Corporation obtaining approval, the name of its representative, and the address of its main office

Monsanto Japan Limited
Seiichiro Yamane, President

Ginza Sanno Bldg. 8F
4-10-10, Ginza, Chuo-ku, Tokyo

Approved Type 1 Use Regulation

Name of the type of Living Modified Organism:	Coleoptera resistant maize (*cry3Bb1, Zea mays* L.) (MON863, OECD UI:MON-ØØ863-5)
Content of the Type 1 Use of Living Modified Organism:	Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them.
Method of the Type 1 Use of Living Modified Organism:	-

Outline of the Biological Diversity Risk Assessment Report

I. Information collected prior to assessing Adverse Effect on Biological Diversity

1. Information concerning a recipient organism or the species to which the recipient organism belongs

(1) Taxonomical position and state of distribution in natural environment

The academic name for maize is *Zea mays* L. The origin is considered to be the area from the Southwest region of the United States to Mexico, Central America, and South America, but there is no conclusive theory. There is no report of natural distribution in Japan.

(2) History and present state of Use

The origin of maize is considered to be the area from the Southwest region of the United States to Mexico, Central America, and South America, but there is no conclusive theory. It is generally understood that the earliest cultivation could date back 9,000 years. The first introduction to Japan is said to be in 1579 to Nagasaki or Shikoku, and maize has long been cultivated since then. At present, it is used mainly for feed, but also for food and various food products including cooking oil and starch. Currently, it is the most widely cultivated grain in the world and can be grown in the area from 58 degrees north latitude to 40 degrees south latitude mainly in the US, China, Brazil, Argentina, and European countries and others.

Japan currently imports about 16 million tons of maize for feed and food.

(3) Physiological and ecological properties

i) Environmental conditions allowing inhabiting or growth

The optimum germination temperatures of maize seed are 32-36ºC and the minimum germinating and minimum growing temperatures are 6-10ºC. In practice, the optimum sowing season is considered to be the period when the temperatures are 13-14ºC or more than those, and usually maize is sown in spring and harvested in autumn as an annual plant.

The dormancy of the seed is extremely low, and even when seeds fall to the ground, they do not germinate until the soil temperature reaches 10ºC. In most cases the seeds would decay and die before germinating.

ii) Mode of propagation or reproduction

Maize is a monoecious annual plant which propagates by seed, and can be self-pollinated but in most cases cross-pollinated as a typical wind pollinated flower. It is not reported that maize seeds possess dormancy. The dispersion distance of maize pollen differs by the presence of barriers such as woods or mountains or others, the direction of the wind or others, but is considered to be approximately 300-500 m.

Related species of maize is teosinte of the same *Zea* as well as some other species classified into the genus *Tripsacum*. Maize can be hybridized only with teosinte in nature, and hybridization in nature with any species of genus *Tripsacum* is not known. In Japan, the growth of teosinte and wild species of the genus *Tripsacum* has not been reported.

iii) Production of harmful substances

Regarding maize, production of harmful substances that can affect the growth or habitat of other wild fauna and flora has not been reported.

iv) Other information

It has not been reported so far that maize seeds which were spilled during transportation and others on locations other than cultivation fields have grown.

2. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

i) Composition and origins of component elements

Composition of donor nucleic acids and origins of component elements used for the development of the Coleoptera resistant maize (*cry3Bb1, Zea mays* L.)(MON863, OECD UI No.: MON-ØØ863-5) (hereinafter referred to as this recombinant maize) is shown in Table 1.

ii) Functions of component elements

The *cry3Bb1* gene, the target gene to confer Coleoptera resistance, is derived from *Bacillus thuringiensis* subsp. *kumamotoensis,* a gram-positive bacterium universally exists in soil. The Cry3Bb1 protein which is encoded by the *cry3Bb1* gene possesses an insecticidal activity against corn rootworms (*Diabrotica* sp.) (hereinafter referred to as CRW), which is one of the major pest insects of order Coleoptera to maize cultivation in the US. This insect damages the root of maize. *B.t* proteins which are produced by the bacterium *B.t* including Cry3Bb1 protein bind to the specific receptors on the midgut epithelium of the target insects and form cation selective pores, which leads to the inhibition of the digestive process and results in the insecticide activity.

The insecticidal spectrum of the Cry3Bb1 protein is extremely narrow, and the Cry3Bb1 protein shows the insecticidal activity only against the Colorado potato beetle (*Leptinotarsa decimlineata)* (hereinafter referred to as CPB) and CRW, which are respectively classified into two genera *Leptinotarsa* and *Diabrotica* of the family Chrysomelidae, among the order Coleoptera. These related species of the same genera with these two insect species do not inhabit Japan.

Table 1 Component elements of plasmid PV-ZMIR13L and their origins and functions

Component elements	Origin and function
cry3Bb1 gene cassette	
4-AS1	A promoter that contains 4 copies of AS-1 element and a part of 35S promoter from cauliflower mosaic virus (CaMV). Has a function to make target genes expressed in all the tissues constantly.
wt CAB	5'-terminal untranslated region of wheat chlorophyll a/b binding protein. Activate the expression of target genes.
ract1 intron	Intron of rice actin gene. Activate the expression of target genes by enhancing the efficiency of splicing.
cry3Bb1	The gene which encodes modified Cry3Bb1 protein of *Bacillus thuringiensis*. The detail of its function is described in p.2.
lacZ	Partial coding sequence for β-d-galactosidase or lacZ protein. It was used as a selectable marker in cloning experiments in *E.coli*. Only 2 bps of the lacZ gene sequence remain, because most part of this gene was cut out with restriction enzyme treatment at the time of preparation of this gene cassette.
tahsp 17 3'	3'-terminal untranslated region of wheat heat shock protein 17.3. Terminates transcription and induces polyadenylation.
nptII gene cassette	
35S	A promoter from cauliflower mosaic virus (CaMV). Has a function to make target genes expressed in all the tissues constantly.
nptII	A gene isolated from the prokaryotic transposon, Tn5, encoding neomycin phosphotransferase II. Utilized as a selectable marker for transformation since it confers resistance to kanamycin when being expressed in bacteria.
ble	A part of bleomycin resistance gene isolated from Tn5. Encodes 50 amino acids at the N terminal region of the Ble protein, but does not confer bleomycin resistance.
NOS 3'	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens*. Terminates transcription and induces polyadenylation of mRNA.

In order to investigate whether the Cry3Bb1 protein shares functionally important amino acid sequences with known contact allergens, the Cry3Bb1 protein was compared with contact allergens in database. As a result, the Cry3Bb1 protein did not share structurally related sequences with known allergens.

While the *nptII* (neomycin phosphotransferase type II) gene, which is an antibiotic resistance marker gene introduced for the selection of transgenic plants, is derived from *Escherichia coli* transposon Tn5, the encoded NPTII protein confers resistance to aminoglycoside antibiotics (kanamycin and others) by inactivating these antibiotics through phosphorylation. As a result, addition of kanamycin to the medium makes it possible to select transgenic cells.

In order to investigate whether the NPTII protein shares functionally important amino acid sequences with known contact allergens, the NPTII protein was compared with the contact allergens in database. As a result, the NPTII protein did not share structurally related sequences with known allergens.

(2) Information concerning vector

i) Name and origin

The vector used for the production of this recombinant maze is plasmid pUC119 from *Escherichia coli.*

ii) Properties

The total numbers of base pairs of the vectors are 7,292 bp. These vectors contain a kanamycin/neomycin-resistant gene (*nptII* gene) derived from *E.coli* transposon Tn5 as a selectable marker gene for the construction vector.

(3) Method of preparing living modified organisms

i) Structure of the entire nucleic acid transferred in the recipient organism

For the production of this recombinant maize, the plasmid PV-ZMIR13 was constructed by connecting the *cry3Bb1* gene cassette（[4-AS1]-[wtCAB]-[ract1]-[*cry3Bb1*]-[lacZ]-[tahsp17 3’]）and *nptII* gene cassette ([35S]-[*nptII*]-[ble]-[NOS3’]) to a basic vector derived from pUC119 containing the above-mentioned *nptII* gene. When the genes are introduced into plant cells, a linear plasmid (PV-ZMIR13L) was used, after treating this PV-ZMIR13 with restriction enzyme *Mlu*I to remove the plasmid backbone containing the *nptII* gene region.

ii) Method of transferring nucleic acid transferred in the recipient organism

PV-ZMIR13L, a linear plasmid was introduced by particle gun bombardment to the inbred A634 that is classified into dent type.

iii) Processes of breeding living modified organisms

The callus to which PV-ZMIR13L was introduced was grown on a tissue culture media containing 2, 4-D for a certain period of time, and then the recombinant plant was selected on a kanamycin-adding medium. From the selected callus, the regenerated plant was obtained and the expression of the Cry3Bb1 protein was analyzed. Pedigree selection was started in 1997, and field experiments were carried out from 1998 to 1999. Finally, MON863 was selected as an excellent event. In filed experiments carried out at one site in Illinois in 1999, the morphological and growth characteristics of this event were investigated and also analysis of the expression of the Cry3BbI protein and inserted genes were implemented. Based on these results, necessary approval was obtained in the US and general commercial cultivation began in 2003.

(4) State of existence of nucleic acid transferred in cells and stability of expression of traits caused by the nucleic acid

Based on Southern blotting analyses, it was confirmed that 1 copy of the DNA from PV-ZMIR13L which is necessary for the expression of the *cry3Bb1* gene and the *nptII* gene is inserted into the genome of this recombinant maize at 1 site. Also, Southern blotting and Western blotting analyses of multiple generations of the plant indicated that *cry3Bb1* and *nptII* genes on the inserted DNA are stably inherited and expressed in offspring. It was also confirmed that resistance to Coleopteran pest insects is stably expressed in multiple generations.

(5) Difference from the recipient organism or the species to which the recipient organism belongs

i) With the expression of the Cry3Bb1 protein, which is encoded by *cry3Bb1* gene, resistance to corn borers (CRW), which is the major pest insect of the order Coleoptera, was conferred to this recombinant maize. Also, a decrease of feeding damage by CRW was confirmed. Roots of maize are damaged by CRW, but Cry3Bb1 protein expresses in all the tissues constantly in this recombinant maize.

ii) Isolated field tests were carried out using MON863AX, MON863BX and MON863CX which belong to the event of this recombinant maize, as well as MON863AC, MON863BC and MON863CC as the control lines. MON863BX and MON863CX are genetically identical hybrid cultivars that were obtained from the same hybrid combination. It was confirmed that the tested recombinant maize strains were of the MON863 event.

(a) Morphological and growth characteristics

Providing this recombinant maize and the non-recombinant control maize, evaluation was conducted regarding germination rate, uniformity of germination, time of tassel exsertion, time of silking, time of flower initiation, time of flower completion, flowering period, time of maturation, plant type, tiller number, total number of ears, number of productive ears, culm length, height of ear, grain color, grain shape, and fresh weight after harvesting. No statistically significant difference was observed between this recombinant maize and the non-recombinant control maize in all of the items.

(b) Chilling-tolerance and heat-tolerance at the early stage of growth

Sensitivity to a low temperature (4ºC) of the seedlings of this recombinant maize and non-recombinant control maize was evaluated. Almost all of the seedlings died down to the ground on the 14th day, and no difference was observed between this recombinant maize and non-recombinant control maize.

(c) Wintering ability or summer survival of the matured plant

Maize is an annual plant, and after ripening it usually dies down to the ground naturally in winter. Overwintering test for the matured plant of this recombinant maize was not carried out, since it does not regrow and propagate vegetatively, or produce seeds.

(d) Fertility and size of the pollen

To examine the fertility (maturity) and size of the pollens, pollens were stained with potassium iodine solution and observed under a microscope. As a result, no difference was observed between this recombinant maize and non-recombinant control maize.

(e) Production, germination rate, dormancy, and shedding habit of the seed

Ear length, ear diameter, row number per ear, grain number per row, 100-kernel weight, of the ears harvested after sib-mating, and the germination rate of the harvested seeds were examined. As a result, no statistically significant difference was observed between this recombinant maize and non-recombinant control maize in all of the characteristics examined.

(f) Hybridization

Hybridization test was not performed since no wild relatives that can be hybridized grow in Japan.

(g) Production of harmful substances

Plow-in tests, succeeding crop tests, and soil microflora tests were performed using this recombinant maize and a non-recombinant control maize. No statistically significant difference was observed between this recombinant maize and the non-recombinant control maize in all of the items.

3. Information concerning the Use of living modified organisms

(1) Content of the Use

Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them.

(2) Emergency measures which should be taken to prevent Adverse Effect on Biological Diversity in case Adverse Effect on Biological Diversity could arise

Refer to the attached Plans of Emergency Measures.

(3) Information obtained from Use abroad

From 1998 to 1999, field tests were conducted at 19 sites in total in the US to examine morphological and growing characteristics including germination rate, plant vigor, plant height, flowering time, and time of silking, as well as yield, pest sensitivity, and wintering ability. No difference was found between this recombinant maize and the non-recombinant control maize except that this recombinant maize possesses resistance to CRW, an insect of the order Coleoptera.

This recombinant maize has been commercially cultivated overseas including the United States and Canada since 2003.

II. Item-by-item assessment of Adverse Effect on Biological Diversity

1. Dominance in competition

As a result of comparative examination of various traits of the recombinant maize and non-recombinant control maize relating to dominance in competition, statistically significant difference was not observed between them. This recombinant maize has a trait to produce Cry3Bb1 protein, an insecticidal ingredient against pests of the order Coleoptera. This may raise the survival rate of the recombinant plant temporarily. However, since there was no statistically significant difference in other traits relating to dominance in competition between the recombinant maize and the non-recombinant control maize, it cannot be considered that dominance in competition is raised with only the Coleoptera resistance.
Based on the above understanding, it was considered that there is no risk of adverse effect on biological diversity attributable to dominance in competition.

2. Production of harmful substances

As a result of comparative examinations on the production of harmful substances by the plow-in test, succeeding crop test, and soil microflora test, no difference was observed between the recombinant maize and the non-recombinant control maize.

As a result of expression of Cry3Bb1 protein, this recombinant maize is conferred with resistance to corn rootworm (*Diabrotica* sp.*)* (hereinafter referred to as CRW), one of the major maize pests damaging the root of maize. So far, it has been reported that Cry3Bb1 protein exerts insecticidal activity only against the Colorado potato beetle (*Leptinotarsa decimlineata)* (hereinafter referred to as CPB) and CRW, which are respectively classified into genera *Leptinotarsa* and *Diabrotica* of the family Chrysomelidae, among the order Coleoptera, but not against other insects.

Literature review clarified that CPB, CRW, and their related species of the same genera do not inhabit Japan. However, since there is the possibility that the protein exerts insecticidal activity against uninvestigated insects of the order Coleoptera, the following assessment was carried out.

With regard to the insect species of the order Coleoptera which are classified as endangered or sub-endangered and listed in the "Ministry of Environment's Red-list (revised edition 2000)", the possibility of the adverse effect of pollen dispersion of the recombinant maize was examined based on the feeding habit, habitat, behavior pattern, and distribution of each species. As a result, it was concluded that there are no insect species of the order Coleoptera which may be affected by pollen dispersion of this recombinant maize, among the species listed in the Ministry of Environment's Red-List.

In order to verify the possibility further, insects of the order Coleoptera were listed from "Important areas for the conservation of biodiversity of insects (Edited by Nature Conservation Committee, Japan Insects Association)", and the possibility of adverse effect by the pollen of the recombinant maize was examined based on the feeding habit, habitat, behavior pattern, and distribution of each species, in a similar manner to species listed on the "Red-list". As a result, larvae of three species, including *Chrysolina angsticollis, Catapionus virdimetallicus,* and *Epilachna niponica* were identified as insect species which may possibly be affected in some way depending on the level of pollen dispersion, since they feed on plant leaves.

The specifics of the adverse effect were then evaluated. In Japan, since a bioassay method using Coleopteran insects has not been established so far, a bioassay was carried out using CPB, which is a pest insect known to show the highest sensitivity to Cry3Bb1 protein.

CPB larvae within 24 hours after hatching were fed with the pollen of the recombinant maize and those of the non-recombinant control maize and the survival rates of them were then compared. As a result, statistically significant difference was found at a pollen density of 2,000 particles/cm^2.

A comparison on the quantity, shape and size of pollen between the recombinant maize and the non-recombinant control maize found no statistically significant difference.

Since there was no difference in the quantity, shape and size of pollen of the recombinant maize and those of non-recombinant control maize as mentioned above, the distance within which pollen dispersion exert adverse effect was calculated by assigning the pollen density of 2,000 particles/cm^2, a level that exerts effect on the survival of CPB, to the model equation of Kawashima *et al*., which indicates the relationship between the distance from the field and the number of dropped maize pollen. This model equation represents the maximum number of deposited pollen that cannot be exceeded under normal climatic conditions. As a result, it was estimated that the maximum range on which the pollens of recombinant maize deposit at a density of 2,000 particles/cm^2 is 20 m.

Additionally, according to literature survey, the plants consumed by larvae of the 3 species of Coleopteran insects identified as the species subject to the adverse effect of this recombinant maize, inhabit a wide range of areas including open field and mountains. The cultivation fields of maize and surrounding area of the fields are not the main habitat for these plants.

From these results, it was concluded that the possibility where the dispersion of pollen would disturb the maintenance of species or individual populations is extremely low, based on the assessment of the range within which the dispersion of the pollen of the recombinant maize is expected to exert adverse effect and the judgment regarding the habitat of the 3 species.

Based on the above understanding, it was concluded that there is no risk of adverse effect on biological diversity attributable to the production of harmful substances.

3. Hybridization

Related species of maize are teosite of the same genus *Zea* and some other species of genus *Tripsacum*, but teosinte is the only species that can be hybridized with maize in nature. In Japan, the growth of teosinte and wild species in the genus *Tripsacum* has not been reported.

Based on the above understanding, it was considered that there is no risk of adverse effect on biological diversity caused by hybridization.

III. Comprehensive assessment of Adverse Effect on Biological Diversity

Maize, the species to which the recipient organism belongs, has long been used in Japan. Also, there was no difference in the various traits concerning dominance in competition between the recombinant maize and the non-recombinant maize. Based on the above understanding, it was concluded that there is no risk of adverse effect on biological diversity caused by dominance in competition.

Various traits related to the production of harmful substances were evaluated in the plowing-in test, succeeding crop test, and soil microflora test, and no difference was found between the recombinant maize and the non-recombinant maize. In addition, the adverse effect on 3 species of insects of genus Coleoptera which were regarded as wild habitats to be susceptible to the adverse effect of pollen dispersion of this recombinant maize in Japan were examined. It was concluded that the possibility that they are affected by pollen at the individual population level is extremely low because the range within which the pollen of this recombinant maize exert adverse effect is estimated within 20 m around the field and also because the nontargeted Coleopteran insects which originally inhabit the natural ecosystem do not inhabit mainly around the maize cultivating field. Based on the above understanding, it was concluded that there is no risk of adverse effect on biological diversity caused by the production of harmful substances.

Since there is no wild plant which can be hybridized with maize, it was considered that there is no risk of adverse effect on biological diversity attributable to hybridization

Consequently, it was judged that there is no risk of adverse effect on biological diversity in Japan attributable to the use of this recombinant maize for provision as food, for provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them.