# Cultivation of maize line MON89034xNK603

## COGEM advice CGM/091208-01

*This notification concerns the cultivation of the genetically modified maize line MON89034xNK603. This maize line expresses the cp4 epsps and cp4 epsps L214P genes conferring tolerance to glyphosate containing herbicides. In addition, MON89034xNK603 contains the cry1A.105 and cry2Ab2 genes and is therefore resistant to certain lepidopteran insects.*

*Previously, COGEM advised positively on import of maize line MON89034xNK603 and on cultivation of maize line NK603.*

*In Europe, no wild relatives of maize are present and establishment of maize plants in the wild has never been observed. There are no reasons to assume that the inserted traits will increase the potential of the maize line to establish feral populations. In addition, in Europe the appearance of volunteers is very rare.*

*COGEM is of the opinion that the molecular characterization of MON89034xNK603 is adequate.*

*The applicant conducted several laboratory experiments to study whether maize MON89034xNK603 affects non-target organisms (NTOs) adversely. None of the laboratory experiments have been carried out with MON89034xNK603. In most cases either Cry1Ab.105 or Cry2Ab2 pure protein was used. COGEM is of the opinion that the applicant did not sufficiently demonstrate that the Cry1A.105 and Cry2Ab2 proteins do not interact. Five of the nine NTOs that were studied do not occur in the European Union and the applicant did not explain why these organisms are relevant to European maize fields. In addition, although the applicant did not show that Cry2Ab2.820 is biologically identical to Cry2Ab2 some of the experiments have been carried out with Cry2Ab2.820. The laboratory experiments also exhibit other shortcomings: the statistical power of the experiments and the obtained P value are not given, the choice for the statistical test is not explained and the applicant did not provide an explanation for the high mortality (over 15%) in certain control groups.*

*The applicant presented three studies that describe MON89034xNK603 field trials. However, none of these field trials investigated the effect of MON89034xNK603 on NTOs in Europe. COGEM is of the opinion that the provided data are insufficient to allow a conclusion that cultivation of MON89034xNK603 exerts negligible effects on NTOs.*

*Based on these considerations, COGEM cannot advise positively on cultivation of maize line MON89034xNK603. COGEM is of the opinion that additional data from laboratory experiments and field trials have to be supplied to be able to make a reliable environmental risk analysis on cultivation of maize line MON89034xNK603.*

### Introduction

The scope of the present notification (EFSA/GMO/NL/2009/72) by Monsanto Company, as represented by Monsanto Europe S.A. concerns the cultivation of maize line MON89034xNK603.

COGEM has been asked to evaluate the safety of commercial cultivation of this maize line in the European Union with respect to human health and the environment.

MON89034xNK603 was produced by crossing the two parental maize lines MON89034 and NK603 using traditional breeding methods. The maize line contains the *cry1A.105* and *cry2Ab2* genes, which confer resistance to certain lepidopteran pests. In addition, this line contains the *cp4 epsps* and *cp4 epsps L214P* genes, which confer tolerance to glyphosate containing herbicides. MON89034xNK603 maize has not yet been authorized for commercial cultivation in any other country.

**Previous COGEM advices**

In October 2009, COGEM issued a positive advice on the import and processing for use in feed and food of genetically modified maize line MON89034xNK603.[1] COGEM concluded that import and processing of MON89034xNK603 poses a negligible risk to the environment. Three years earlier, in July 2006, COGEM issued a positive advice on the cultivation of maize line NK603.[2] COGEM was of the opinion that cultivation of maize line NK603 poses a negligible risk to human health and the environment.

**Aspects of the crop**

Maize (*Zea mays L.*) is a member of the grass family *Poaceae.* Maize is cultivated as an agricultural crop, originating from Central America. Although insect pollination cannot be completely excluded, maize is predominantly wind pollinated.[3],[4] According to literature, pollen viability varies between 30 minutes and 9 days.[4,5,6] In Europe, no wild relatives of maize are present and, therefore, hybridization with other species cannot occur.

Throughout the world, the appearance of volunteers is very rare. Grains exhibit no germination dormancy, resulting in a short persistence. In addition, only few seeds remain on the field after harvesting of fodder maize.[3] Establishment of maize plants in the wild has never been observed in the Netherlands and COGEM is not aware of any reports of wild maize plants elsewhere in Europe.

**Molecular characterization**

The genetically modified maize line MON89034xNK603 was produced by crossing the two parental maize lines MON89034 and NK603 using traditional breeding methods. The molecular characterization of maize MON89034 and NK603 was previously evaluated by COGEM. COGEM concluded that the molecular characterization of both parental lines was adequate.[7,8] An overview of the construction and inserted genetic elements of both parental lines as well as the properties of the introduced genes is given below.

*Parental maize line MON89034*

The genetically modified maize line MON89034 was produced by *Agrobacterium tumefaciens*-mediated transformation using *A. tumefaciens* strain ABI and the PV-ZMIR245 vector, which consists of two T-DNA regions and the vector backbone. The T-DNA I region contains the

*cry1A.105* and the *cry2Ab2* genes, and the T-DNA II region contains the neomycin phosphotransferase II (*nptII*) gene, which confers resistance to certain aminoglycoside antibiotics, such as neomycin, kanamycin and paromycin. The T-DNA I and T-DNA II regions are both flanked by right and left border sequences which allow the T-DNA regions to be inserted independently. After transformation paromycin resistant plants were selected. These plants contained the T-DNA II region or the T-DNA I and II regions. In subsequent generations the T-DNA-I and T-DNA II regions which were integrated at different loci segregated. The plants that contained the T-DNA II region were eliminated and only the plants containing the T-DNA I region were selected.

The T-DNA I region consisted of:
- Right border region derived from the Ti-plasmid of *A. tumefaciens* used for transfer of the T-DNA;
- *e35S* promoter providing constitutive expression, which was derived from *Cauliflower mosaic virus* (CaMV) and contains the duplicated enhancer region;
- *Cab* leader, leader region from the chlorophyll a/b-binding protein from wheat;
- *Ract1* intron, intron from the rice actin gene;
- Cry1A.105 coding sequence, coding sequence for the *cry1A.105* gene which is a modified version of the *cry1A* gene from *Bacillus thuringiensis. Cry1A.105* encodes Cry1A.105, a modified Cry1A protein, which consists of domains I and II from Cry1Ab/Cry1Ac, domain III from Cry1F and substantially the entire Cterminal domain of Cry1Ac. The codon usage of *cry1A.105* has been optimized for expression in monocots;
- *Hsp17* terminator from the wheat heat shock protein 17.3, which ends transcription and directs polyadenylation;
- *FMV* promoter providing constitutive expression from *Figwort mosaic virus* (FMV);
- *Hsp70* intron from the heat shock protein 70 gene of maize;
- *SSU-CTP* targeting sequence, chloroplast targeting sequence of the small subunit of ribulose 1,5-bisphosphate carboxylase from maize;
- Cry2Ab2 coding sequence, coding sequence for the *cry2Ab2* gene, which encodes the Cry2Ab2 protein. The Cry2Ab2 protein has been isolated from *B. thuringiensis* var. *kurstaki*. The codon usage of *cry2Ab2* has been optimized for expression in monocots;
- *nos* terminator sequence from the nopaline synthase gene of *A. tumefaciens,* which ends transcription and directs polyadenylation;
- Left border region derived from the Ti-plasmid of *A. tumefaciens* used for transfer of the T-DNA.

*Properties of the introduced genes conferring insect resistance*

Maize line MON89034 was genetically modified by the insertion of the *cry1A.105* and the *cry2Ab2* genes. The *cry1A.105* and *cry2Ab2* genes encode δ-endotoxins specific for insects of the order Lepidoptera. The δ-endotoxins are solubilized in the midgut of susceptible insects and are activated by midgut proteases to release a toxin fragment. The toxin fragment binds to specific

receptors on the epithelial surface of the midgut. Subsequently, pores are formed in the membranes of the gut cells of the insect, enabling midgut bacteria to enter the body cavity, which leads to septicemia and death.[9]

*Parental maize line NK603*
The genetically modified maize line NK603 was produced by particle bombardment. A restriction fragment of plasmid PV-ZMGT32L, containing two *cp4 epsps* expression cassettes was inserted into maize line NK603.

The two expression cassettes contain the following sequences:
• *cp4 epsps* expression cassette 1:
- P-ract1/ract1 intron, promoter, transcription start site and intron derived from *Oryzae sativa*; intron promotes transcription
- *ctp2*, gene from *Arabidopsis thaliana*; encoding a chloroplast transit peptide
- *cp4* epsps, gene derived from *Agrobacterium tumefaciens CP4*; encoding 5 enolpyruvylshikimate-3-phophatesynthase (CP4 EPSPS)
- Nos 3', terminator from *A. tumefaciens*; stops transcription

• *cp4 epsps* expression cassette 2:
- E35S, constitutive promotor from CaMV
- *hsp70*, intron derived from *Z. mays*; stabilises transcription
- *ctp2*, *gene* derived from *A. thaliana*; encoding a chloroplast transit peptide
- *cp4 epsps L214P*, gene derived from *A. tumefaciens CP4*; encoding CP4 EPSPS
- Nos 3', terminator from *A. tumefaciens*; stops transcription

*Properties of the introduced genes conferring herbicide tolerance*
Maize line NK603 was genetically modified by the insertion of the *cp4 epsps* and *cp4 epsps L214P* genes, which encode CP4 EPSPS proteins. EPSPS is an enzyme involved in the biosynthesis of aromatic amino acids. Glyphosate inhibits EPSPS, resulting in a lack of amino acids essential for growth and development of plants. Maize line NK603 expresses CP4 EPSPS proteins, which are not inhibited by glyphosate[10] and is therefore tolerant to glyphosate containing herbicides.

**Environmental risk assessment**
In the opinion of COGEM, there is no reason to assume that the traits expressed in maize MON89034xNK603 will increase the potential of maize to establish feral populations. With regard to potential adverse effects of MON89034xNK603 on non-target organisms (NTOs), the applicant refers to laboratory, greenhouse and field studies. These studies will be discussed below.

*Laboratory and greenhouse studies*

The applicant performed several experiments to study whether MON89034xNK603 has an adverse effect on NTOs. Studies on collembola (*Folsomia candida*), soil microorganisms and the northern bobwhite quail (*Colinus virginianus*) were carried out with plant material of parental maize line MON89034. All other laboratory experiments used either the Cry1A.105 or the Cry2Ab2 purified protein. In none of the laboratory experiments or greenhouse studies maize MON89034xNK603 was used. COGEM is of the opinion that experiments to study the effect of MON89034xNK603 on NTOs should be carried out with MON89034xNK603 and not with other maize lines (e.g. MON89034). Whenever possible, instead of pure proteins plant material of MON89034xNK603 should be used.

As stated above, the majority of the laboratory experiments were carried out with either Cry1A.105 or Cry2Ab2 pure protein. Only one study using target organisms (the European corn borer and the corn earworm) was presented to demonstrate the absence of interaction between these two proteins. Moreover, in the study that examined the potential for interaction between the Cry1A.105 and Cry2Ab2 proteins the Cry2Ab2.820 protein was used. According to the applicant, the Cry2Ab2.820 protein contains three additional chloroplast transit peptide amino acids at the N-terminus. Furthermore, the Cry2Ab2.820 protein includes an additional amino acid after the cleavage site of the protein. As the Cry2Ab2.820 protein appears to be different from the Cry2Ab2 protein that is present in maize MON89034xNK603, the applicant should provide information to show that Cry2Ab2.820 is biologically identical to Cry2Ab2. Because the applicant did not demonstrate that Cry2Ab2.820 is biologically identical to Cry2Ab2 the information from the study on the interaction between Cry1A.105 and Cry2Ab2.820 may not be used to conclude that the Cry1A.105 and the Cry2Ab2 protein do not interact. In conclusion, COGEM is of the opinion that the applicant did not sufficiently demonstrate that the Cry1A.105 and Cry2Ab2 proteins do not interact. Therefore, the combination of both the Cry1A.105 and the Cry2Ab2 proteins should have been used in the laboratory experiments that are carried out with pure proteins.

The applicant used several NTOs, namely collembola (*F. candida*), soil microorganisms, earthworm (*Eisenia fetida*), ladybird beetle (*Coleomegilla maculata* and *Hippodamia convergens),* minute pirate bug *(Orius insidiosus)*, honey bee *(Apis mellifera)*, parasitic wasp *(Ichneumon promissorius)*, and northern bobwhite quail (*C. virginianus*), in laboratory experiments or greenhouse studies.

Five of these NTOs, i.e. *C. maculata*, *H. convergens, O. insidiosus, I. promissorius* and *C. virginianus* do not occur in the European Union. COGEM is of the opinion that NTOs that are relevant to the crop ecosystem in Europe should be used. Therefore, if non-European NTOs are used, the applicant should explain why these organisms are relevant to European maize fields. To facilitate the selection of relevant NTOs, a research report in which guidelines for the selection of NTOs relevant to the North-West European situation were developed, was written in commission of COGEM.[11,12] The guidelines in this report could be used to select NTOs relevant to the European situation.

In laboratory experiments that studied the effect of the Cry2Ab2 protein on minute pirate bugs and parasitic wasps the Cry2Ab2.820 protein was used. As this protein appears to be different from the Cry2Ab2 protein that is present in maize MON89034xNK603 and because the applicant did not show that Cry2Ab2.820 is biologically identical to Cry2Ab2, in COGEM's view the results obtained with this Cry2Ab2.820 protein cannot be used for conclusions on (the absence of) an effect caused by Cry2Ab2.

Most laboratory experiments were carried out with only four to six replicates with each replicate containing ten to fifty organisms. The number of replicates in combination with the variability within the experiment determines the ability to detect effects accurately. COGEM is of the opinion that an effect that is present should be detected in at least 80% of the cases, therefore experiments should have a statistical power of 0.8 or more.[13] However, information on the statistical power of the experiments is not given and it is therefore unclear how well the experiments are able to detect an effect. If the statistical power of the experiments is below 0.8 the number of replicates should be increased to ensure an accurate detection of any effect that might be present.

Different statistical tests have been used without explanation for the chosen method. COGEM is of the opinion that the applicant should clarify why a certain statistical test was chosen. In addition, in most experiments the obtained P value is not given. The applicant should give information about the obtained P-values.

In several of the laboratory experiments that used honey bees or ladybird beetles mortality in the control groups exceeded 15%. The applicant did not provide an explanation for the high mortality in some of the control groups. COGEM points out that a high mortality in control groups could indicate problems with the experimental setup[14] and could mask an effect that is present. Preferably, mortality in control groups should not exceed 15%.

Most of the laboratory experiments investigated sublethal effects (behaviour, weight and development to adult) in addition to mortality. Unfortunately, in most cases population growth was not studied. COGEM is of the opinion that it is important to study whether maize MON89034xNK603 has sublethal effects on non-target organisms because sublethal effects can affect population size significantly. In a previous advice COGEM stated that she considers measurements of population growth the method of choice when studying whether a genetically modified crop has an adverse effect on non-target organisms, because both mortality and sublethal effects are reflected in this parameter.[13]

*Field studies*

The applicant provided a number of studies with information on field trials. The majority of these studies referred to field trials that were carried out with other maize lines such as MON89034, NK603 or MON89034xMON88017. Only three studies referred to field trials with maize line

MON89034xNK603. In two of the studies field trials were described in which the effect of MON89034xNK603 on target organisms was studied. In the study that referred to eight European field trials (three sites in Germany and five in Spain) data on damage caused by wireworm (*Agriotes* sp.), aphids (*Aphis* sp.), cutworm, fruit fly and the European corn borer was presented. In the study that referred to Argentinean field trials (five sites) that were carried out in 2004/2005 the effect of MON89034xNK603 on *Spodoptera frugiperda, Tropinopus laevipes* and *Helicoverpa zea* was examined. All the above-mentioned organisms are target organisms and therefore these studies do not provide information to assess the effect of MON89034xNK603 on NTOs.

Only in the study that referred to the Argentinean field trials (three sites) that were carried out in 2005/2006, the abundance of NTOs (*Chrysopa* spp. Coccinellidae, *Doru* spp., *O. insidiosus, Trichogramma* spp.) was investigated. In two out of twelve observations the abundance of *Doru* spp. was higher for MON89034xNK603 when compared to the control. No statistical differences were detected for the other organisms. The applicant concludes that MON89034xNK603 does not have an altered environmental impact when compared to conventional maize. However, the NTOs that are present in Europe differ from the NTOs present in Argentina. Therefore, the results of the Argentinean field trial are not sufficient to conclude that MON89034xNK603 does not adversely affect European NTOs. In addition, COGEM is of the opinion that all relevant ecological groups (predators, parasitoids, pollinators/nectar feeders, soil organisms and protected/ endangered butterflies) should be represented by the NTOs that are studied. In the Argentinean field trials not all relevant ecological groups were represented.

The maize lines in the field trials were planted in plots. It is unclear what the number of maize plants in a plot is. On basis of the data presented it cannot be excluded that the number of MON89034xNK603 maize plants in the plots is too low to draw legitimate conclusions on the effect of MON89034xNK603 on NTOs.

Overall, COGEM is of the opinion that the studies that have been carried out do not provide enough information to endorse the conclusion of the applicant that MON89034xNK603 does not affect NTOs adversely.

**General surveillance**

General surveillance has been introduced to be able to observe unexpected adverse effects of genetically modified crops on the environment. The setting or population in which these effects might occur is either not, or hardly predictable. The central tool for general surveillance in case of cultivation of MON89034xNK603 maize is an annual farmer's questionnaire which is addressed to a subset of farmers that cultivate MON89034xNK603 maize. In COGEM's view the questionnaire should not only contain questions about the performance of MON89034xNK603 maize on the field, but should also contain questions about unexpected effects of the MON89034xNK603 maize on the whole of the farmer's premises. COGEM is also of the opinion that the part of the farm questionnaire dealing with animals is too general. Birds, deer and insects

are assigned to one category “wildlife”. Information about the occurrence of wildlife should be obtained by different questions for specific groups of organisms (e.g. mammals, (predatory) birds, and insects). In addition, the farmer should be asked whether unusual quantities of other animals were observed and whether dead animals were found. The questions in the farm questionnaire refer to the usual situation, but the usual situation is not well defined. It would be better to rephrase the questions to acquire data that can be used to detect negative or positive trends in populations of organisms relevant to the monitoring scheme.

**Advice**

The present application concerns the cultivation of the genetically modified maize line MON89034xNK603. This maize line expresses the *cp4 epsps* and *cp4 epsps L214P* genes conferring tolerance to glyphosate containing herbicides. In addition, MON89034xNK603 contains the *cry1A.105* and *cry2Ab2* genes and is therefore resistant to certain lepidopteran insects. In the past, COGEM advised positively on the import of this particular maize line.

There are no wild relatives of maize in Europe and the appearance of volunteers is rare. Furthermore, there are no reasons to assume that the inserted traits will increase the now absent potential of the maize line to establish feral populations. COGEM is of the opinion that the molecular characterization is adequate.

The applicant conducted several laboratory and field studies and refers to these studies with regard to the absence of potential adverse effects of MON89034xNK603 on non-target organisms (NTOs).

None of the laboratory experiments have been carried out with MON89034xNK603 and in most cases either Cry1Ab.105 or Cry2Ab2 pure protein was used. COGEM is of the opinion that the applicant did not sufficiently demonstrate that the Cry1A.105 and Cry2Ab2 proteins do not interact. In addition, five out of nine of the studied NTOs (*C. maculata*, *H. convergens, O. insidiosus, I. promissorius* and *C. virginianus*), do not occur in the European Union and the applicant did not explain why these organisms are relevant to European maize fields. Although the applicant did not show that Cry2Ab2.820 is biologically identical to Cry2Ab2 some of the experiments have been carried out with Cry2Ab2.820. In addition, the laboratory experiments also exhibit some other shortcomings: the statistical power of the experiments and the obtained P value are not given, the choice for a certain statistical test has not been explained and the applicant did not provide an explanation for the high mortality (over 15%) in certain control groups.

The applicant presented three studies that describe MON89034xNK603 field trials. However, none of these field trials studied the effect of MON89034xNK603 on NTOs in Europe. One study referred to field trials that investigated the effect of MON89034xNK603 on NTOs. However, the NTOs that are present in Europe differ from the NTOs in Argentina. Therefore, the results of the Argentinean field trial are not sufficient to conclude that MON89034xNK603 does not adversely affect European NTOs. In addition, the NTOs that were studied in the Argentinean field trials did not include NTOs from all relevant ecological groups (i.e. predators, parasitoids, pollinators/nectar feeders, soil organisms and protected/endangered butterflies). COGEM

is of the opinion that the data provided are not sufficient to conclude that cultivation of MON89034xNK603 exerts negligible adverse effects on NTOs.
Furthermore, the General Surveillance plan could be improved on several points.

*Conclusion*
COGEM is of the opinion that she cannot adequately perform a risk analysis with regard to the cultivation of MON89034xNK603. As a result of the concerns mentioned, COGEM currently cannot issue a positive advice on the cultivation of maize line MON89034xNK603.

COGEM is of the opinion that additional data should be submitted from laboratory experiments and field studies that study the effect of MON89034xNK603 maize on NTOs, and which are relevant to European maize fields.

Preferably, the additional data on laboratory experiments should refer to experiments with maize line MON89034xNK603. If pure proteins were used in the laboratory experiments the applicant should demonstrate that the Cry1A.105 and Cry2Ab2 proteins do not interact or the two Cry proteins should have been used in combination. Furthermore, if pure proteins were used the Cry proteins that are present in maize MON89034xNK603 should have been studied; in case other proteins were used the applicant should demonstrate that the studied Cry protein (e.g. Cry2Ab2.820) is biologically identical to the Cry protein that is present *in planta* (e.g. Cry2Ab2).

The additional data on laboratory experiments should refer to studies with NTOs that are relevant to the crop ecosystem in Europe. If non-European organisms were used, the applicant should explain why these organisms are relevant to European maize fields. In addition, the additional data should refer to laboratory experiments with a statistical power of 0.8 or more, and the obtained P-values and an explanation for the statistical test should be presented.

Most importantly, additional data on field trials that were carried out in Europe with maize MON89034xNK603 should be provided. The additional data should refer to European field trials that study the effect of MON89034xNK603 on NTOs. In these field trials all relevant ecological groups (i.e. predators, parasitoids, pollinators/nectar feeders, soil organisms and protected/endangered butterflies) should be represented.

**Referenties**

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