COMMISSIE
COGEM
GENETISCHE
MODIFICATIE

BEZOEKADRES:
A. VAN LEEUWENHOEKLAAN 9
3721 MA BILTHOVEN

POSTADRES:
POSTBUS 578
3720 AN BILTHOVEN

TEL.: 030 274 2777
FAX: 030 274 4476
INFO@COGEM.NET
WWW.COGEM.NET

Aan de staatssecretaris van
Infrastructuur en Milieu
dhr J.J. Atsma
POSTBUS 30945
2500 GX Den Haag

DATUM 3 augustus 2011
KENMERK CGM/110803-01
ONDERWERP Advies 'additional information concerning the application for cultivation of MON89034xNK603 maize'

Geachte heer Atsma,

Naar aanleiding van een adviesvraag betreffende de vergunningaanvraag voor teelt, import en verwerking van de genetisch gemodificeerde maïslijn MON89034xNK603 van Monsanto Europe S.A. (EFSA/GMO/NL/2009/72), deelt de COGEM u het volgende mee:

Samenvatting
De COGEM heeft in 2009 geadviseerd over de vergunningaanvraag voor teelt, import en verwerking van de genetisch gemodificeerde maïslijn MON89034xNK603. Deze maïslijn brengt de *cp4 epsps*, *cp4 epsps L214P*, *cry1A.105* en *cry2Ab2* genen tot expressie en is hierdoor tolerant voor glyfosaat bevattende herbiciden en resistent tegen bepaalde insecten uit de orde van de Lepidoptera.
In haar eerdere advies concludeerde de COGEM dat er onvoldoende gegevens waren om eventuele effecten van de maïslijn op niet-doelwitorganismen te kunnen beoordelen. Op basis van het COGEM advies is de aanvrager om aanvullende informatie gevraagd. De aanvrager heeft meer gegevens aangeleverd over de bij de laboratoriumexperimenten gebruikte eiwitten en de statistische analyse van de laboratoriumexperimenten. Daarnaast heeft de aanvrager in Spanje een veldproef uitgevoerd naar het effect van MON89034xNK603 op niet-doelwitorganismen.
Hoewel de aangeleverde informatie een aantal van de vragen van de COGEM beantwoordt, zijn niet alle vragen voldoende beantwoord. Daarnaast geeft de aangeleverde informatie aanleiding tot nieuwe vragen.
De COGEM vindt beantwoording van de openstaande vragen noodzakelijk om het eventuele effect van de teelt van MON89034xNK603 op niet-doelwitorganismen te kunnen beoordelen. Ook is de COGEM nog in afwachting van een op grond van haar eerdere opmerkingen aangepast 'general surveillance' plan. Concluderend is de COGEM van mening dat er op dit moment onvoldoende informatie aanwezig is om tot een definitief oordeel te kunnen komen.

De door de COGEM gehanteerde overwegingen en het hieruit voortvloeiende advies treft u hierbij aan als bijlage.

Hoogachtend,

Prof. dr. ir. Bastiaan C.J. Zoeteman
Voorzitter COGEM

c.c.
Drs. H.P. de Wijs
Dr. I. van der Leij

Additional information concerning the application for cultivation of MON89034xNK603 maize

COGEM advice CGM/110803-01

This advice concerns the application for cultivation, import and processing of genetically modified MON89034xNK603 maize. This maize line expresses the cp4 epsps, cp4 epsps L214P, cry1A.105 *and* cry2Ab2 *genes conferring tolerance to glyphosate containing herbicides and resistance to certain lepidopteran insects.*

In its previous advice on this application, COGEM concluded that insufficient data were provided to allow conclusions on the effect of MON89034xNK603 on non-target organisms. COGEM was of the opinion that additional data from laboratory experiments and field trials had to be provided.

Based on COGEM's previous advice the applicant was asked for additional information. The applicant provided more information on the Cry1A.105 and Cry2Ab2 proteins that were used in the laboratory experiments and on the statistical analysis of the obtained data. In addition, a Spanish field trial that examined the effect of MON89034xNK603 on NTOs was provided. Although some of COGEM's questions are answered by the provided information, some new questions arose and other questions were not sufficiently answered.

COGEM is of the opinion that the applicant should justify the extrapolation of the results from the interaction study to non-target organisms, and should provide more information on the statistical analysis of the obtained data and the protein concentrations used in the laboratory study. In addition, the applicant should elaborate on the observed effect on the anthocorid bug Oirus insidiosus. *Furthermore, COGEM is of the opinion that the applicant should provide more information on the use of "maintenance" pesticides in the Spanish field trial. In addition, the effect of MON89034xNK603 on butterflies was not examined in laboratory experiments or the Spanish field trial. Instead the applicant estimated whether butterflies would be affected. COGEM strongly prefers the use of laboratory experiments to examine the presence of an effect of MON89034xNK603 on non-target lepidopteran species over theoretical exposure analysis as in the latter case flaws can be introduced by the use of assumptions, estimations and extrapolations. In addition, laboratory experiments allow the examination of sublethal effects on population growth.*

In conclusion, COGEM is of the opinion that the above mentioned questions need to be addressed to allow conclusions on the effect of cultivation of MON89034xNK603 on non-target organisms.

In addition, in response to COGEM's previous questions the applicant still has to provide a revised general surveillance plan.

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

Previous COGEM advice

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.

In December 2009, COGEM examined the application for cultivation of MON89034xNK603 maize and concluded that that the provided data were insufficient to allow a conclusion on the effect of MON89034xNK603 on NTOs.[9] COGEM was of the opinion that additional data from laboratory experiments and field trials had to be supplied to allow a reliable environmental risk analysis on cultivation of MON89034xNK603 maize.

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 pollinating insects visit maize plants and therefore 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. Seeds exhibit no 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.[7,8,9]

Properties of the introduced genes conferring insect resistance
Parental 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.[10]

Properties of the introduced genes conferring herbicide tolerance
Parental 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. MON89034x NK603 expresses CP4 EPSPS proteins, which are not inhibited by glyphosate[11] 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.

In its previous advice, COGEM concluded that additional data from laboratory experiments and field trials had to be supplied to allow a reliable environmental risk analysis on cultivation of MON89034xNK603 maize. In response to COGEM's questions the applicant has provided some additional information and clarifications. The provided information will be discussed below.

Laboratory and greenhouse studies
Test substances
In order to make an accurate risk assessment, COGEM prefers the use of plant material of the GM crop of concern i.e. MON89034xNK603, instead of the use of pure proteins. In its previous advice COGEM noted that none of the laboratory or greenhouse studies were carried out with plant material of MON89034xNK603. Instead the applicant provided studies on the effect of MON89034 on Collembola (*Folsomia candida*), soil microorganisms and northern bobwhite quail (*Colinus virginianus*) and studied the effect of the Cry1A.105 and Cry2Ab2 pure proteins on earthworms (*Eisenia fetida*), ladybird beetles (*Coleomegilla maculata* and *Hippodamia convergens*), the anthocorid bug (minute pirate bug: *Orius insidiosus*), honey bee *(Apis mellifera*) and parasitic wasp *(Ichneumon promissorius*). The two Cry proteins were administered separately.

Cry2Ab2 proteins used in laboratory studies
The Cry2Ab2.820 protein that was used for most of the laboratory studies appeared to be different from the Cry2Ab2 protein produced by MON89034xNK603. According to the applicant, the Cry2Ab2.820 protein contains three additional chloroplast transit peptide amino acids at the N-

terminus. Therefore, COGEM asked the applicant to clarify whether the Cry2Ab2.820 protein which was used in several of the laboratory experiments was biologically identical to the Cry2Ab2 protein produced by MON89034xNK603 (question 1.2).

The applicant compared the putative N-terminal sequences of the Cry2Ab2 proteins produced by MON89034 and the Cry2Ab2.820 protein produced by *Escherichia coli* in their response to questions of the Belgian competent authority regarding the application for cultivation of MON89034xMON88017.[12] Although the cleavage site in the chloroplast transit peptide could not be experimentally determined the comparison showed that the two proteins are probably identical.

In response to the questions of COGEM the applicant provided characterization studies of the *E. coli* produced Cry2Ab2.820 protein and the Cry2Ab2 protein produced by MON89034.[13] The physicochemical and functional properties of the two proteins were compared. No differences in biochemical properties were identified. In addition, an insect bioassay with *Helicoverpa zea* (target organism) was performed. COGEM is of the opinion that the results from this study demonstrate that the biological activity of the plant produced and the *E. coli* produced Cry2Ab2 proteins is equivalent.

The Cry2Ab2 protein which was used in the studies with honey bees and earthworms was not produced by *E. coli*, but by *Bacillus thuringiensis.* The comparison between the putative N-terminal sequences of the Cry2Ab2 proteins produced by MON89034, *E. coli* and *B. thuringiensis,* which is present in the response of the applicant on questions of the Belgian competent authority regarding the application for cultivation of MON89034xMON88017, showed that the Cry2Ab2 protein that is produced by *B. thuringiensis* is different from the Cry2Ab2 protein produced by MON89034.

The applicant provided a study on the functional equivalence of *B. thuringiensis* and *E. coli* produced Cry2Ab2 protein. A diet-incorporation insect bioassay was carried out with a target organism (the corn earworm *H. zea*).[14] In COGEM's view the results from this study demonstrate that the biological activity of the *B. thuringiensis* and the *E. coli* produced Cry2Ab2 proteins is equivalent.

Based on the results discussed above, COGEM is of the opinion that the applicant has sufficiently demonstrated that the biological activities of the Cry2Ab2 proteins produced by *E. coli* and *B. thuringiensis* that were used in the laboratory studies are equivalent to the Cry2Ab2 protein produced by MON89034.

In the laboratory experiments that examined the effect of pure proteins on non-target organisms (NTOs) the pure proteins were not administered in combination, but the Cry1A.105 and the Cry2Ab2 proteins were administered separately. COGEM is of the opinion that laboratory experiments should be carried out with the two pure proteins in combination, when the absence of interaction is not sufficiently demonstrated. In the original application a study was provided that examined the interaction between the Cry1A.105 and the Cry2Ab2.820 proteins.[15] As mentioned previously, COGEM asked the applicant to clarify whether the Cry2Ab2.820 protein which was

used in several of the laboratory experiments was biologically identical to the Cry2Ab2 protein produced by MON89034xNK603 (question 1.2). In addition, COGEM pointed out that the study was carried out with target organisms (the European corn borer *Ostrinia nubilalis* and the corn earworm *H. zea*). Apparently, the applicant assumes that the results from these two target organisms can be extrapolated to other organisms, i.e. NTOs, but an explanation for this assumption is lacking. Because of these two remarks COGEM concluded that the applicant did not sufficiently demonstrate that the Cry1A.105 and Cry2Ab2 proteins do not interact. Therefore, the applicant was asked to provide sufficient information to allow a conclusion on the absence of an interaction between the two proteins to be drawn (question 1.1).

In view of the data provided, COGEM is of the opinion that the applicant sufficiently demonstrated that the biological activity of the Cry2Ab2.820 protein is equivalent to the Cry2Ab2 protein produced by MON89034. Therefore, COGEM considers this question sufficiently answered. In its response the applicant does, however, not explain why it is assumed that the results from these two target organisms can be extrapolated to other organisms, i.e. NTOs. Therefore, in COGEM's view this issue is not adequately addressed.

Non-target organisms

The applicant performed laboratory experiments or greenhouse studies with several NTOs, namely Collembola (*F. candida*), soil microorganisms, earthworm (*E. fetida*), ladybird beetle (*C. maculata* and *H. convergens),* anthocorid bug *(O. insidiosus),* honey bee *(A. mellifera),* parasitic wasp *(I. promissorius),* and northern bobwhite quail (*C. virginianus*). The applicant stated that the test organisms are viewed as surrogate species and represent groups of insects and soil organisms commonly observed in European maize fields.

Five of these NTOs, i.e. *C. maculata*, *H. convergens, O. insidiosus, I. promissorius* and *C. virginianus* do not occur in the European Union. In addition, the applicant did not perform field trials to study possible effects of MON89034xNK603 on NTOs in Europe. COGEM is of the opinion that to assess the effect of a GM crop, NTOs that are relevant to the crop's ecosystem in Europe should be used. Therefore, COGEM asked the applicant to explain the relevance of these non-European NTOs to European maize fields (question 2).

In the response of the applicant to our questions, the previously provided explanation for the selection of these organisms was repeated. The article of Knecht *et al.* (2010)[16] was used as a reference for the occurrence of Ichneumonid species and *O. insidiosus* in the EU. COGEM points out that the article itself does not provide any information on the occurrence of these species in the EU and the database that is mentioned in the article is not accessible to COGEM. However, COGEM notes that the applicant provided results from a Spanish field trial in which the effect of MON89034xNK603 on European NTOs is studied. Therefore, COGEM is of the opinion that European NTOs are sufficiently represented in the risk assessment. However, since butterflies were not observed in the field trial a number of questions on the effect of MON89034xNK603 on non-target lepidopteran species remain. These will be discussed later in this advice.

Data analysis

In its previous advice, COGEM noted that most laboratory experiments were carried out with 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. As information on the statistical power of the experiments was not provided in the original application it was unclear if they were sensitive enough to allow detection of an effect if this effect would be present. Therefore, COGEM asked the applicant to provide information on the statistical power of the experiments (question 3).

In response to our questions the applicant performed a retrospective power analysis at a statistical power of 80% and determined the minimal detectable differences between test and control groups. COGEM considers the provided information sufficient.

In addition, COGEM is of the opinion that the P value which was obtained in the laboratory experiments should be reported. In the original application for most laboratory experiments the P value had not been given. COGEM asked the applicant to report the obtained P values (question 3). In response to our questions the applicant re-did the statistical analysis with comparable statistical tests and reported the obtained P value. COGEM considers this issue sufficiently answered.

COGEM noted that for the laboratory experiments different statistical tests were used without an explanation for the chosen method. Therefore, COGEM asked the applicant to clarify why a certain statistical test was chosen (question 3).

In the response of the applicant to COGEM's questions the applicant explained that if test guidelines were available the recommended statistical procedures were followed, but no further explanation on the selected statistical method was provided.

For the assessment of nominal data (e.g. survival of larvae) COGEM prefers the use of tests such as Chi-square or G-tests on the raw data. The data from the laboratory experiments are often analyzed with more complex statistical methods, but detailed information on the statistical analysis is lacking: it is e.g. unclear whether the assumptions of the selected method are met. For instance, in the laboratory study on the effect of Cry2Ab2 on honey bee larvae[17] the only information that is provided is the sentence stating that the test substance treatments were compared to the control substance treatment using Dunnett's test. Because no further information is provided, it is unclear whether all assumptions of the Dunnett's test are met.

COGEM is of the opinion that the applicant should provide insight in the statistical methodology, including the steps which were taken to ensure that the data matches the assumptions of the selected statistical method.

In conclusion, COGEM is of the opinion that with regard to the statistical analysis of the laboratory experiments some clarification is still needed.

Protein concentrations

According to the reports of the laboratory studies the studies were carried out with protein concentrations that ensured "a safety factor of at least 10x" (maximum dose). The applicant does, however, not explain how the maximum doses were established. To examine the effect of Cry1A.105 on *C. maculata, O. insidiosus* or *I. promissorius* the applicant used 240 µg Cry1A.105/g diet and 100-120 µg Cry2Ab2/g diet (maximum dose). These organisms eat maize pollen, but as they are parasitoids or predators they may accumulate Cry proteins. In addition, some of these organisms occasionally feed on plant tissue (i.e. *O. insidiosus).* Duan *et al.* (2008) exposed *O. insidiosus* to a maximum hazard exposure dose which was 10 times greater than the expected environmental concentration of Cry protein in maize plant tissue.[18] The concentration of Cry proteins in corn plant tissue was used as a conservative estimate of the potential environmental exposure concentration as data on the concentration of Cry proteins in their prey was lacking.

The concentration of Cry1A.105 and Cry2Ab2 in over season leaf (a combination of leaf samples collected at four different growth stages) is on average approximately 30 µg/g fresh weight (according to the Argentinean (2004/2005) and European (2007) field trials). In order to ensure a safety factor of 10 times the maximum environmental exposure dose the above mentioned organisms should be exposed to higher doses of Cry1A.105 and Cry2Ab2 proteins than those used in the presented laboratory experiments.

In conclusion, COGEM is of the opinion that the applicant should clarify for the different laboratory experiments how the maximum doses were established and should explain how the tested maximum dose relates to the maximum environmental exposure dose, especially, but not limited to, the above mentioned organisms.

Laboratory study exposing Orius insidiosus to Cry1A.105

In the study on the effect of Cry1A.105 on *O. insidiosus* a significant effect on mortality was observed with the 240 µg Cry1A.105/g diet. The survival of *Orius insidiosus* was 47% when exposed to the Cry1A.105 protein, but 88% in the control groups. Therefore, the applicant also performed dose-response studies (three replicates). The mean survival rate was 55% when exposed to 240 µg Cry1A.105/g diet and 89% in the control group.

No significant differences in survival were detected between concentrations of ≤ 120 µg Cry1A.105 /g diet and the control group (doses tested 240, 120, 60 and 30 µg Cry1A.105 /g diet). No significant effect on the percent of nymphs developing to adults was detected for all concentrations tested. The applicant does not elaborate on the cause for the observed effect at 240 µg Cry1A.105 /g diet and does not clarify whether the observed effect is considered to be biologically significant during cultivation of MON89034xNK603. In COGEM's view the applicant should explain whether the observed effect is considered a reason of concern.

Mortality in control groups

In the laboratory experiments that used adult honey bees (*A. mellifera*) or the ladybird beetle *H. convergens* mortality in the control groups exceeded 15%. In the original application no

explanation was provided for the high mortality in the control groups. COGEM points out that a high mortality in control groups could indicate problems with the experimental setup[19] and could mask an effect that is present. Preferably, mortality in control groups should not exceed 15%. COGEM asked the applicant to clarify the observed mortality in the control groups (question 4).

The applicant provided information on the minimal detectable differences between test and control groups. This information shows that the honey bee control mortality did not interfere with the possibility of the study to detect an effect if this would be present. In addition, the applicant clarifies the observed mortality in the adult honey bee control groups.[20,21] Furthermore, the applicant refers to OECD guidelines for testing of chemicals where the maximum duration of acute oral toxicity tests with adult honey bees is 96 hours.[22] The laboratory studies with honey bees were terminated at day 18 when the control mortality exceeded 20% which is longer than the maximum duration of laboratory studies with adult honey bees according to the OECD guidelines.

The applicant also informed COGEM that the study with the ladybird beetle *H. convergens* was superseded by other studies that examined the effect of Cry1A.105 and Cry2b2 proteins on the ladybird beetle *C. maculata.*

COGEM considers its question on the mortality of the control groups to be sufficiently addressed.

Field studies

In the original application the applicant provided a number of field trial studies. 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, but in two of them only the effect of MON89034xNK603 on target organisms was studied. 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. Based on this field trial the applicant concluded that MON89034xNK603 does not have an environmental impact different from conventional maize.

In its previous advice COGEM pointed out that European NTOs differ from the NTOs present in Argentina. In addition, in the Argentinean field trials not all ecological groups that are considered relevant by COGEM (predators, parasitoids, pollinators/nectar feeders, soil organisms and protected/ endangered butterflies) were represented. Based on the above mentioned considerations, COGEM concluded that the results of the field trials were not sufficient to conclude that MON89034xNK603 does not adversely affect European NTOs.

European field trial studying non-target organisms

COGEM is pleased that the applicant carried out a European field trial in response to COGEM's questions. This field trial was carried out in Spain (one site) during two years and examined the effect of MON89034xNK603 on several NTOs using pitfall traps, sticky traps and visual counts. [23] Predators, parasitoids, detrivores and herbivores were represented in the taxa collected. The applicant concluded that no biologically meaningful differences between MON89034xNK603 and

the conventional control hybrid were observed in the Spanish field trial. COGEM agrees with this conclusion. However, COGEM notices that during the study 'maintenance pesticides' were applied as needed at the sites. No information is provided on the type and amount of pesticides used. If used wrongly, pesticides could obscure effects on NTOs. Therefore, COGEM is of the opinion that information on the type and amount of pesticides and the time of application should be provided.

Pollinators/nectar feeders and butterflies
In the Spanish field trial pollinators/nectar feeders and protected/endangered butterflies, were not among the observed species. The applicant states that they are not sufficiently present in the field and not representative of a commercial maize field.

Pollinator/nectar feeders were not observed in the field trial, but the effect of the Cry1A.105 and Cry2Ab2 proteins which are produced by MON89034xNK603 on honey bees was examined in laboratory experiments. Based on the results from these experiments, COGEM is of the opinion that there is no indication that the Cry1A.105 or the Cry2Ab2 proteins would have an adverse effect on honey bees when administered separately. However, in MON89034xNK603 both proteins are present. As previously mentioned, COGEM still has some questions regarding the study that examined the presence of an interaction between the two proteins (question 1.1). Therefore, COGEM will await the response of the applicant on this issue before finalizing its opinion on the effect of MON89034xNK603 on honey bees.

Butterflies were not observed in the field trial and no laboratory experiments were carried out to examine the effect of MON89034xNK603 on non-target lepidopteran species.

Instead the applicant provided a list with lepidopteran species that occur in European maize habitats. The majority of these species do not feed on the maize plants themselves, but may be indirectly exposed to the Cry1A.105 and Cry2Ab2 proteins when pollen is deposited on the leaves of neighbouring host plants. In the technical dossier the applicant determines the possible exposure of non-target lepidopteran species to the Cry proteins in MON89034xNK603 pollen and estimates whether these species will be affected by cultivation of MON89034xNK603 using the LC50 of the European corn borer. COGEM strongly prefers the use of laboratory experiments to examine the effect of MON89034xNK603 on non-target lepidopteran species over a theoretical exposure analysis as in the latter case flaws can be introduced by the use of assumptions, estimations and extrapolations. In addition, the presence of sublethal effects on population growth can be examined with laboratory experiments.

COGEM points out that the list of lepidopteran species in European maize habitats that is provided by the applicant is based on a database[16] that is not accessible to COGEM. COGEM notices that the provided list only contains a subset of the species that could be affected by Bt maize pollen deposition according to Schmitz *et al.* (2003).[24] The differences between the two lists should be clarified.

In addition, the applicant focuses on three endangered lepidopteran species that inhabit cultivated areas or field margins. COGEM points out that in the description of the host range of these three species the applicant mentions genera, but refers to these genera as if they are single species. As a consequence it is unclear to which species the applicant exactly refers, which leads to unclear or possibly incorrect information. E.g. from the sentence '*Silene* is not a very common species' it is unclear which *Silene* species is meant. If one would assume that all *Silene* species are meant, the information would be incorrect, because some *Silene* species are common in Europe. COGEM is of the opinion that the information provided should be revised with regard to the above mentioned aspects.

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 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.

In the response of the applicant on our questions the applicant states that the MON89034xNK603 questionnaire will be updated when the EFSA opinion on the harmonized Farmer Questionnaire is published. Therefore, at present the applicant considers it premature to revise their questionnaire. The applicant points out that some of the above mentioned aspects were included in the harmonized questionnaire. COGEM lacks confidence that all its remarks will be included in the applicant's revised questionnaire. From the response of the applicant it is for instance unclear whether effects on the farmer's premises but outside the cultivated area (e.g. storage areas) will be detected. In addition, several of the aspects that were mentioned by COGEM are not mentioned in the response of the applicant at all. Therefore, COGEM will await the revised questionnaire to determine whether all its points are included.

Advice

This 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.

In its previous advice on this application, COGEM concluded that the provided data was not sufficient to conclude that cultivation of MON89034xNK603 would have negligible adverse effects on NTOs. In addition, COGEM was of the opinion that the General Surveillance plan could be improved on several points.

Based on COGEM's previous advice, the applicant was asked to provide additional information. The applicant provided information that demonstrated that the Cry proteins that were used in the laboratory experiments are biologically equivalent to the Cry proteins produced by MON89034xNK603. The absence of interaction between the Cry1A.105 and Cry2Ab2 proteins is, however, still not sufficiently demonstrated by the applicant. COGEM is of the opinion that the applicant should justify why the results obtained with target organisms can be extrapolated to NTOs.

Some of the laboratory experiments were carried out with non-European NTOs, but the applicant presented a Spanish field trial in which the effect of MON89034xNK603 on European NTOs is studied. Therefore, COGEM is of the opinion that European NTOs are sufficiently represented in the risk assessment.

The applicant provided sufficient justification for the >15% mortality in the control groups of the laboratory studies with adult honey bees and the ladybird beetle *H. convergens*.

In its previous advice, COGEM stated that further information on the statistical analysis should be provided. Although some of COGEM's questions are answered, it remains unclear whether the criteria (i.e. assumptions) for the statistical procedures which were used were met. COGEM is of the opinion that for all laboratory experiments the applicant should describe exactly which steps were taken in the statistical analysis of the data, including the steps which were taken to ensure that the data meets the requirements of the statistical method selected.

COGEM noted that survival of the anthocorid bug *Orius insidiosus* was affected when they were exposed to 240 µg Cry1A.105 /g diet, but not when exposed to 120 µg Cry1A.105 /g diet. The applicant did not discuss these results. COGEM is of the opinion that the applicant should discuss the significance of the observed effect in detail.

In addition, according to the applicant the Cry1A.105 and Cry2Ab2 protein concentrations that were used in the laboratory studies ensure a safety factor of at least 10x (maximum dose). It is, however, unclear how the maximum doses were established. In COGEM's view, the applicant should clarify how the maximum doses for the different NTOs were established and should explain how they relate to the maximum environmental exposure dose.

The applicant provided results from a Spanish field trial on the effect of MON89034xNK603 on NTOs. Predators, parasitoids, detrivores and herbivores were represented in the taxa collected. According to the applicant "maintenance" pesticides were applied. COGEM is of the opinion that information on the type and amount of pesticides is necessary to assess the results of the field trial.

Pollinators/nectar feeders and protected/endangered butterflies were not observed in the Spanish field trial. The effect of the Cry1A.105 and Cry2Ab2 proteins produced by MON89034xNK603 on honey bees was examined in laboratory experiments. In contrast laboratory experiments to examine the effect of MON 89034xNK603 on non-target lepidopteran species were not carried out. COGEM strongly prefers the use of laboratory experiments to examine the effect of MON89034xNK603 on non-target lepidopteran species over theoretical exposure analysis as in the latter case flaws can be introduced by the use of assumptions, estimations and extrapolations. In addition, laboratory experiments allow the examination of sublethal effects on population growth.

COGEM is of the opinion that the above mentioned questions need to be addressed to allow conclusions on the effect of cultivation of MON89034xNK603 NTOs. In addition, COGEM awaits a revised general surveillance plan to determine whether all points that were raised in its previous advice are included.

References

1. COGEM (2009). Additional advice on the import and processing of MON89034 x NK603. Advies CGM/091020-01

2. COGEM (2006). Teelt van maïslijn NK603. Advies CGM/060704-01

3. Hin CJA (2001). Rapport Landbouwkundige risico's van uitkruising van GGO-gewassen Centrum voor Landbouw en Milieu (CLM)

4. Treau R & Emberlin J (2000). Pollen dispersal in the crops Maize (*Zea mays*), Oil seed rape (*Brassica napus* ssp. *Oleifera*), Potatoes (*Solanum tuberosum*), Sugar beet (*Beta vulgaris* ssp. *vulgaris*) and Wheat (*Triticum aestivum*)- Evidence from publications. Soil Association (=leading organization for organic certification UK)

5. Coe EHJR, Neuffer MG & Hoisington DA (1988). The genetics of Corn. pp. 81-258. In: Sprangue GF, Dudley JW, Editors. Corn and Corn Improvement, Third Edition. American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, Madison, Wisconsin. 986 pp

6. Luna VS, Figueroa MJ, Baltazar MB *et al.* (2001). Maize pollen longevity and distance isolation requirements for effective pollen control. Crop Science 41: 1551-1557

7. COGEM (2009). Molecular characterization of maize MON89034. Advies CGM/090126-01

8. COGEM (2003). Markttoelating 'NK603 maize tolerant to glyphosate'. Advies CGM/030319-08

9. COGEM (2009).Cultivation of maize line MON89034xNK603. Advies CGM/091208-01

10 .Broderick NA, Raffa KF & Handelsman J (2006). Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. Proceedings of the National Academy of Science 103: 15196-15199

11. Funke T, Han H, Healy-Fried ML *et al.* (2006). Molecular basis for the herbicide resistance of Roundup Ready crops. Proceedings of the National Academy of Sciences of the United States of America: 103: 13010-13015

12. Monsanto (2010). Responses to Belgian CA/EFSA questions-application EFSA-GMO-BE-2009-71 30th July 2010

13. Karunanandaa K, Thorp JJ, Goley ME *et al.* (2006). Characterization of the Cry2Ab2 protein purified from the corn grain of MON89034 and comparison of the physicochemical and functional properties of plant produced and *E. coli*-produced Cry2Ab2 proteins. Monsanto Technical Report MSL 20071

14. Levine SL & Uffman J (2006). Evaluation of the functional equivalence of the Cry2Ab2 protein produced in *E. coli* and *Bt* against a sensitive lepidopteran species. Monsanto Technical Report MSL 20132, 1-23

15. MacRae T, Brown C & Levine S (2005). Evaluation of the potential for interactions between the Bacilllus thuringiensis proteins Cry1A.105 and Cry2Ab2. Monsanto Technical Report MSL 19859

16. Knecht S, Romeis J, Malone L *et al.* (2010).A faunistic database as a tool for identification and selection of potential non-target arthropod species for regulatory risk assessment of GM maize. GMOs in Integrated Production. IOBC/wprs Bulletin 52: 65-69

17. Maggi V (2000c). Evaluation of the dietary effect(s) of purified *Bacillus thuringiensis* Cry2Ab2 protein on honey bee larvae. Monsanto Technical Report MSL16961

18. Duan JJ, Teixera D, Huesing J *et al.* (2008). Assessing the risk to nontarget organisms from *Bt* corn resistant to corn rootworms (Coleoptera: Chrysomelidae): Tier-1 testing with *Orius insidiosus* (Heteroptera: Anthocoridae). Environmental Entomology 37: 838-844
19. COGEM (2008). Designing experimental protocols to investigate the impact of GM crops on non-target arthropods. Onderzoeksrapport CGM 2008-01
20. Maggi V (2000b). Evaluation of the dietary effect(s) on insect protection protein 2 on adult honeybees (*Apis mellifera* L.). Monsanto Technical Report MSL16176
21. Richards K (2006b). Evaluation of the dietary effects of a Cry1A.105 protein on adult honeybees (*Apis mellifera* L.) Monsanto Technical Report CA-2005-072
22. OECD (1998) Guideline for the testing of chemicals No. 213: Honeybees, Acute Oral Toxicity Test. OECD, Paris
23. Brown CR, Ahmad A, Kendrick DL *et al.* (2011). Evaluation of non-target arthropods from lepidopteran-protected and glyphosate-tolerant maize MON89034xNK603 and the single components MON89034 and NK603 in Spanish field trials during 2008 and 2009. Monsanto Technical Report MSL0023330
24. Schmitz G, Bartsch D & Pretscher P (2003). Selection of relevant non-target herbivores for monitoring the environmental effects of *Bt* maize pollen. Environ. Biosafety Res. 2: 117-132