Application for cultivation of MON89034 maize

COGEM advisory report CGM/120710-02

The present application concerns the cultivation of the genetically modified maize line MON89034. This maize line expresses the genes cry1A.105 *and* cry2Ab2 *conferring resistance to certain lepidopteran insects. In the past, COGEM advised positively on the import of maize line MON89034. Two applications for cultivation of the stacked lines MON89034xNK603 and MON89034xMON88017 were assessed by COGEM. COGEM concluded for both stacked lines that the data on the effects of non-target organisms (NTOs) were insufficient to draw conclusions.*

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 maize to establish feral populations. COGEM is of the opinion that the molecular characterisation is adequate.

The applicant conducted several laboratory and field studies and refers to these studies in order to demonstrate the absence of potential adverse effects of MON89034 on NTOs. COGEM is of the opinion that the applicant did not sufficiently demonstrate that interaction between the Cry1A.105 and Cry2Ab2 proteins is absent in MON89034, since no studies were done on synergistic effects in non-target organisms. Therefore, a study in which honey bees were exposed to the Cry1A.105 and Cry2Ab2 proteins separately does not suffice to conclude that MON89034 cultivation will not affect honey bees. In a study on the NTO Orius insidiosus*, the potential exposure of this anthocorid bug to Cry1A.105 via prey and plant material other than pollen is not taken into account. In the field trials, differences in Coccinellidae numbers between years and differences in numbers between collection methods were not addressed by the applicant. In addition, the theoretical exposure analysis for non-target lepidopteran species did not take into account that the LC50 value in the field may vary up to a factor of ten.*

COGEM is therefore of the opinion that the data provided are not sufficient to conclude that cultivation of MON89034 exerts negligible adverse effects on NTOs. Furthermore, the General Surveillance plan should be improved on several points. As a result of these concerns, COGEM cannot issue a positive advice on the cultivation of maize line MON89034.

Introduction

The scope of the present notification (EFSA/GMO/BE/2011/90) by Monsanto Company, as represented by Monsanto Europe S.A., concerns the cultivation of maize line MON89034. MON89034 expresses the *cry1A.105* and *cry2Ab2* genes, which confer resistance to certain lepidopteran pests. COGEM was asked to evaluate the environmental risks of commercial cultivation of this maize line.

Previous COGEM advice

COGEM advised negatively on the import of MON89034 maize in 2007.[1] In 2009, COGEM considered additional information provided by the applicant and issued a positive opinion on import of MON89034 maize, concluding that import of MON89034 poses a negligible risk to the environment.[2]

COGEM has previously examined two applications for cultivation of stacked maize lines with MON89034 as one of its parents; MON89034xMON88017 and MON89034xNK603. COGEM concluded that provided data on effects on non-target organisms (NTOs) for both applications were insufficient to draw conclusions.[3,4]

For the application for cultivation of MON89034xNK603, additional information was provided by the applicant twice. Amongst other things, the results of a Spanish field trial that assessed the effects of MON89034xNK603, NK603 and MON89034 on NTOs was added to the dossier.[5,6] Since several questions have remained unanswered, COGEM has not finalised the risk assessment of cultivation of MON89034xNK603 yet.

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.[7,8] According to literature, the time over which pollen remains viable varies from 30 minutes to 9 days.[8,9,10] In Europe, no wild relatives of maize are present and, therefore, hybridisation 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.[7] 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 characterisation

The molecular characterisation of maize MON89034 was previously evaluated by COGEM.[2] COGEM concluded that the molecular characterisation of MON89034 is adequate. The dossier for MON89034 cultivation included an updated bioinformatics analysis of the inserted sequences and putative polypeptides spanning the genomic DNA-insert junctions, which did not reveal any new concerns. An overview of the construction of MON89034, the inserted genetic elements as well as the properties of the introduced genes is given below.

The genetically modified maize line MON89034 was by produced by *Rhizobium radiobacter* (formerly known as *Agrobacterium tumefaciens*)-mediated transformation using *R. radiobacter* 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 consists of:

- Right border region derived from the Ti-plasmid of *R. radiobacter* 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 Cry1A.105, a modified Cry1A protein from *Bacillus thuringiensis*. It consists of domains I and II from Cry1Ab/Cry1Ac, domain III from Cry1F and the C-terminal domain of Cry1Ac. The codon usage of *cry1A.105* has been optimised 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 optimised for expression in monocots;
- *nos* terminator sequence from the nopaline synthase gene of *R. radiobacter,* which ends transcription and directs polyadenylation;
- Left border region derived from the Ti-plasmid of *R. radiobacter* 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 that target insects of the order Lepidoptera. The δ-endotoxins are solubilised 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.[11]

Environmental risk analysis

In the opinion of COGEM, there is no reason to assume that the traits introduced in maize MON89034 will increase the potential of maize to establish feral populations. With regard to the potential adverse effects of MON89034 on non-target organisms (NTOs), the applicant refers to laboratory, greenhouse, field studies and a theoretical exposure analysis.

Most of the studies present in the dossier of MON89034 were evaluated previously by COGEM during the assessment of the application for cultivation of MON89034xNK603.[3,5,6] Compared to the MON89034xNK603 dossier, a laboratory study with *Daphnia magna* was added to the MON89034 dossier, as well as results of agronomic field trials conducted in Spain and Germany in the spring of 2007. The Spanish field trial studying effects on non-target organisms (NTOs), which COGEM assessed as part of the MON89034xNK603 dossier, was not included. Since the results of this field trial are relevant to the current application, COGEM has considered the study in its risk assessment.

Validity of administrating the Cry proteins separately to NTOs in laboratory studies

The applicant provided laboratory studies on the effect of MON89034 plant material on Collembola (*Folsomia candida*), soil microorganisms and northern bobwhite quail (*Colinus virginianus*). Purified Cry1A.105 and Cry2Ab2 proteins produced in *Escherichia coli* were used in studies on ladybird beetles (*Coleomegilla maculata* and *Hippodamia convergens*), an anthocorid bug (minute pirate bug: *Orius insidiosus*), and a parasitic wasp *(Ichneumon promissorius*). For studies on earthworms (*Eisenia fetida*) and honey bee *(Apis mellifera*), purified Cry1A.105 expressed in *E. coli* was used, as well as purified Cry2Ab2 proteins produced in *B. thuringiensis*. Most of the other laboratory studies made use of the Cry2Ab2.820 protein produced in *E. coli*. The applicant has previously shown for the assessment of MON89034xNK603 that the activity of the various purified Cry2Ab2 proteins used in the laboratory studies is biologically equivalent to the Cry2Ab2 protein produced by MON89034.

In case absence of interaction between proteins is not sufficiently demonstrated, COGEM is of the opinion that laboratory experiments have to be carried out with the proteins in combination, in order to make an accurate risk assessment.

The applicant provided a study that examined the interaction between the Cry1A.105 and the Cry2Ab2.820 proteins.[12] The interaction study was carried out with target organisms (the European corn borer *Ostrinia nubilalis* and the corn earworm *Helicoverpa zea*), and showed that it is plausible that the combined effect of the Cry1A.105 and Cry2Ab2.820 proteins consists of dose-additive activity on these target organisms.

As mentioned in its previous opinions on cultivation of MON89034xNK603, COGEM questions whether the results from the interaction study using target organisms can be extrapolated to non-target organisms.[5,6] The applicant did not sufficiently substantiate its claim that extrapolation to NTOs in this case is justified, nor does he present data on the absence of synergism between the Cry1A.105 and Cry2Ab2 proteins using a non-target insect species.

Because combination toxicity mechanisms may vary between species, synergistic activity of the two proteins from MON89034 should be tested in at least one non-target insect species to conclude that synergistic effects are not present.

Laboratory study exposing Orius insidiosus *to Cry1A.105*

In the laboratory study on the effect of Cry1A.105 on the minute pirate bug (*O. insidiosus*) a significant effect on mortality was observed at a dose of 240 µg Cry1A.105 per gram diet. No significant differences on survival were detected with concentrations of ≤ 120 µg Cry1A.105 per gram diet. For the risk assessment of MON89034xNK603, the applicant explained the calculation of the dose of 240 µg Cry1A.105/g diet that was used in the laboratory study. This dose is based on the expression level of Cry1A.105 in MON89034 pollen.

According to the applicant, pollen is the major route by which *O. insidiosus* may be exposed to the Cry protein. The applicant calculated the 'maximum expected environmental concentration' (MEEC) to which *O. insidiosus* could be exposed and states that the 'no observed effect concentration' exceeds the MEEC fourteen times. COGEM points out that *O. insidiosus* feeds on insects as well as plant tissues such as pollen.[13,14] The expression of Cry proteins in MON89034 pollen is low when compared to the expression in other plant tissues, and Cry proteins may accumulate in prey species. As a result, the exposure of *O. insidiosus* under field conditions may be higher than would be expected based on the expression level of Cry1A.105 in MON89034 pollen.

Since the potential exposure of *O. insidiosus* to Cry1A.105 via other exposure routes such as additional plant tissues and prey is not taken into account, COGEM cannot conclude whether the observed effect on *O. insidiosus* in the laboratory will be of biological significance during cultivation of MON89034.

Field trials

In the application, two studies refer to field trials in which the effect of MON89034 on target organisms is studied, but none is included on the effects on non-target organisms. For the assessment of MON89034xNK603, the applicant included the results from a European field trial. This field trial was carried out in Spain (one site) for two years and examined the effect of MON89034xNK603, NK603 and MON89034 on several NTOs.[15] Field studies on potential effects on NTOs are vital for the environmental risk analysis of Bt crops. Since MON89034 was assessed in the above-mentioned field trial, COGEM takes this study into consideration for the risk assessment of MON89034.

In the Spanish field trial, NTOs were studied using pitfall traps, sticky traps and visual counts. Predators, parasitoids, detrivores and herbivores were present in the taxa collected. The applicant concludes that in the Spanish field trial no biologically meaningful differences were observed between MON89034 and the conventional control.

COGEM points out that the report on the Spanish field study appears to contain flaws and inconsistencies, in particular with regard to the data on *Coccinellidae*. The sticky trap data

show that lower numbers were present in MON89034 than in the conventional control. In contradiction to these data, the 'visual observations' indicate that higher numbers were present in MON89034. No explanation is given for these differences.

The differences in numbers of *Coccinellidae* between MON89034 and its control fluctuate markedly over the two trial years. The applicant does not address this variance for MON89034. It therefore remains unclear whether the observed difference between the two years has a biological significance. Therefore, COGEM cannot exclude the possibility of an effect of MON89034 on *Coccinellidae* in the field.

Pollinators/nectar feeders and butterflies

In addition to predators, parasitoids and herbivores which were collected in the Spanish field trial, COGEM also considers pollinators/nectar feeders and protected/endangered butterflies as relevant ecological groups. The latter two groups were not studied in the Spanish field trial. The applicant declares that they were not sufficiently present in the field and not representative of a commercial maize field.

The effect of the Cry1A.105 and Cry2Ab2 proteins produced by MON89034 was examined in laboratory experiments on honey bees. 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 have an adverse effect on honey bees when administered separately. However, in MON89034 both proteins are present.

As previously mentioned, COGEM has questions regarding the extrapolation of results from the study that examined the presence of an interaction between the two proteins in target organisms to non-target organisms. Therefore, COGEM cannot exclude the possibility that MON89034 will have an effect on honey bees.

Butterflies were not observed in the field trial and no laboratory experiments were carried out to examine the effect of MON89034 on non-target lepidopteran species. In the technical dossier a theoretical exposure analysis was performed to assess the exposure of non-target lepidopteran species to MON89034 pollen. In the assessment of MON89034xNK603, COGEM declared that it strongly prefers the use of experimental data to examine the effect of an event on non-target lepidopteran species over a theoretical exposure analysis. COGEM pointed out that in the latter case flaws can be introduced by the use of assumptions, estimations and extrapolations.

The theoretical exposure analysis is based on the LC50 values detected in laboratory bioassays with target organisms. COGEM notes that reported LC50 values vary widely, depending amongst others on the way of administration, design of the experiments, and origin and history of the insects tested. In some instances the reported LC50 values vary more than ten-fold.[16] In order to make a theoretical exposure assessment based upon the LC50 value of the target organism, it is a prerequisite that this LC50 is the lowest value reported to avoid underestimation of the possible effect. Since it is not clear if the LC50 used in the calculation

is representative for the lowest actual LC50, COGEM cannot exclude the possibility that non-target lepidopteran species will be affected by cultivation of MON89034.

Daphnia magna study

In the application for cultivation of MON89034, a study was included on the effects of MON89034 pollen on *Daphnia magna*, a freshwater cladoceran. COGEM is of the opinion that the experimental setup and analysis of the study on *Daphnia magna* using MON89034 pollen has serious shortcomings. In its present state, the study has no value for the environmental risk analysis.

In current literature, the environmental risk assessment of aquatic environments concerning the cultivation of GM crops is under discussion.[17] For example, caddisflies fed Bt maize resulted in increased mortality in a laboratory study.[18] However, the authors could not confirm this finding in waterways in the field.[19]

Two essential questions play a role. Firstly, are aquatic non-target organisms exposed to relevant concentrations of Bt toxins? The exposure to Bt toxins depends amongst other things on the deposition of pollen in waterways.[17] Secondly, the choice of an appropriate surrogate test species is a topic of debate. Can non-aquatic species act as indicator species for susceptible aquatic NTOs exposed to Bt proteins? *Dapnia magna* is the model system for ecotoxicological studies, but is not routinely used in the risk assessment of GM plants. Besides COGEM has doubts about the design and analysis of the study on *Daphnia magna*, as the applicant does not explain the choice of this organism as an aquatic test species above e.g. caddisfly larvae, which are more closely related to Lepidoptera than Crustaceae.

Conclusion

Taking into account the considerations described above, COGEM concludes that several issues in the studies on NTOs should be addressed before COGEM can exclude the possibility that NTOs will be significantly affected by cultivation of MON89034.

General surveillance

General surveillance (GS) 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 monitoring plan that was submitted for cultivation of MON89034 is a standard plan, which has been used for several applications. In 2010, COGEM published a report on the principles that COGEM considers should be followed for general surveillance.[20] Several of COGEMs principles were incorporated into the EFSA guidance on Post Market Environmental Monitoring (PMEM), which has recently been published. [21]

COGEM is of the opinion that the General Surveillance plan submitted for MON89034 cultivation should be improved. As mentioned in previous reports,[20,21,22] COGEM is of the opinion that:

- the authorisation holder must guarantee that a sufficient number of observations are collated;
- the number of distributed and completed questionnaires should be reported;
- the cultivation areas of the GM crop should be stated;
- the farm questionnaire should be expanded to include questions on changes in the persistence and invasiveness of the GM plants and on unanticipated effects in the farmyard;
- the questions on the presence of animals should be subdivided to obtain information on specific types of animals;
- comparator(s) should be defined for use in the farm questionnaire;
- all observations collated by the authorisation holder should be retrievable;
- the authorisation holder must ensure that third parties (farmers, monitoring networks, member states) will participate in the execution of the monitoring plan;
- the Member States should be directly informed of cases in which adverse effects linked with the cultivation of a GM crop require immediate measures to protect human health and the environment.

Conclusion

The present application concerns the cultivation of the genetically modified maize line MON89034. This maize line expresses the genes *cry1A.105* and *cry2Ab2* conferring resistance to certain lepidopteran insects. In the past, COGEM advised positively on the import of maize line MON89034. Two applications for cultivation of stacked lines MON89034xNK603 and MON89034xMON88017, with MON89034 as on of the parent lines, were assessed by COGEM. COGEM concluded for both stacked lines that the data on the effects on non-target organisms (NTOs) were insufficient to draw conclusions.

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 characterisation 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 MON89034 on non-target organisms (NTOs). COGEM is of the opinion that the applicant did not sufficiently demonstrate that interaction between the Cry1A.105 and Cry2Ab2 proteins is absent in MON89034, since no studies were done on synergistic effects in non-target organisms. Due to this uncertainty, the study in which honey bees were exposed to the Cry1A.105 and Cry2Ab2 proteins separately does not suffice to conclude that MON89034 cultivation will not affect honey bees. In the study on *Orius insidiosus*, the potential exposure of this anthocorid bug to Cry1A.105 via other exposure routes such as prey and plant tissues other than pollen is not taken into account. The *Daphnia magna* study using MON89034 pollen is of limited value to the environmental risk analysis. In the field trials, differences in *Coccinellidae* numbers between years and differences in numbers between collection methods were not addressed by the applicant. In

addition, the theoretical exposure analysis to assess the exposure of non-target lepidopteran species did not take into account that the LC50 value in the field may vary up to a factor of ten.

COGEM is therefore of the opinion that the data provided are not sufficient to conclude that cultivation of MON89034 exerts negligible adverse effects on NTOs. Furthermore, the General Surveillance plan should be improved on several points.

As a result of the concerns mentioned above, COGEM cannot issue a positive advice on the cultivation of maize line MON89034.

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