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Potential synergies that can enhance Bt toxicity in SmartStax

Analyses of Levine et al., 2008a and MacRae 2008
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Testbiotech e. V.
Institute for Independent Impact Assessment in Biotechnology

Christoph Then

Summary:
It is well known that synergistic and additive effects both between Bt toxins and other compounds do occur. In general, synergistic effects can be characterised by findings that exceed those that can be predicted from those of the single components. These effects are under discussion as to whether they could be used commercially to enhance the toxicity of Bt toxins in pest insects. However, it is also known that in some cases toxicity in non-target organisms may be enhanced, causing unexpected risks for the environment and human health.

Synergistic effects may not only arise from the interaction of Bt toxins, but also from plant components or abiotic stressors (such as residues from spraying or toxic heavy metals e.g. cadmium). For example, some plant enzymes that diminish the digestion of proteins (protease inhibitors) can strongly enhance the toxicity of Bt toxins. Even the presence of very low levels of protease inhibitors can multiply the insecticidal activity of some Cry toxins. It is known that maize produces such inhibitors.

It is also known that synthetically produced Bt toxins can show much higher toxicity than native proteins. Even small changes in the structure of the proteins can cause huge changes in their toxicity. In SmartStax, the structure of the proteins is modified, Cry1A.105 is even produced synthetically.

These effects render higher toxicity and give rise to unexpected risks. They can only be investigated by experimental investigations since the specific mixture of Bt toxins does not occur in nature, and the structure of the Bt toxins is different from native sources.

The investigations presented by the applicants, were commissioned and paid for by Monsanto. They were conducted in Monsanto Laboratories. No independent laboratories were involved. The results were not published in peer- reviewed magazines.

Levine et al and McRae investigated synergistic effects in pest insects only. Levine et al tested combinations of Bt toxins as produced in SmartStax on the European corn borer. MacRae worked with some Bt toxin sub-combinations on the Southern corn rootworm. The investigations did reveal additive effects, but no synergistic or antagonistic effects in the pest insects.

Levine et al and McRae did not perform any testing aimed at showing there is no toxicity risk for

humans and animals from the interaction of the Bt toxins. Further potential synergies with the residues from herbicide spray, the plant components or external additional factors were not considered. There was also no data made available to establish whether the combined ingestion of the toxins and residues from spraying can lead to a change in the composition of the intestinal flora, and therefore impact on the health of humans and animals.

EFSA does not even mention the investigations of Levine et al and McRae in their opinion. It is only very vaguely stated that EFSA considers it unlikely that interactions between the Bt toxins would occur that would give rise to safety concerns.

Contrary to EFSA, experts from EU Member States such as Austria, Belgium and Germany are advocating the need to carry out feeding studies to test for synergistic effects.

1. Background of the investigation

Smartstax produces six different Bt toxins. This combination of toxins does not occur in nature:

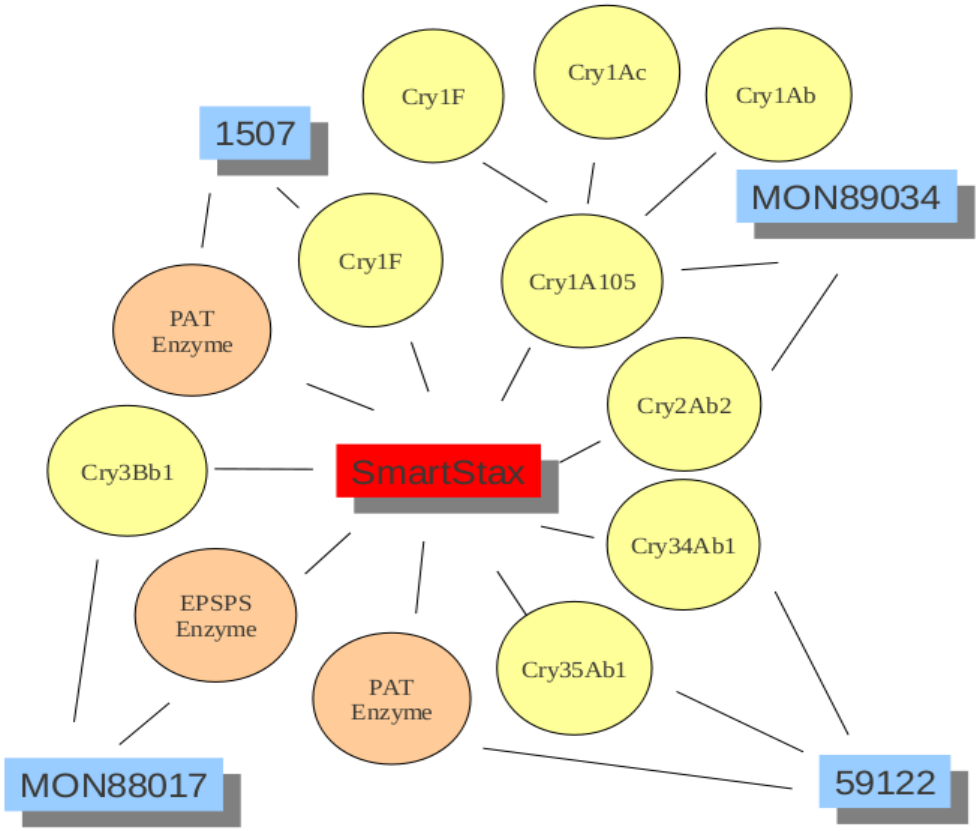
- The Bt toxins are derived from at least four different subspecies of *Bacillus thuringiensis.*
- The Bt toxins produced in the plants display modified DNA and changes in the structure of their proteins. They are, therefore, fundamentally different from naturally occurring toxins.
- The Bt toxins in the plants are produced in an activated form. They are solubilized and shortened and are not produced in their natural inactive and crystallized form.
- One of the Bt toxins in SmartStax (Cry1A.105) is a synthetic protein that did not previously exist in nature.

Furthermore, SmartStax contains two gene constructs that confer herbicide tolerance to glufosinate (brand names such as Liberty or Basta) and one gene construct that confers herbicide tolerance to glyphosate (brand names such as Roundup). All in all, SmartStax produces nine foreign proteins on the basis of gene constructs that are derived from seven species or subspecies or specific strains. Since the gene constructs themselves contain further elements such as promotors, the net result is that even more species are involved than listed in Table 1.

Table 1: Overview of foreign proteins produced in SmartStax and the source of their original DNA

Foreign proteins produced in SmartStax	Source of the original DNA
Cry1A.105 toxin	*Bacillus thuringiensis*, synthetic protein showing analogies with three Bt toxins (Cry1Ac, Cry1Ab, Cry1F) from two different strains (*kurstaki* and *aizawai*).
Cry1F toxin	*Bacillus thuringiensis*, subspecies *aizawai*
Cry2Ab2 toxin	*Bacillus thuringiensis*, subspecies *kurstaki*
Cry3Bb1 toxin	*Bacillus thuringiensis*, subspecies *kumamotoensis*
Cry34Ab1 and Cry35Ab1 toxins	*Bacillus thuringiensis*, strain PS149B1
EPSPS enzyme	*Agrobacterium* sp. strain CP4
PAT enzyme (2x)	*Streptomyces viridochromogenes*

Fig. 1: Overview of all Bt toxins and herbicide tolerances in SmartStax



2. Relevant risks described in literature

It is very well known that synergistic, antagonistic and additive effects occur both between the Bt toxins and between the Bt toxins and other compounds. These effects are used intentionally to enhance toxicity in pest insects (overview: Prado Lopez et al., 2009). However, it is also known that toxicity in non- target organisms can be enhanced (for overview see Then, 2010). In general, synergistic effects can be characterised by emerging effects that exceed those that can be predicted from additive linear dosage-response effect, while additive effects follow a predictable dose-response relationship. Both effects are relevant for assessing the toxicity of Bt plants: Efficacy, selectivity in target and non-target organisms can be impacted by interactivity amongst the toxins or between the toxins and other external factors such as enzymes, pesticides or bacteria (for overview see Then, 2010).

For example, a recently published study carried out by Sharma et al. (2010) found synergistic effects of Cry1Ab and Cry1Ac in target pest insects. Further synergistic effects between Cry1Ac and other Bt toxins such as Cry2Ab2 and Cry1F are discussed in Lee et al. (1996), Chakrabarti et al (1998), Stewart et al. (2001) and Kashdan et al (2007). Synergistic effects can become highly problematic for non-target organisms: Interaction between the toxins can cause unexpectedly higher

toxicity. Even a lower selectivity can be observed, thus the range of possibly affected organisms might be widened (Then, 2010). In this case, the effects might also affect human or animal health.

Furthermore, synergistic effects with the plants own components (such as trypsin inhibitors that can enhance toxicity of Bt proteins) and other abiotic factors (such as residues from spraying, cadmium) have to be taken into account. For example, it is known that protease inhibitors (such as trypsin inhibitors) can strongly enhance the toxicity of Bt toxins. Even the presence of extremely low levels of protease inhibitors enhances the insecticidal activity of some Cry toxins up to 20-fold (Pardo-Lopez et al., 2009). It is further known that maize produces such inhibitors (Shulmina et al., 1985) and that interaction with cadmium can cause toxicity in non-target organisms (Kramarz et al, 2007).

Moreover, as Pardo Lopez et al. (2009) and Pigott et al. (2008) show, synthetically derived and modified Bt toxins can show much higher toxicity than native proteins. Even small changes in the structure of the proteins can cause huge changes in their toxicity. This is especially relevant in the case of Cry1A.105: Its toxicity cannot be assessed by comparison with naturally occurring Bt toxins. Moreover, Cry1A.105 is not the only protein that is changed in its structure, since all of the Bt toxins produced in the plants are technically modified to improve their expression and their efficacy on pest insects.

3. Overview of investigations and findings of Levine et al (2008a) and MacRae (2008)

Monsanto commissioned and paid for the investigations. Both research teams only tested for potential interactions in certain pest insects. While McRay tested some sub-combinations, Levine et al. worked with the full mixture of toxins present in SmartStax.

- **MacRae, 2008:**

MacRae tested some sub-combinations of the Bt toxins produced in SmartStax on the southern corn rootworm (*Diabrotica undecimpunctata howardi*). Cry34Ab1 and Cry35Ab1 were tested in combination with Cry3Bb1.

Specific findings:

With the methods and material as used by MacRae, the findings showed some additive effects but no significant synergistic effects in the southern corn rootworm.

- **Levine et al (2008a):**

Levine et al tested combinations of the Bt toxins produced in SmartStax on the European corn borer, ECB (*Ostrinia nubilalis*).

In a first experimental setting, they tested the Bt toxins that are known to be active in *Lepidoptera* for additive effects. For this purpose, they used material from genetically engineered plants that produce Cry1A.105 and Cry2Ab2 and Cry1F. For purposes of comparison, they also used material that only produces Cry1F.

In a second experimental setting, the aim was to find out whether the effects of the toxins as used in the first experiments are enhanced in the European corn borer when combined with Cry3b1, Cry34Ab1 and Cry35Ab1. In result, by combining these toxins, the combination as present in

SmartStax was used.

Findings:
With the methods and materials used by Levine et al., no significant synergistic interaction between the toxins produced in SmartStax was found in the European corn borer.

Levine et al. (2008a) gave an overview of prior investigations to examine interaction between the proteins:

> *"The Cry1A.105 and Cry2Ab2 proteins were shown to have additive activity against two lepidopteran pests of corn (MacRae et al., 2005), the European corn borer (ECB, Ostrinia nubilalis (Hübner)) and the corn earworm (CEW, Helicoverpa zea (Boddie)). Subsequently, it was demonstrated that combined Cry1A.105 and Cry2Ab2 activity is not altered in the presence of the Cry3Bb1 protein and vice-versa in sensitive insect dietbioassays (MacRae et al. 2006) and that protection against root feeding damage is additive when combining MON 88017 and DAS-59122-7 by conventional breeding (Levine and Uffman, 2008). In addition, no interaction was observed between the Cry1F and Cry34/35Ab1 proteins in sensitive insect diet-bioassays (Herman and Storer, 2004). Recently, the Cry3Bb1 and Cry34/35Ab1 proteins were shown to have additive activity against southern corn rootworm species in diet bioassays (MacRae, 2008). "*

This overview is interesting from several points of view:

- All mentioned publications are confidential, produced by industry and not available to the general public. They were never published in peer- reviewed magazines.
- None of these publications were mentioned by EFSA.
- Some of these publications indeed show at the very least additive effects in the toxicity of the Bt toxins produced in SmartStax.

4. Assessment of the investigations

4.1 Evidence of insufficient testing

Some of the most apparent deficiencies of the investigations are:

- Both studies were only conducted with target organisms. No specific tests related to risks for food and feed e.g. on mammalian cell systems were performed. Therefore, risk assessment of the impact on food and feed cannot be conducted on the basis of these existing studies.
- Only interaction between the Bt toxins was investigated. Potential synergies with EPSPS and PAT Proteins or with residues from herbicide spraying were left aside. Further, other relevant compounds that can trigger synergistic effects such as components from food or feed (such as proteinase inhibitors) stressors, bacteria and pharmaceutical compositions (like antibiotics) were completely ignored (for a list of some relevant factors see Then, 2010).

The tests were not performed in independent research facilities under the supervision of independent experts and institutions. No independent institution was involved in quality control. The results were not published in peer- reviewed articles.

4.2. Assessment by EFSA and experts from EU Member States

EFSA is aware that the studies conducted by Levine et al. (2008a) and McRae (2008) do not deal with health risks. In a letter they sent to the applicants they say[1]:

> *"The applicant has provided various studies showing the absence of interactions between the newly introduced Cry proteins in their effects on insects that are sensitive towards these Cry proteins. Yet the applicant has not provided a risk assessment addressing the potential interactions among the newly expressed proteins, including the Cry proteins (Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, Cry34Ab1/Cry35Ab1), CP4 EPSPS, and PAT, with regard to possible effects on human and animal health. The applicant is requested to provide such a risk assessment of potential interactions among newly expressed proteins."*

In response to this observation by EFSA, the applicants give various reasons why further studies are not thought to be necessary[2]. These reasons include:

- The safety of the single toxins has already been assessed.
- Mammals do not have the specific receptors to activate the toxins.
- If no synergies are observed in target pest insects, it is unlikely they will occur in other organisms.
- The content of the toxins would be too low.

By using these kinds of arguments, the applicants avoid addressing the fact that synergistic interaction between Cry2Ab2 and Cry1Ab and between Cry2Ab2 and Cry1Ac has already been discussed as being likely in Mattila et al. (2005) and Stewart et al. (2001). Further, they do not mention that alternative modes of actions conferring toxicity of Bt toxins that also might be relevant for mammals (Soberon et al., 2009). They also neglect possible interaction with the plants´ components that might increase the toxicity of the Bt toxins (Pardo Lopez et al., 2009) and lead to indirect health effects possibly resulting from a change in the intestinal flora.

Residues from herbicide spraying are also omitted. As a recent overview of the scientific literature shows (PAN AP, 2009), the toxicity of glyphosate, its metabolites and its additive POEA (polyoxyethylene alkylamine) need to be re-evaluated. Thus, there needs to be a detailed investigation of the residues from spraying and their potential interaction with other components of the genetically engineered plants.

EFSA (2010 a and 2010 b) simply defers to the answer of the applicants and concludes that synergies are not to be expected. Specific risks are not discussed in detail. In its opinion, EFSA (2010a) only states very vaguely:

> *"On the basis of the known functional characteristics and modes of action of the newly expressed proteins (Cry1A.105, Cry2Ab2, Cry1F, PAT, Cry3Bb1, CP4 EPSPS, Cry34Ab1, and Cry35Ab1), the EFSA GMO Panel considers it unlikely that interactions between these proteins would occur that would raise any safety concern."*

Contrary to EFSA, experts from EU Member States such as Austria, Belgium and Germany are urging that synergistic effects should be tested in feeding studies. As the experts from Austria state:

> *"But the safety of all newly expressed proteins in animal models applied simultaneously and*

1 Quoted from: Responses to EFSA questions (Ref. PB/SM/Is (2009) 3980589) (20 May 2009) 19 June 2009

2 Responses to EFSA questions (Ref. PB/SM/Is (2009) 3980589) (20 May 2009) 19 June 2009

combined was not assessed in the dossier. Insecticidal Cry proteins produced by GM plants as well as transproteins conferring tolerance to herbicides constitute a sum of new plant constituents possibly interacting within the organism. So far, there is absolutely no scientific knowledge about such those in the respective new combinations and possibly resulting additive and/or synergistic effects."

By referring to the concerns of experts from the Members States, EFSA does not really address the substance of the problem. They mostly refer to their own Guidance and to the answer of the applicant in June 2009.

Table 2: Relevant comments from the experts of Member States and the answer from EFSA 2010b

Member State	Statement	answer from EFSA
Austria	A stacked organism has to be regarded as a new event, even if no new modifications have been introduced. The gene-cassette combination is new and only minor conclusions could be drawn from the assessment of the parental lines, since unexpected effects (e.g. synergistic effects of the newly introduced proteins) cannot automatically be excluded. (page 1)	In case of this stack the applicant is requested to follow the Guidance Document of the EFSA Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants containing stacked transformation events (2007). (page 1)
Austria	a) There is no history of safe use of the new recombinant protein expressed by an artificially arranged insert such as Cry1A.105. b) As far as all Bt toxins are concerned, a history of safe use cannot be argued on the basis of the safety of Bt sprays applied in organic farming. The inserted genes are truncated and arranged with expression modulating DNA parts originating from different organisms and permanently expressed compared to a tight timely Bt spraying schedule. (page 22)	The safety of the newly expressed proteins was previously evaluated by the EFSA GMO Panel in its opinions on the single parental events for this stacked event (MON 89034, 1507, MON 88017, 59122). (page 22)
Austria	Furthermore, according to EFSA, a potential for increased toxicity and/or allergenicity to humans and animals or for modified nutritional value due to the stacked events may arise from additive, synergistic or interactions among the single events with regard to antagonistic effects of the gene products or by these produced metabolites (EFSA 2007). But the safety of all newly expressed proteins in animal models applied simultaneously and combined was not assessed in the dossier. Insecticidal Cry proteins produced by GM plants as well as transproteins conferring tolerance to herbicides constitute a sum of new plant constituents possibly interacting within the organism. So far, there is absolutely no scientific knowledge about such those in the respective new combinations and possibly resulting additive and/or synergistic effects. (page 24)	At the request of the EFSA GMO Panel the applicants provided a risk assessment of potential interactions among the single events with regard to antagonistic effects of the gene products or by these produced human and animal health, in its response dated 23 June 2009. (…) On the basis of the known functions and modes of action, the EFSA GMO Panel considers it unlikely that interactions between these newly expressed proteins (Cry1A.105, Cry2Ab2, Cry1F, PAT, Cry3Bb1, CP4 EPSPS, Cry34Ab1, and Cry35Ab1) would occur that would raise any safety concern. (Page 24)
Belgium	The safety aspects of the multiple challenge, due to	No event-related effects on human and animal

Member State	Statement	answer from EFSA
	the combination of the newly inserted proteins, are rather weakly demonstrated (page 34)	health have previously been identified in the EFSA GMO Panel` s opinions on the single parental events. At the request of the EFSA GMO Panel the applicants provided a risk assessment of potential interactions among the single events with regard to antagonistic effects of the gene products or by these produced human and animal health, in its response dated 23 June 2009. (…) On the basis of the known functions and modes of action, the EFSA GMO Panel considers it unlikely that interactions between these newly expressed proteins (Cry1A.105, Cry2Ab2, Cry1F, PAT, Cry3Bb1, CP4 EPSPS, Cry34Ab1, and Cry35Ab1) would occur that would raise any safety concern.
	In case of MON 89034 × 1507 × MON 88017 × 59122 there may be a multiple challenge, which can be more harmful than any individual newly inserted proteins. It is highly desirable to refer to studies that have demonstrated that the combination of all these newly inserted proteins is not detrimental. However, the modes and sites of biological activity are different for the Cry, PAT and CP4 EPSPS proteins and there is no known or conceivable mechanism of interaction between these proteins which could lead to adverse health effects in animals or humans. Does this observation really guarantee full safety ?	No event-related effects on human and animal health have previously been identified in the EFSA GMO Panel`s opinions on the single parental events. At the request of the EFSA GMO Panel the applicants provided a risk assessment of potential interactions among the single events with regard to antagonistic effects of the gene products or by these produced human and animal health, in its response dated 23 June 2009. (…) On the basis of the known functions and modes of action, the EFSA GMO Panel considers it unlikely that interactions between these newly expressed proteins (Cry1A.105, Cry2Ab2, Cry1F, PAT, Cry3Bb1, CP4 EPSPS, Cry34Ab1, and Cry35Ab1) would occur that would raise any safety concern.
Germany (BfN)	Generally, more studies based on plant-derived materials of MON89034x1507xMON88017x 59122 maize are requested. MON89034x1507xMON88017x59122 maize combines several classes of Bt proteins active against both Lepidoptera and Coleoptera. For the environmental risk assessment interactions between these proteins should be addressed in more detail. (page 50)	At the request of the EFSA GMO Panel. The applicants provided a risk assessment of potential interactions among the single events with regard to human and animal health, in its response dated 23 June 2009. The EFSA GMO Panel concludes in its opinion, section 5.1.4.3, that “The EFSA GMO Panel considered all the data available for maize MON 89034 x 1507 x MON 88017 x 59122 and the newly expressed proteins (Cry1A.105, Cry2Ab2, Cry1F, PAT, Cry3Bb1, CP4 EPSPS, Cry34Ab1, and Cry35Ab1) and is of the opinion that interactions between the maize events that might impact on the food and feed safety of maize MON 89034 x 1507 x MON 88017 x 59122 are unlikely. Therefore, the EFSA GMO Panel does not consider additional animal safety studies with the whole GM food/feed necessary.” (page 50)
Germany (BfN)	Data from the parental lines can be informative but not sufficient for the risk assessment (see Andow & Hilbeck 2004; Hilbeck et al 2008). Experiments should account for the high total amount of Bt protein in MON89034x1507xMON88017x59122 maize and for possible interactions of the mixture of Cry1.105, Cry2Ab2, Cry1F, Cry3Bb1 and Cry34Ab1/Cry35Ab1. The two studies submitted on possible interactions of Cry Proteins (MacRae	Considering the scope of the application (that excludes cultivation) and the intended uses of maize MON 89034 x 1507 x MON 88017 x 59122 (which include its segregating progeny), it can be concluded that the exposure of potentially sensitive non-target organisms to the Cry1A.105, Cry1F, Cry2Ab2, Cry3Bb1, Cry34Ab1 and Cry35Ab1 proteins is likely to be very low and of no ecological relevance. (page 59)

Member State	Statement	answer from EFSA
	2008 and Levine et al. 2008) focus on the target organisms. Known differences between the sensitivity to Bt-Toxin within the taxonomic order related to the target organisms as well as methodological details of the studies do not allow assessment of interactions of the present Bt proteins in general. (page 59)	

5. Conclusions:
It is not possible to assess risks to human health due to possible synergies of the Bt proteins simply by referring to experiments with pest insects or by testing single compounds. There have been no studies on potential health impacts due to combinations of the toxins or synergies with external factors such as protease inhibitors, or with residues from spraying. Not even the toxicity of single components such as the synthetic toxin Cry1A.105 has been sufficiently determined. In general, the mode of action of Bt toxins is not fully understood and is controversially debated (Pigott & Ellar, 2007, Soberon et al., 2009). Thus, risks for human health caused by the Bt toxins in SmartStax cannot be excluded.

The opinion from EFSA (2010a) that mainly defers to the Monsanto perspective must be rejected because it is inadequate to protect human health and the environment.

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