Mixture toxicity regarding the application for cultivation of MON89034xNK603 maize

COGEM advisory report CGM/130604-02

This advisory report 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 opinions concerning this application, COGEM concluded that the data on the combined effect of the two Cry proteins in MON89034xNK603 were insufficient to draw conclusions on non-target organisms (NTOs). COGEM was of the opinion that additional data from laboratory experiments had to be provided. The applicant recently provided additional information on the studies that assess the effect of MON89034xNK603 on NTOs. Although three out of four of COGEMs remaining questions are satisfactorily addressed in this information, one important question was not sufficiently addressed. COGEM questions the rationale of the applicant regarding mixture effects of a combination of Cry proteins. The provided information does not substantiate the claim that the data obtained from laboratory experiments on target organisms can be extrapolated to exposure to NTOs.
In conclusion, COGEM is of the opinion that interaction between Cry proteins in NTOs needs to be addressed in a laboratory experiment to allow conclusions on the effect of cultivation of MON89034xNK603 on NTOs.

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 opinions

In October 2009, COGEM issued a positive opinion 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 advised positively 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 the provided data were insufficient to draw conclusions on the effect of MON89034xNK603 on non-target organisms (NTOs).[3] In April 2011 and February 2012, the applicant provided additional information on the experiments that had been carried out.[4,5] In 2011, the results of a Spanish field trial that assessed the effect of MON89034xNK603 on NTOs were added to the dossier. COGEM studied the additional information provided on these occasions and concluded that not all its questions had been answered.

Recently, the applicant provided further information on the questions raised by COGEM. The Dutch Ministry of Infrastructure and the Environment asks COGEM whether the information answers its previous questions and lifts its objections. Of the four questions COGEM posed in her last opinion, questions two to four have been answered satisfactorily by the applicant. These questions concerned mortality of *Orius insidiosus*, data from the Spanish field trial, and a theoretical exposure analysis of non-target butterflies. COGEMs considerations regarding the first question will be discussed below.

Question 1. Assessment of the interaction between Cry proteins in NTOs

In the dossier, a study by McRae was provided that examined the interaction between the Cry1A.105 and the Cry2Ab2.820 proteins.[6] The biological activity of the latter protein was shown to be equivalent to the Cry2Ab2 protein which is produced by MON89034xNK603. 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 consisted of an additive activity on these target organisms. In laboratory experiments presented, the effect of the Cry proteins on NTOs was examined separately, assuming that there was no interaction effect between the Cry proteins.

COGEM previously stated that it is of the opinion that the laboratory experiments on NTOs should be carried out with the two pure proteins in combination, when the absence of interaction is not sufficiently demonstrated. The information provided by the applicant did not prove that the data from laboratory experiments, carried out with the separate Cry proteins, can be extrapolated to the situation where NTOs are exposed to Cry1A.105 and Cry2Ab2 proteins at the same time. A combination effect between the two Cry proteins could lead to an altered specificity towards non-target insects. COGEM concluded that the applicant should substantiate its claim that the results from interaction studies with target organisms can be extrapolated to NTOs or should provide data on type of interaction effect between the Cry1A.105 and Cry2Ab2 proteins using non-target insect species.

In its latest response, the applicant did not provide additional experimental results. The applicant states that there is no argumentation that warrants additional experiments in NTOs, when the proteins have been shown to act independently in a susceptible organism.

In the various responses to COGEM's questions on the interaction of Cry proteins, the applicant seems to confuse the toxicological definitions of 'concentration addition' and 'independent joint action'. The experiment by McRae on target organisms shows that the combined effect of the two Cry proteins in MON89034xNK603 results in concentration additivity. However, the applicant discusses

these results as if they are a case of independent action. This raises doubts regarding the argumentation of the applicant that additional experiments on NTOs are not needed to prove the absence of interaction between the Cry proteins.

In view of the nature of the answer of the applicant to question one, a short consideration is given below on testing of mixture toxicity effects of Bt proteins on NTOs.

Considerations regarding mixture toxicity of GM plants expressing multiple Bt toxins

When GM plants express more than one Cry protein, the question arises whether these proteins may cause any effects that are difficult to predict from the single proteins. Especially the various interaction effects (synergisms and antagonisms) deserve attention. Although a modeling framework for assessment of mixture toxicity is available, there is still often a great deal of confusion on terminology in the scientific literature and elsewhere.[7] Here, the principles that guide COGEM in the assessment of mixtures arising from multiple Bt toxins are indicated.

Concentration addition

The usual reference for assessment of mixture effects is concentration addition. Assume that for each chemical in the mixture some toxicological endpoint is available, e.g. LC50. Then there is concentration addition if the effect of the mixture can be predicted by the sum of the concentrations, each divided by its LC50. The ratio between concentration and LC50 is also known as *toxic unit*. Under the concentration-addition model there will be 50% mortality if the sum of toxic units of a mixture equals unity:

At 50% effect of the mixture: $$\sum_{i=1}^{n} \frac{c_i}{LC50_i} = 1$$

where *n* is the number of toxins in the mixture.

Please note that a mixture may cause an effect even if each of the compounds is below its own toxic threshold. For example, if a mixture contains ten compounds each present in 10% of their LC50, none of the compounds is likely to have an effect in isolation, but the mixture will have an effect of 50%.

Conclusion 1:

If Bt proteins follow the concentration-addition model, the toxicity of a mixture can be easily predicted from the single compounds if for each compound the toxicological endpoint (e.g. LC50) is known, as well as its level of expression in the GM plant.

Conclusion 2:
Under the concentration-addition model a Bt protein may contribute to the toxicity of a GM plant expressing more than one Bt protein, even if it is not present in a toxic concentration itself.

Simple similar action

Concentration additivity is expected when two toxins have the same mode of action, e.g. bind to the same receptor. In that case a certain amount of toxin A can be replaced without change of effect by toxin B. The only difference might be the rate at which they penetrate to this receptor, which is corrected for by the LC50. Examples are organosphosphates acting upon AChE, chlorobenzene congeners causing membrane disturbance, etc.

Synergism and antagonism

Synergism and antagonism can be seen as two different violations of the concentration-addition model. Two or more toxins show antagonism if the sum of toxic units necessary to cause 50% effect is greater than unity. Synergism is present if the sum of toxic units necessary to cause 50% effect is smaller than unity. There are also more complicated cases in which mixtures tend to be antagonistic in low doses, additive in intermediate doses and synergistic at high dose. For these more complicated cases special models have been derived that describe the complete dose-effect relationship.[7]

Antagonism and synergism are expected when toxins have different modes of action. For example, a strong synergism is seen when a fungicide binds to an enzyme degrading an insecticide. When these two toxins are combined the insecticide can become much more toxic than without the fungicide. Antagonistic effects are seen when two compounds influence each other's uptake. For example, zinc and cadmium are often antagonistic at low does because zinc blocks the uptake of cadmium.

Conclusion 3:
If Bt proteins have different modes of action there can be synergism or antagonism. The precise effect cannot be predicted, as there are many ways in which mixtures can deviate from concentration additivity.

Response addition or independent joint action

A completely different way to describe the joint action of toxins is the response addition model. Under this model the effect of a mixture can be predicted by multiplying the fractional effects:

$$E_{mix} = 1 - \prod_{i=1}^{n} (1 - E_i)$$

where E_i is the effect of toxin i (scaled between 0 and 1) and E_{mix} is the effect of the mixture.

Note that, although the model is referred to as “addition” the effects are actually multiplied, not added. E.g. if toxin A alone kills 60% of the animals, and toxin B alone kills 40% of the animals, the mixture kills 76% of the animals.

Response addition is expected if two toxins have completely different modes of action that cannot be compared to each other. The model is also denoted as *independent joint action*. The joint action is explained from the effects they cause rather than from their concentrations. The terms synergism and antagonism do not have a meaning in this model. Response addition can often also be described as synergistic or additive in terms of the concentration addition model. The two approaches do not exclude each other.

Conclusion 4:
If Bt proteins have different modes of action, their joint effects can be described as response addition. It is not possible to extrapolate between toxins, however, as each toxin has its own dose-effect relationship.

Deviations from response addition
Deviations from response addition are seen if the sensitivities of the targets are correlated, either positively or negatively. For example if toxin A alone kills 60% of the animals, and toxin B alone kills 40% but the animals insensitive to A are also insensitive to B the mixture may kill less than 76%. Similarly, if the sensitivities are anticorrelated the mixture may kill more than 76%. If the correlation is +1 the mixture of A and B kills 100%, if the correlation equals –1, the mixture kills no more than 60%. Pure response addition is seen if the correlation equals zero.

Conclusion 5:
If Bt proteins have different modes of action, their joint effects can deviate from response addition if the sensitivities are correlated among the targets. Such deviations are not due to antagonism or synergism since the toxins are assumed not to interact, so they cannot antagonize nor reinforce each other.

Extrapolation between species
Both the correlation structure of sensitivities and the properties of biochemical receptors may differ between species. Therefore the type of mixture effect might also differ between species, especially between pest species (that for obvious reasons are especially sensitive to the mixture) and non-target organisms (that are generally less sensitive but might show unexpected interaction effects due to a combination of biochemical targets not present in the pest).

Conclusion 6:
Mixture effects are a function of toxin properties as well as properties of the biological receptor. Therefore, mixture effects may not extrapolated simply between species.

Overall conclusion:
The model of concentration additions seems to be the best starting point for judging the effects of plants expressing more than one Bt toxin.

Request regarding the assessment of interaction of Cry proteins in NTOs

In view of the considerations above, COGEM points out that effects of toxin mixtures may differ between species, due to properties of the toxin and of the biological receptor *in vivo*. Therefore, results of tests of the interaction of Cry proteins on sensitive organisms may not be extrapolated to NTOs. COGEM requests the applicant to investigate if the Cry proteins in MON89034xNK603 have a concentration-addition effect in NTOs by performing an experiment on NTOs, similar in set-up to the experiment of McRae. A ladybird beetle would be an appropriate NTO for this test.

Conclusions

COGEM is of the opinion that the applicant does not sufficiently address the issue of the extrapolation of the results from the assessment of an interaction between the two Cry proteins in MON89034xNK603 to NTOs in its latest response. According to COGEM, this issue is of paramount importance for the risk assessment of MON89034xNK603 maize and may concern all Bt crops expressing multiple Cry proteins.

Questions two to four are sufficiently addressed by the applicant in its latest response. COGEM is willing to fulfil the request of the applicant to postpone the assessment of the General Surveillance plan until a revised General Surveillance plan is supplied by the applicant. A General Surveillance plan is mandatory for any market application of a GM plant. COGEM notes that cultivation of MON89034xNK603 maize may only be authorised after the approval of the GS plan.

References

1. COGEM (2009). Additional advice on the import and processing of MON89034 x NK603. Advisory report CGM/091020-01
2. COGEM (2006). Cultivation of maize line NK603. Advisory report CGM/060704-01
3 COGEM (2009). Cultivation of maize line MON89034xNK603. Advisory report CGM/091208-01
4. COGEM (2011). Additional information concerning the application for cultivation of MON89034 x NK603 maize. Advisory report CGM/110803-01
5. COGEM (2012). Additional information concerning the application for cultivation of MON89034xNK603 maize. Advisory report CGM/120327-01

6. MacRae T *et al*. (2005). Evaluation of the potential for interactions between the Bacilllus thuringiensis proteins Cry1A.105 and Cry2Ab2. Monsanto Technical Report MSL 19859
7. Van Gestel CAM eds. *et al*. (2011). *Mixture Toxicity. Linking Approaches from Ecological and Human Toxicology*. SETAC Press and CRC Press, Taylor & Francis Group, Boca Raton, FL.