Event report
International scientific workshop ‘Stacked Bt genes: assessment of effects on non-target organisms’

15th of October, 2014
Amsterdam, the Netherlands

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Summary
In recent years, insect resistant GM plants expressing multiple Bt proteins have become increasingly common. The production of multiple Bt proteins by these GM plants raises new questions for the assessment of potential risks to non-target organisms. The main question is whether the effect of a Bt protein could change due to the presence of other Bt proteins. In other words, is it possible that Bt proteins interact and that due to this interaction the impact of a GM crop on NTOs changes when compared to a GM crop expressing a single Bt protein.

The Advisory Committee on Releases to the Environment (ACRE), La Comisión Bioseguridad (CNB), the Scientific Institute of Public Health (WIV-ISP) and the Netherlands Commission on Genetic Modification (COGEM) organised a scientific workshop to discuss aspects that are important when assessing the effects of multiple Bt proteins and to identify practical approaches to assess these effects.

The presentations given, remarks made and discussion points put forward during the workshop are summarised in this event report.

Introduction

There is considerable experience with the safety studies needed to assess potential non-target organism (NTO) effects in the case of genetically modified (GM) crops producing single Bt proteins. In recent years, insect resistant GM crops expressing multiple Bt proteins have become increasingly common. The production of multiple Bt proteins by these GM crops raises new questions for the assessment of potential risks to NTOs. The main question is whether the effect of a Bt protein could change due to the presence of other Bt proteins. In other words, is it possible that Bt proteins interact and that due to this interaction the impact of a GM crop on NTOs changes when compared to a GM crop expressing a single Bt protein.

To address and discuss this question the Advisory Committee on Releases to the Environment (ACRE), La Comisión Bioseguridad (CNB), the Scientific Institute of Public Health (WIV-ISP) and the Netherlands Commission on Genetic Modification (COGEM) organised a scientific workshop on this topic.

The workshop was open for those interested in the topic. Participants from academia, risk assessment bodies, industry, and non-governmental organisations attended the workshop. Forty-three participants from sixteen countries were present.

Programme[1]

Opening

Prof. Nico van Straalen (chair of the subcommittee 'Agriculture' of COGEM) welcomed the participants of the workshop and briefly introduced the topic of the workshop.

Interaction between *Bacillus thuringiensis* toxins: A review of the available data

Dr Nelly van der Hoeven (Ecostat, the Netherlands)

Van der Hoeven started her presentation with an overview of the seven structurally related Bt protein groups (i.e. the 3-domain Cry, Cyt, Bin-Cry, Mtx-Cry, Vip3, Cry6&Cry55 and Mtx1 protein groups) and presented the results from her literature review of all scientific publications involving toxicity of multiple Bt proteins. If Bt proteins in a mixture interact, the measured combined toxicity deviates from the toxicity expected when assuming an additive effect of the individual Bt proteins. The synergy factor (SF) was calculated for each combination of Bt proteins for which sufficient data was available in the publications. The SF is calculated by dividing the predicted ED50 of the Bt protein mixture (assuming an additive effect) by the observed ED50.

Van der Hoeven evaluated the calculated SFs and reported that forty-seven percent of the data on combined toxicity did not reveal any synergism (SF < 2), whereas in fifty-three percent of the cases synergism (SF>2) was reported. In eighteen percent of these cases synergy factors of more than 10

1. An overview of the programme can be found in appendix 1.

were observed. The majority (53%) of these studies involved resistant strains of target organisms. Strong synergy (SF>50) was exclusively observed if one of the Bt proteins was a Cyt, Mtx or Bin protein and the insect strain was resistant or insensitive to the other Bt protein in the mixture. A synergistic effect (SF>10) was almost never observed in the case of sensitive (non-resistant) organisms and mixtures of 3-domain Cry proteins, although also for this group of organisms a large synergistic effect could not be excluded (SF up to 33). Interestingly, the reported data showed that the type of interaction of a Bt protein mixture can be species specific. The same Bt protein mixture had an additive effect in one organism, whereas an antagonistic or synergistic effect could be observed in another organism.

Van der Hoeven underlined that almost no data on the toxicity of Bt protein mixtures to NTOs was available in scientific literature.

Van der Hoeven emphasised that in the case of resistant target organisms strong synergistic effects may be observed (even up till SF>250) and postulated that high synergistic effects of Bt protein mixtures on NTOs cannot be excluded if the mechanism associated with the insensitivity of NTOs is similar to the mechanism of resistance in pest organisms.

Remarks and questions
In response to a question, Van der Hoeven clarified that the classification into classes of synergism (no (SF<2), weak (2<SF<10), strong (SF>10)) was developed by herself. A participant pointed out that high doses of proteins are used in toxicity studies with resistant organisms. Exposing organisms to high doses may be difficult, because high amounts of proteins can be difficult to dissolve. This might complicate the calculation of correct dose response curves.

Transfer of multiple Cry proteins through the arthropod food web and assessment of combined effects on susceptible herbivores – laboratory experiments, challenges, and first results
Dr Michael Meissle (Agroscope, Institute for Sustainability Sciences, Switzerland)

Meissle informed the audience about the results of his experiments on the behaviour and effect of multiple Bt proteins and pointed out some challenges that may be encountered when studying the combined effects of Bt proteins on NTOs.

One of the challenges that Meissle observed is that different Cry proteins may behave differently in the food chain, because of differences in expression in the plant, mode and site of feeding, and protein digestion and excretion. The transmission of Cry proteins in the food web was investigated with Cry1Ac and Cry2Ab expressing lepidopteran resistant cotton (Bolgard II). Both Cry proteins diluted along the food chain, with one exception. In spider mites (*Tetranychus urticae*) higher than expected Cry1Ac protein concentrations (but not Cry2Ab) were observed. In addition, the studies also revealed that the ratio of the two Cry proteins in different NTOs was highly variable.

Another challenge that Meissle encountered is the variable activity of different batches of Cry proteins (Cry1Ac and Cry2Ab2) and fluctuating susceptibility of strains of the test organism *Heliothis virescens* (tobacco budworm; Lepidoptera: Noctuidae).

The bioactivity of the Cry1Ac and Cry2Ab2 proteins in dry leaves, fresh leaves and different prey organisms (*Spodoptera littoralis* and *T. urticae*) was investigated, but did not seem to correlate with the values obtained with ELISA assays. The reason for the deviation is currently being investigated.

Meissle also presented the results from his studies on the combined effect of Cry1Ac and Cry2Ab2 proteins. To study the presence of interaction, different ratios of Cry proteins and a susceptible test organism (*H. virescens*) were used. The results from the interaction study corresponded with additivity.

Meissle concluded his presentation with a question: "what is the best mixture for testing?"

Remarks and questions
A participant asked how the combined effect of multiple Cry proteins should be studied. Meissle replied that the presented approach could also be used to study the effect of more Cry proteins. Meissle mentioned that he is currently trying to assess the effect of GM maize expressing four Cry proteins, but that it is already difficult to determine the concentration of all proteins. A member of the audience pointed out that not all combinations need to be tested if experiments are designed cleverly.

Effects of insecticidal Cry proteins on nematodes – implications for the ecological risk assessment of a stacked Bt-maize variety (MON89034-MON88017)
Dr. Sebastian Höss (Ecossa, Germany)

Höss stressed the importance of assessing the effect of Bt maize on nematodes. Nematodes have a key position in soil food webs and are potentially exposed to Cry proteins in the rhizosphere. Also, the mode of action of nematicidal Cry proteins (Cry5B, Cry14B) is similar to that of insecticidal Cry proteins.

To study the effect of the stacked Bt maize variety MON89034xMON88017 a tiered approach was used involving laboratory, microcosm and field experiments. Höss first led the audience along a series of laboratory experiments with *Caenorhabditis elegans* resulting in the following conclusions:

- The nematidical Cry5B protein is present in the gut of bacterial feeders, but not in the gut of hyphal feeders, indicating that not all nematodes may be exposed to Cry proteins
- *C. elegans* is susceptible to insecticidal Cry proteins (Cry1.105, Cry2Ab2 and Cry3Bb1), but the insecticidal Cry toxins are less toxic (factor 3) than the nematicidal Cry5B toxin
- Cry1.105 and CryAb2 (but not Cry3Bb1) act via the same receptors as Cry5B
- An equimolar mixture of the three insecticidal Cry toxins was less toxic than expected indicating antagonism
- MAPK 'defense' genes, which are indicative of a specific stress response, are upregulated upon exposure to the insecticidal Cry protein mixture, but not when exposed to the single insecticidal Cry proteins, which may explain the antagonism observed in the mixture

Secondly, the results from microcosm experiments were presented. These indicated that the indigenous nematode community was considerably more sensitive than the single species tested in the lab.

Thirdly, Höss reported on field experiments. The concentration of Cry proteins in field soil was low (<1 ng/g soil dry weight) and no effect of the Cry proteins on the nematode population was observed.

Finally, Höss mentioned that Cry proteins are a potential risk to soil nematodes and stressed the importance to assess possible effects on nematodes. Based on the data presented, Höss concluded that the potential risk of cultivation of MON89034xMON88017 to nematodes is well acceptable.

Remarks and questions
A participant suggested experiments to investigate the effect of the protein mixture at a 10% effect level. The dose response curves of the insecticidal Cry proteins differed and therefore the mixture effect may change depending on the effect level studied.

Approaches and considerations for assessing potential interactions of Bt proteins in support of environmental safety assessments for genetically modified crops
Dr. Peter Jensen (Monsanto Company, Saint Louis (Mo), USA)

Jensen briefly introduced the possible types of interactions between substances (additivity, antagonism, potentiation and synergy) and listed the conditions that US EPA stipulated to allow the use of data from single events in the risk assessment of stacked events: the stability of the transgene needs to be confirmed, the expression of the transgenes has to be comparable and the absence of synergism has to be demonstrated using sensitive insect bioassays. Alternatively, effects on NTOs may be studied with the combined substances.

The mode of action of Bt proteins involving binding to specific receptors was brought to the attention of the audience along with two accepted models that describe an additive response 1) the concentration addition model, and 2) the response addition model, were presented. As Bt proteins have the same general mode of action, the concentration addition model is considered the most appropriate.

Jensen highlighted some aspects of and considerations for studies evaluating interaction of multiple Bt proteins. Interaction studies are carried out under the null hypothesis of no interaction (i.e. additivity). Concentration response relationships are determined of the individual and the combined agents. In the bioassays purified Bt proteins or plant tissue incorporated in diet may be used and lethal or sublethal endpoints may be scored. According to Jensen, appropriate Bt protein ratios should be used (they should mimic the concentrations in the plant). Jensen also mentioned that the bioassays should be conducted using insect species sensitive to at least one of the Bt proteins in the stacked event. An adequate number of replications should be used (at least three), and the data should be analysed in a proper way.

To illustrate the approach followed to evaluate the interaction of multiple Bt proteins, Jensen brought the results of interaction studies on Bt proteins with different and similar activity spectrums and the results of binding studies using brush border membrane vesicles of *Heliothis virescens* (tobacco budworm; Lepidoptera: Noctuidae) to the attention of the audience.

Jensen familiarised the participants with the decision table for NTO testing which is used by Monsanto to assess whether further studies are needed.

Observed synergy in sensitive species?	Known NTO adverse effect from single(s) at expected environmental concentration	Test NTO species for adverse effects from stacks
No	No	No
No	Yes	Case specific
Yes	No	No
Yes	Yes	Yes

Jensen reiterated that the effect of the Bt proteins can be assessed independently if there is evidence that the Bt proteins do not interact using a bioassay with a sensitive target species. Consequently, existing data on the effect of the single Bt proteins may be used in the environmental risk assessment for the stacked event. Additionally, Jensen argued that the assessment of potential interactions within a stacked event with multiple Bt proteins addresses all potential interactions in events containing subcombinations of these Bt proteins.

Remarks and questions
A member of the audience pointed out that the insensitivity of organisms to certain Bt proteins may be the result of different causes. The pH of the gut, the absence of certain proteases or the absence of certain receptors are all factors that may be the reason why an organism is not sensitive to a Bt protein.

The extrapolation of study results from one (pest) organism to another (non-target) organism was a topic of discussion. The same combination of Bt proteins was observed to have a different effect on different organisms. A participant remarked that this observation meant that testing more organisms would not lead to more certainty in the risk assessment. Another person suggested that further testing might not be necessary. The potential occurrence of synergism may also be taken into account in risk assessment using other approaches.

Interaction between Bacillus thuringiensis toxins: a review and implications for risk/safety assessment
Dr. Adinda De Schrijver (Biosafety and Biotechnology Unit, Scientific Institute of Public Health, Belgium)

De Schrijver introduced the two questions that were the topic of her research project:
- Can interactions between Bt proteins be predicted?
- To what extent are studies on interactions relevant for risk assessment?

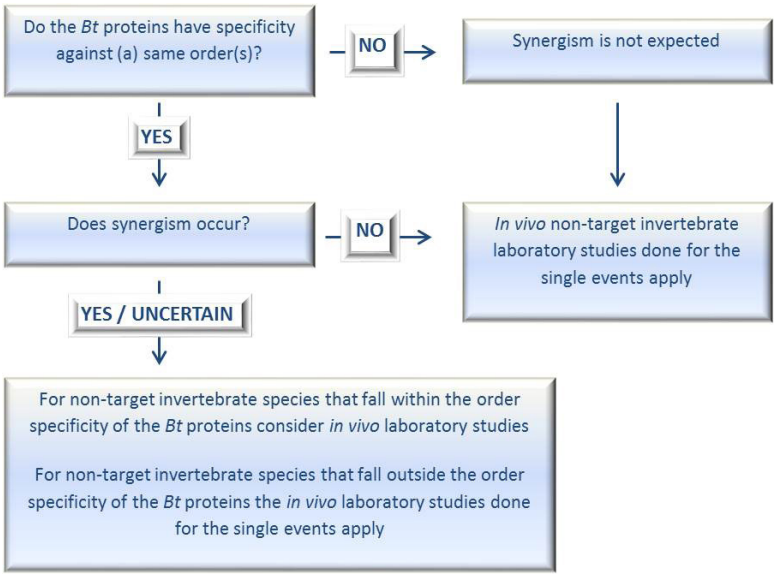
De Schrijver evaluated the results from published interaction studies showing that interactions have been demonstrated to occur between Bt proteins active against Diptera, Lepidoptera or Nematoda. De Schrijver pointed out that the type of interaction occurring seems to be species-dependent. In

addition, De Schrijver reported that there are no indications that interactions between Cry proteins with different primary activity occur. These observations led De Schrijver to conclude that interaction is not expected when Cry proteins have a different specificity. On the other hand, if Bt proteins have the same specificity the potential occurrence of interaction cannot be predicted.

De Schrijver reminded the participants that there are indications that Cry proteins may display cross-activity besides their primary activity.

Taking into account the data published in literature, De Schrijver concluded that testing for interactions between Bt proteins with different order specificity seems to be of less relevance to the risk assessment of transgenic plants. It is more relevant to conduct interaction studies if the Bt proteins are active against the same order. In addition, De Schrijver pointed out that studying interactions in target organisms seems to be of little relevance in assessing potential risks to NTOs.

The following decision tree was presented as a tool to assess the need to conduct additional in vivo studies.



Remarks and questions
One of the participants mentioned that in the presented approach the trigger for additional studies is the known activity of the Bt proteins. Interaction studies are considered necessary if the Bt proteins are active against the same order. If a Bt protein is active against a certain organism and this organism is reclassified to another order, this could influence the need for additional studies and the type of non-target species to consider for further studying.

Discussion
The participants of the workshop discussed whether interactions could occur between Bt proteins expressed by GM plants. They also discussed whether such interactions are predictable.

The presented interaction studies led the participants to conclude that interaction (i.e. deviations from additivity) between Bt proteins may occur. The concentration addition model was considered the best model to use as a starting point when studying the potential interaction of Bt protein mixtures.

The participants concluded that it is extremely difficult to predict whether interactions are expected in a specific case. The importance of research to gain further insight in the occurrence of interaction between Bt proteins was stressed. If new information becomes available the occurrence of interactions might be predictable in the future.

Subsequently, the role of interaction studies in risk assessment was discussed. From a risk assessors point of view the possible occurrence of synergism is considered of particular importance because this might lead to greater effects than expected. Some participants argued that observed antagonism should also be a trigger for further studies, because the available data show that an antagonistic mixture effect in one species does not exclude a synergistic mixture effect in another species.

The presentation of De Schrijver was highlighted. In her presentation De Schrijver mentioned that there are no indications that interactions occur if the Bt proteins are not active in the same order. It was emphasised that to reach a conclusion on the absence of interaction, possible cross-order activity of the Bt proteins should be taken into account. Therefore, information on known cross-order activity should be part of applications.

A participant argued that based on the observation of De Schrijver further studies would only be required if the Bt proteins are active in the same order. In such a case, the effect of the Bt protein mixture on a NTO from that order should be studied. Another participant was of the opinion that the available information is insufficient to conclude that Bt proteins that are not active in the same order will not interact.

It was mentioned that to detect interaction, studies on a species that is sensitive to one of the Bt proteins present in the mixture are the most promising. Bt proteins are more likely to interact and display a greater effect than expected in a species that is already sensitive to one of the toxins.
A participant argued that it is not necessary to detect potential interactions between Bt proteins in a mixture. This participant was of the opinion that potential effects on NTOs should be studied using the combination of Bt proteins instead of studying the potential effects of the individual Bt proteins separately.

A participant pointed out that it is not possible to study all NTOs that might be exposed to the Bt proteins. It was suggested to use worst case scenarios and higher margins of safety to ensure that the exposure of NTOs to the expressed Bt proteins is such that they would not be adversely affected if interactions would occur. Organisms of special interest could be studied on a case-by-case basis.

One of the participants sketched a future scenario in which crops with many Bt genes would need to be assessed. This would make it even more difficult to predict whether interactions might occur.

The possibility to use other approaches (such as worst case scenarios, models, mitigation measures etc.) to take the possible interaction between Bt proteins into account in the risk assessment was put forward as a possible way to deal with the possible occurrence of interactions which cannot be predicted.

Concluding remarks of the chair

Based on the views, arguments and suggestions of the participants the chair concluded that potential interactions between Bt proteins should be taken into account in the risk assessment of stacked events. The relative concentration addition model seems to be a good null model when analysing whether Bt proteins interact. It is however difficult to predict whether interactions between Bt proteins will occur because these interactions seem to be species specific.

Closing

Van Straalen thanked the speakers and participants for their willingness to share their knowledge and insight on the topic and expressed his appreciation for the valuable suggestions made by the participants to deal with potential interactions in risk assessment. He closed the workshop and wished everyone a good journey home.

Appendix 1: workshop programme

10.00	**Registration, coffee & tea**
10.30-10.45	**Welcome** Prof. Nico van Straalen (COGEM), chair workshop
10.45-11.15	**Interaction between *Bacillus thuringiensis* toxins: A review of the available data** Dr. Nelly van der Hoeven, Ecostat, the Netherlands
11.15-11.45	**Transfer of multiple Cry-proteins through the arthropod food web and assessment of combined effects on susceptible herbivores – laboratory experiments, challenges, and first results** Dr. Michael Meissle, Agroscope; Institute for Sustainability Sciences, ISS, Switzerland
11.45- 13.45	*Lunch*
13.45-14.15	**Effects of insecticidal Cry proteins on nematodes – implications for the ecological risk assessment of a stacked Bt-maize variety (MON89034—MON88017)** Dr. Sebastian Höss, Ecossa, Germany
14.15-14.45	**Approaches and Considerations for Assessing Potential Interactions of Bt Proteins in Support of Environmental Safety Assessments for Genetically Modified Crops** Dr. Peter Jensen, Monsanto Company, Saint Louis (M0), USA
14.45-15.15	*Coffee break*
15.15-15.45	**Interaction between *Bacillus thuringiensis* toxins: a review and implications for risk/safety assessment** Dr. Adinda De Schrijver, Biosafety and Biotechnology Unit, Scientific Institute of Public Health, Belgium
15.45-17.00	*Discussion*