de Rubisco en arroz (<i>Oryza sativa</i>) intensifica la traducción, termina la transcripción y dirige la poliadenilación (terminación).
Método de introducción	Los genes se insertan en los eventos individuales (caso 1, 2 y 3) mediante la transformación mediada por <i>Agrobacterium</i> , con el tiempo los nuevos rasgos de cada línea parental se combinaron mediante los métodos de fitomejoramiento tradicional para producir este nuevo híbrido (evento apilado).
Sitio y tipo de liberación	<p>Sitio: las regiones de cultivo de maíz en México</p> <p>Tipo: la comercialización generalizada de cultivo (no confinado)</p>
Uso previsto/justificación	Incrementar la producción de maíz

1. GENERAL INFORMATION

Maize, known in many English-speaking countries as corn or mielie/mealie, is a grain domesticated by indigenous peoples in Mesoamerica in prehistoric times, the same region established as the centre of origin and diversity for maize (Watson & Dallwitz, 1992; cited in MoEF & MoST [2011]). Maize is a cereal crop that is grown widely throughout the world in a range of agro-ecological environments, with more maize produced annually than any other grain. There are various types of maize differing in colour, texture, grain shape and size. White, yellow and red are the most common types. The white and yellow varieties are preferred by most people, depending on the region. Ninety percent of white maize consumption is in Africa and Central America. Yellow maize is preferred in most parts of South America and the Caribbean. It is also the preferred animal feed in many regions, as it gives a yellow colour to poultry, egg yolks and animal fat (IITA, 2009).

2. HOST ORGANISM DATA

Please refer to the companion maize biology document.

3. RECEIVING ENVIRONMENT

3.1. Production

In 2012, maize production in Mexico reached just over 22 million tonnes, making it the second most produced commodity in Mexico, after sugar cane and ahead of sorghum (Figure 1). It also made Mexico the world 4th producer of maize after USA, China, Brazil and Argentina (FAOSTAT, 2013). Yet, the maize production remained relatively constant in the last 6 years, below its potential production capabilities (Servicio de Informacion Agroalimentaria y Pesquera, 2012).

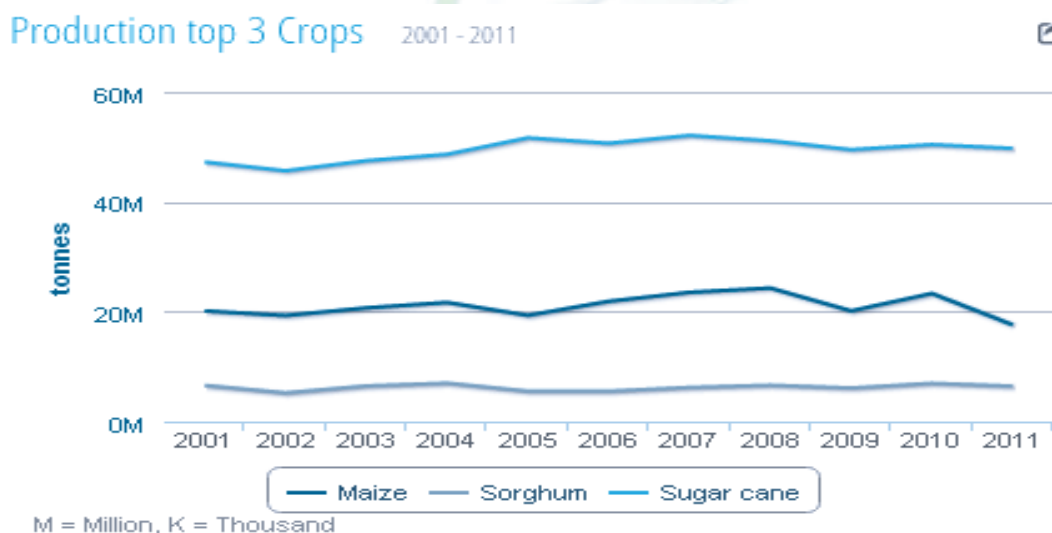


Figure 1. Maize production in Mexico, 2000-2011 (FAOSTAT, 2013).

3.2. Consumption and uses of the crop plant

Maize and cornmeal (ground dried maize) constitute a staple food in many countries. Maize meal is made into a thick porridge in many cultures. Maize meal is also used as a replacement for wheat flour, to make cornbread and other baked products. Masa (cornmeal treated with lime water) is the main ingredient for tortillas, atole and many other dishes of Mexican and Latin American food.

Maize is also a major source of cooking oil (corn oil) and of maize gluten. Maize starch can be hydrolysed and enzymatically treated to produce syrups, particularly high fructose corn syrup, a sweetener; and can also be fermented and distilled to produce grain alcohol.

Latin American countries, specifically Mexico, possess a great wealth of maize genetic diversity. There have been more than 40 landraces of maize in Mexico.

Maize is the most important crop in Mexico in terms of food, economy and socio-cultural traditions.

In Mexico, white maize is produced for human food, while yellow maize is produced for animal feed and industrial processing (Servicio de Informacion Agroalimentaria y Pesquera, 2012).



3.3. Cultivation of maize in Mexico

In many countries of Latin America, maize is produced on small units of land. For example, in Mexico there are approximately 2 million maize producers, and most of the land planted with maize (77 %) is less than 5 hectares in size, which contributes 67 % of total production. Producers are classified as:

- Micro-producers. Production is mainly for self-consumption and occasionally for local trade. Machinery and agrochemicals are not normally used,
- Small- and medium-size producer. Production is oriented to satisfy a particular client's needs. Technology and improved local seeds are often used,
- Major producer. Production is oriented to satisfy market demands. High yields are always present, and major producers are normally advised by experts.

Despite the adoption of improved modern varieties of maize, landraces still occupy more than 20% of the cultivated land, and are grown by 25% of farmers (mainly small income farmers). The main states in Mexico that cultivate white maize are: Sinaloa, Jalisco, Michoacan, Chiapas and Guerrero. On the other hand, the main states growing yellow corn are: Chihuahua, Jalisco, Tamaulipas and Chiapas (Servicio de Informacion Agroalimentaria y Pesquera, 2012).

3.4. Interaction of maize with related species in Mexico

It is generally agreed that teosinte (*Z. mexicana*) is an ancestor of maize, although opinions vary as to whether maize is a domesticated version of teosinte. Teosinte is a wild grass found in Mexico and Guatemala. Because it has differentiated into various races, species and plant habits, taxonomic classification is still a matter of controversy (CFIA, 1994).

Maize (*Zea mays* ssp. *mays*) hybridises freely with two annual teosintes, with *Zea mays* ssp. *mexicana* at a low rate (<1%), and with *Z. mays* ssp. *parviglumis* hybridises at a high rate (>50%). These wild relatives of maize are native to Central America and are present in different regions of Mexico (Figure 2). Particularly *Zea mays* ssp. *mexicana* is considered a weed in some regions when growing with *Zea mays* ssp. *mays*. Hybridisation events could occur with all types of maize, transgenic or not, so transfer of crop alleles towards wild populations is possible, especially in situations of sympatry.

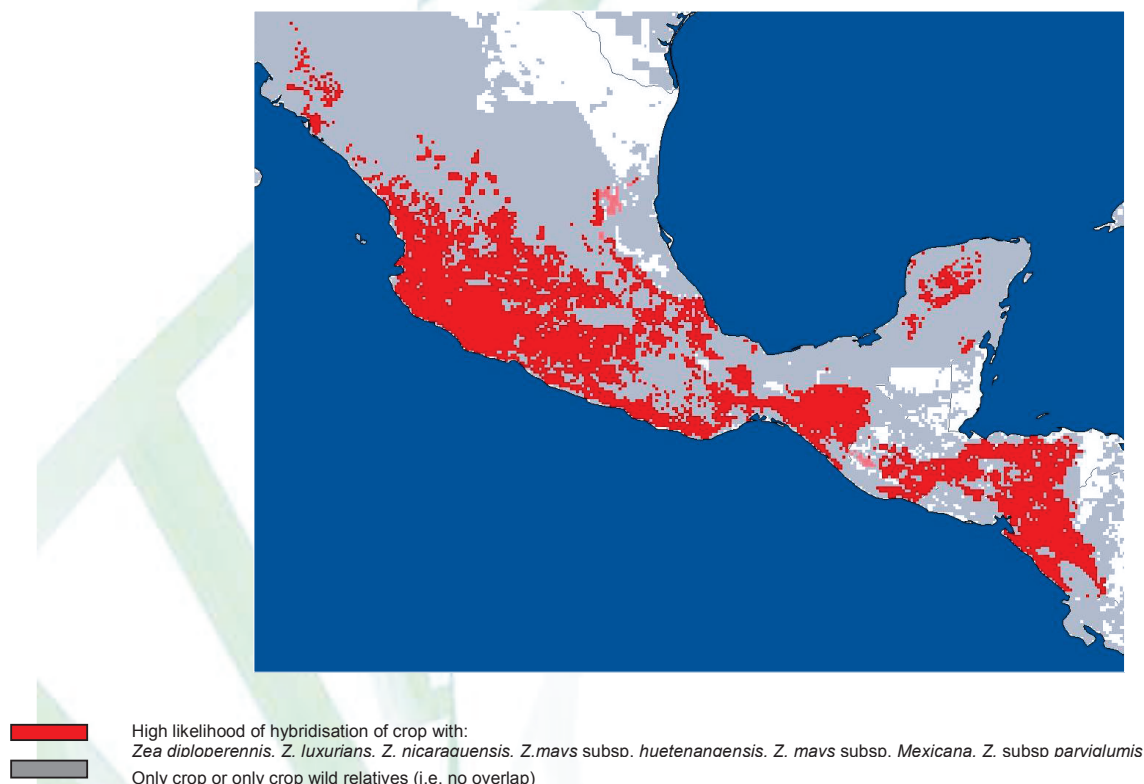


Figure 2. Presence of maize crop and wild relatives in Mexico

3.5. Potential interactions of maize with other life forms during its life cycle

The main organisms which interact with maize are listed in Table 1.

Table 1. Organisms which interact with maize.

Other life forms (Common Name)	Disease/Pest	Interaction with <i>Z. mays</i> (Pathogen; Symbiont or Beneficial Organism; Consumer; Gene Transfer)
<i>Colletotrichum graminicola</i>	Anthrachnose stalk rot and leaf blight	Pathogen
<i>Erwinia stewartii</i>	Bacterial leaf blight	Pathogen
<i>Fusarium</i> spp.	Fusarium seedling, stalk and kernel rot	Pathogen
<i>Gibberella zeae</i>	Gibberella stalk and ear rot	Pathogen
<i>Pseudomonas syringae</i>	Holcus spot	Pathogen
<i>Puccinia sorghi</i>	Leaf rust	Pathogen
<i>Pythium</i> spp.	Pythium seedling blight and root rot	Pathogen
<i>Rhizoctonia</i> spp.	Rhizoctonia seedling blight and root rot	Pathogen
<i>Setosphaeria turcicum</i>	Northern corn leaf blight	Pathogen
<i>Sphacelotheca reiliana</i>	Head smut	Pathogen
<i>Ustilago maydis</i>	Corn smut	Pathogen
<i>Agrotis ipsilon</i>	Black cutworm	Consumer
<i>Chaetocnema pulicaria</i>	Corn flea beetle	Consumer
<i>Delia patura</i>	Seedcorn maggot	Consumer
Earthworms		Symbiont or Beneficial Organisms
Soil microorganisms		Consumer, Symbiont or Beneficial Organisms

4. TRANSGENE DONOR ORGANISMS

4.1. *Cauliflower mosaic virus (CaMV)*

The promoter used in the maize transformation was from Cauliflower mosaic virus (CaMV) which infects plants in the family Brassicaceae. CaMV is a member of the caulimoviruses, one of six genera in the Caulimoviridae family, pararetroviruses that infect plants. Pararetroviruses replicate through reverse transcription just like retroviruses, but the viral particles contain DNA instead of RNA, and the viral genome is not inserted into the host genome. The promoter of the 35S RNA is a very strong constitutive promoter that is well known for its use in plant transformation. It causes high levels of gene expression in dicot plants. However, it is less effective in monocots, especially cereals.

4.2. *Agrobacterium tumefaciens*

The soil bacterium *Agrobacterium tumefaciens* is a plant pathogen that causes crown-gall tumours when it infects dicotyledonous plants. *A. tumefaciens* is an alpha-proteobacterium of the family Rhizobiaceae, which includes the nitrogen-fixing legume symbionts. Unlike the nitrogen-fixing symbionts, tumour-producing *Agrobacteria* are pathogenic and do not benefit the plant. The wide variety of plants affected by *Agrobacterium* makes it of great concern to the agriculture industry (Hoekema *et al.*, 1980). Tumours are induced by genes borne on the tumour-inducing (Ti) plasmid. The Ti plasmid integrates a segment of its DNA, known as T-DNA, into the chromosomal DNA of its host plant cells. Apart from the T-DNA, another region of the Ti-plasmid, called the vir-region, is essential for tumour induction.

4.3. *Bacillus thuringiensis (Bt)*

Bacillus thuringiensis (*Bt*) is a naturally occurring spore-forming soil bacterium that produces crystal proteins (Cry proteins), which are toxic to many species of insects. It belongs to the same family as *Bacillus cereus*, of which certain strains produce toxins that cause gastroenteritis (food poisoning) in humans. *Bt* is differentiated from *B. cereus* because it contains a plasmid bearing genes encoding the crystal proteins that are toxic to insects. *Bt* does not cause food poisoning.

Bt can be found in soils almost everywhere in the world. It has been found in all types of terrain, including beaches, desert, and tundra habitats. There are thousands of different *Bt* strains, producing over 200 Cry proteins that are active against an extensive range of insects and certain other invertebrates. Therefore if sprayed on plants, it is toxic to certain pest insects. For years, farmers and home gardeners have used *Bt* as a microbial spray pesticide to control caterpillars, certain types of

beetles, as well as mosquitoes and black flies. More recently, scientists have developed techniques by which traits from the *Bt* bacterium, including its ability to kill pests, can be introduced into a plant. Specifically, scientists have identified the genes that produces the toxins in *Bt*, and through the use of biotechnology, have incorporated them into the genetic material of several plants. These *Bt* plants synthesise their own bacterial protein to kill pests. (EPA, 2002; University of California San Diego, 2012)

4.4. *Triticum aestivum*

Common wheat, also known as bread wheat, is a cereal grain, originally from the Levant region of the Near East and Ethiopian Highlands, but now cultivated worldwide.

4.5. *Oryza sativa*

Oryza sativa, commonly known as Asian rice, is the plant species most commonly referred to in English as rice. *Oryza sativa* is the cereal with the smallest genome, consisting of just 430 Mb on 12 chromosomes. Compared to other cereal species, it is easy to genetically modify, and is a model organism for cereal biology.

4.6. *Escherichia coli*

The *Hyg^R* (hygromycin phosphotransferase) coding region was isolated from *Escherichia coli* plasmid pLG90 (van den Elzen *et al.*, 1985).

5. PRODUCT CHARACTERISATION.

5.1 Host Organism

Common name; Maize or corn

Scientific name; *Zea mays* L

Class: Angiosperma

Subclass; Monocotyledonea

Order; Graminales

Family: Poaceae

Subfamily: Panicoideae

Tribe: Maydeae

Genus: *Zea*

Species: *mays* sp *mays*

5.2. Traits

Traits: Resistance to coleopterans, lepidopterans and tolerance to the herbicide glyphosate. The stacked hybrid (Event 1 + Event 2 + Event 3) expresses two insecticidal proteins: Cry3Bb1, a delta-endotoxin that is toxic to certain coleopterans, and the delta-endotoxin Cry1Ab that is toxic to certain lepidopterans. Cry3Bb1 is produced by the *cry3Bb1* gene integrated in Event 1, Cry1Ab, by the *cry1Ab* gene integrated in Event 2, and the CP4 EPSPS protein is produced by the *cp4epsps* gene integrated in Event 3.

5.3. Trait Introduction Method

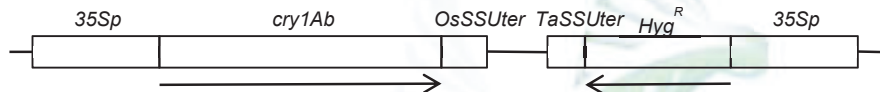
Genes were inserted in the single events (Events 1, 2 and 3) using *Agrobacterium*-mediated transformation, and the novel traits of each parental line were combined using traditional plant breeding methods to produce this new hybrid (stacked event).

5.4. Data on the Gene Constructs

The Event 1 construct is composed as follows:

- 35Sp, the 35S promoter of CaMV
- *cry1Ab* from *Bacillus thuringiensis* ssp. *kurstaki*. The transgene coding sequence was taken from plasmid vector PV-ZMBK07.
- *OssSUTer*, the 3' non-coding region of the small subunit of rubisco of rice (*Oryza sativa*) confers translation enhancement, ends transcription and directs polyadenylation (termination).

- 35Sp, the 35S promoter of CaMV
- *Hyg^R*, A fragment bearing the hygromycin resistance gene (*hph*) from *E. coli*
- TaSSUter, the 3' non-coding region of the small subunit of rubisco of wheat (*Triticum aestivum*) confers translation enhancement, ends transcription and directs polyadenylation (termination).



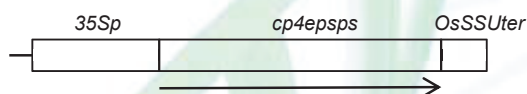
The Event 2 construct is composed as follows:

- 35Sp, the 35S promoter of CaMV
- *cry3Bb1* from *Bacillus thuringiensis* subsp. *kumamotoensis*. The transgene sequence was taken from plasmid vector PV-ZMIR13.
- *OsSSUter*, the 3' non-coding region of the small subunit of rubisco of rice (*Oryza sativa*) confers translation enhancement, ends transcription and directs polyadenylation (termination).
- 35Sp, the 35S promoter of CaMV
- *Hyg^R*, the the hygromycin resistance gene (*hph*) from *E. coli*
- TaSSUter, the 3' non-coding region of the small subunit of rubisco of wheat (*Triticum aestivum*) confers translation enhancement, ends transcription and directs polyadenylation (termination).



The Event 3 construct is composed as follows:

- 35Sp, the 35S promoter of CaMV
- *cp4epsps* from *Agrobacterium* strain CP4. The transgene sequence was taken from plasmid vector PV-ZMGT32
- *OsSSUter*, the 3' non-coding region of the small subunit of rubisco of rice (*Oryza sativa*) confers translation enhancement, ends transcription and directs polyadenylation (termination).



Southern blot analyses of genomic DNA isolated from plants of the stacked hybrid over six generations of crossing and three generations of self-pollination were used to confirm that the introduced DNA was stably inherited and each event segregated as a single locus according to Mendelian genetics.

Multi-generational stable expression of the resistance to lepidopterans and rootworms and glyphosate tolerance traits were demonstrated using bioassay and enzyme linked immunosorbent assay (ELISA) to measure protein concentrations.

Expression of full-length (approx. 47 kDa) CP4 EPSPS protein was confirmed by Western immunoblot analysis and protein concentrations were estimated using ELISA.

5.5. Phenotypic expression

Protein assay found activity in the roots, leaves, inflorescence, and seeds. Activity was highest in inflorescence and leaves. No activity was detected in pollen.

5.6. Characterisation of the proteins

The gene products have a history of safe use, and have undergone review and approval by several regulatory agencies. No interactions among the gene products or negative synergistic effects are expected in the stacked hybrid. Neither Cry1Ab, nor Cry3Bb1 are enzymes and therefore have no effect on plant metabolism.

EPSPS is one of the enzymes that catalyse the shikimate pathway for aromatic amino acid biosynthesis that is specific to plants and micro-organisms, and is located in chloroplasts or plastids in plants. The shikimate pathway is an important metabolic pathway that is considered to be involved in one fifth of carbon fixation by plants. This pathway is regulated by 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, which is involved in the first step of the pathway. It has been clarified to be extremely unlikely that the stages from DAHP to the synthesis of chorismic acid, through the

production of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) catalysed by EPSPS, are inhibited or suppressed by metabolic intermediates or end products of this pathway. This suggests that EPSPS is not the rate-determining enzyme, and as such it is not considered that enhanced EPSPS activity will increase the concentration of aromatic amino acids, the end products of this pathway. In practice, it is reported that plant cells that produce 40 times as much EPSPS as compared to normal do not synthesise excessive aromatic amino acids.

5.7. Agronomic characteristics

The flowering period, vegetative vigour, time to maturity and seed production of the stacked hybrid were within the normal range currently found in commercial maize hybrids.

There were no statistically significant differences in the levels of resistance to the target insects between the stacked hybrid and either of the parental *Bt* lines bearing Events 1 and 2 separately.

There were no significant differences in the tolerance to applications of glyphosate in the stacked line, compared to the parental lines.

5.8. Compositional analyses

The stacked event was compared with the genetically modified parental lines (Events 1, 2 and 3), a control line that has not been genetically modified (i.e. DKC46-26), and four commercial reference lines, also not genetically modified.

Field trials were carried out in four locations in two geographically different regions in Mexico during a single season. In each location, three replicates were present. The choice for the four commercial reference lines could differ from one location to another, and 13 commercial lines in total were employed. For the compositional analysis of the maize plants tested, samples of whole plants and hand-pollinated ears were collected from each plot in each location. Whole plants were used for analysis of forage, while the ears were used for analysis of kernels.

Forage was analysed for macro-nutrients, calcium, and phosphorous, while kernels were analysed for macro-nutrients, micro-nutrients, anti-nutrients, and secondary metabolites. The compositional data obtained were analysed statistically and comparisons were made between the stacked event and its control, as well as between the stacked event and the commercial reference lines. These comparisons were carried out for the data from each location, as well as for all data combined.

A number of statistically significant differences were observed in the comparisons of the composition of kernels from the stacked event and its control. These differences, however, were small and not consistent throughout the various test sites. Consistent differences were observed only in oleic acid, linoleic acid, and niacin contents. For example, the levels of oleic acid increased from 29.48 % of total fatty acids in kernels of control maize to 31.73 % in those of the stacked maize. These values were within the background range of commercial varieties tested in the same experiment (22.02 - 35.24 %).

5.9. Toxicity and allergenicity

The GM maize has no endogenous toxins or significant levels of anti-nutritional factors. Although there have been some reported cases of maize allergy, the protein(s) responsible have not been identified.

The insecticidal spectrum of the modified Cry3Bb1 protein is extremely narrow, and the modified Cry3Bb1 protein shows insecticidal activity particularly against the Colorado potato beetle and corn root-worm. While Coleopterans are susceptible to oral doses of Cry3Bb1 protein, there is no evidence of toxic effects in laboratory mammals or birds. There are no significant mammalian toxins or allergens associated with the host organism.

Cry1Ab protein shows insecticidal activity against order Lepidoptera, and it does not possess insecticidal activity against the insects other than order Lepidoptera. While target insects are susceptible to oral doses of Cry proteins, no evidence of toxic effects was observed in laboratory mammals or birds given up to 10 µg Cry protein/g body weight.

Cry3Bb1 + Cry1Ab proteins combined have the same functions to inhibit the digestive process by binding to the specific receptors on the midgut epithelium of the target insect. However, the pH in midgut of order Lepidoptera is alkaline (pH 10.5- 11.0), and that of order Coleoptera is neutral (pH 6.5- 7.0), and thus the proteins show insecticidal activity under different chemical conditions. Moreover, it is known that non-target insects do not show sensitivity to Cry1Ac protein, and Cry3Aa protein. Non-target insects remain unharmed, even if a mixture of Cry protein which targets the two different families was given to the insect, and it was confirmed that the non-target insect does not receive a synergistic effect by being exposed to Cry1Ac protein and Cry3A protein at the same time. See 5.10.4 (below for further details concerning non-target insect studies).

A. tumefaciens, from which CP4 EPSPS derives, is a common soil bacterium that is responsible for causing crown gall disease in susceptible plants. Although it is not a normal human pathogen, as with many bacteria, there are occasional reports of *Agrobacterium* infecting immuno-compromised humans.

These materials already have the approval from the Mexican Ministry of Health for human consumption (wet mill, dry mill or seed oil), and meal and silage for livestock feed. No potential toxicity was found in the stacked event.

5.10. Environmental safety considerations

5.10.1. Potential to become a weed

The flowering period, vegetative vigour, time to maturity and seed production of the stacked hybrid were within the normal range currently found in commercial maize hybrids. Maize does not possess the potential to become weedy due to traits such as a lack of seed dormancy, the non-shattering nature of maize cobs and the poor competitive ability of seedlings. Resistance to maize rootworm does not, in itself, render maize weedy or invasive of natural habitats as none of the reproductive or growth characteristics were modified.

No competitive advantage was conferred by Event 3, other than that conferred by resistance to glyphosate herbicide. Cultivated maize is unlikely to establish in non-cropped habitats and there have been no reports of maize surviving as a weed. In agriculture, maize volunteers are not uncommon, but are easily controlled by mechanical means or by using herbicides that are not based on glyphosate as appropriate. *Zea mays* is not invasive and is a weak competitor with very limited seed dispersal.

It was determined that it is very unlikely that the stacked hybrid would become a weedy pest of agriculture.

5.10.2. Potential for gene flow

The stacked hybrid did not change the physiological characteristics of its pollen production and pollen viability nor were there any changes in the plant's sexual compatibility with other maize varieties. Pollen dispersal and out-crossing frequency should be equivalent to that of untransformed maize lines, therefore gene exchange between the stacked hybrid maize and other maize varieties will be similar to that which occurs between non-transformed lines.

Studies on pollen flow, overviewed by Andersson and de Vicente (2010) determined that under normal climatic conditions maize pollen is deposited within 100 m from the source, but actual pollen dispersal can reach 1.8 km in the air. Outcrossing was seen as far as 4 km from the pollen source, yet they registered very low frequencies (under 0.01%). In addition, even if viable pollen reaches large distances, it might face strong competition by pollen shedding in the receptor plants. However, large maize fields have larger pollen dispersal due to higher pollen concentration in the air at a given moment.

Wind and atmospheric conditions at the time pollen was released into the atmosphere were critical factors affecting pollen movement and longevity (Luna *et al.*, 2001; Baltazar *et al.*, 2005). Other studies showed that pollination could be as high as 60% between contiguous rows, but pollination from more remote rows decreased with increasing distance such that no out-crosses were found 32 m away from the pollen source (Castillo and Goodman, 1997). However, wind speed and direction, turbulence, and pollen settling speed affect pollen movement (Di-Giovanni and Kevan, 1991; Aylor *et al.*, 2003), and variability in these factors can change the distance of cross-pollination.

5.10.3. Potential impact on biodiversity

The stacked hybrid has no novel characteristics that would extend its use beyond the current geographic range of maize production. The likelihood of out-crossing in Mexico is considerable, since it was determined that the possibility of transfer of novel traits to unmanaged environments where compatible wild relatives inhabit is likely when sympatric distribution occurs.

5.10.4. Potential impact(s) on non-target organisms

The Cry proteins expressed in Event 1 (Cry1Ab) and in Event 2 (Cry3Bb1) were shown to be equivalent to microbial *Bt* used in sprays for over thirty years. The history of use and literature suggest that the bacterial Cry proteins are not toxic to humans, other vertebrates and non-insect non-target invertebrates. Cry3Bb1 is active only against specific coleopteran insects. Both proteins were shown to degrade readily in the environment. CP4 EPSPS protein is not a known toxin and analogous proteins are found in all plants and micro-organisms.

Field studies indicated that the stacked hybrid did not have a negative effect on the abundance of non-target organisms compared to a non-*Bt* hybrid. Species studied included arthropods, earthworms and soil microbes. Coleopteran families including Carabidae, Staphylinidae and Coccinellidae were observed. Consideration was also given to the exposure of fireflies (family Lampyridae) since fireflies are coleopterans. It was concluded that the possibility of firefly exposure to Cry3Bb1 and Cry1Ab proteins from the stacked hybrid would be very unlikely, as these insects generally do not occur sub-surface and do not feed on maize. Feeding trials on non-target invertebrates, including honeybees, ladybird beetles, daphnia, collembola and earthworms, were also conducted. In addition, feeding trials were conducted on the non-target vertebrates, catfish and quail. The stacked hybrid was demonstrated to be safe to these species. When compared with currently commercialised maize lines, the stacked hybrid did not present an elevated risk or impact on interacting organisms, including humans, with the exception of certain target insects.

7. REFERENCES

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