TESTBIOTECH Background 13 - 02 - 2019

Testbiotech comment on EFSA's assessment of genetically engineered maize MON 89034 x 1507 x MON 88017 x 59122 x DAS-40278-9 and sub-combinations independently of their origin for food and feed uses, import and processing under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2013-113) by Dow

Testbiotech e. V.
Institute for Independent Impact Assessment in Biotechnology

Christoph Then & Andreas Bauer-Panskus

Introduction

The GMO panel assessed the five-event stacked maize MON 89034 x 1507 x MON 88017 x 59122 x DAS-40278-9, which is derived from crossing five genetically engineered maize events (EFSA, 2018). The maize contains genes conferring resistance to three herbicides and produces six insecticidal proteins.

- MON 87427 expressing CP4 EPSPS protein for tolerance to glyphosate-containing herbicides;
- MON 89034 expressing Cry1A.105 and Cry2Ab2 insecticidal proteins;
- 1507 expressing the Cry1F insecticidal protein and phosphinothricin acetyl transferase (PAT) protein for tolerance to glufosinate-containing herbicides;
- MON 88017 expressing the Cry3Bb1 and CP4 EPSPS protein for tolerance to glyphosate-containing herbicides;
- 59122 expressing the Cry34Ab1 and Cry35Ab1 insecticidal proteins and the PAT protein for tolerance to glufosinate-containing herbicides and
- DAS-40278-9 expressing the aryloxyalkanoate dioxygenase 1 (AAD-1) protein.

Consequently, the stacked maize produces six insecticidal toxins; Cry1A.105, Cry2Ab2 and Cry1F that target *lepidoptera* insects, and Cry3Bb1, Cry34Ab1 and Cry35Ab1 that target *coleoptera)*. The maize is also resistant to four groups of complementary herbicides (glyphosate, glufosinate and quizalofop- and 2,4-D-containing herbicides). Even though Implementing Regulation 503/2003 has been in force since 2014, EFSA has not applied it in this case.

1. Molecular characterisation

The process of genetic engineering involved several deletions and insertions in the parental maize plants. In order to assess the sequences encoding the newly expressed proteins or any other open reading frames (ORFs) present within the insert and spanning the junction sites, it was assumed that the proteins that might emerge from these DNA sequences would raise no safety issues; and therefore no detailed investigations were carried out in this regard. Furthermore, other gene products, such as miRNA from additional open reading frames, were not assessed. Thus, uncertainties remain about other biologically active substances arising from the method of genetic engineering and the newly introduced gene constructs.

Previous research indicated that expression of Cry1A.105, Cry2Ab2 and EPSPS proteins in genetically engineered maize can induce changes in the overall proteome of the respective GM maize line with impacts on associated endogenous metabolic pathways (Agapito-Tenfen et al. 2014). Similar transgenes are also present in the stacked maize MON89034 x 1507 x MON88017 x 59122 x DAS-40278-9. Thus, robust data should have been presented to assess whether metabolic changes with relevance to biosafety occur in the stacked maize (see comments from Member States).

Environmental stress can cause unexpected patterns of expression in the newly introduced DNA (see, for example, Trtikova et al., 2015). More specifically, Fang et al (2018) showed that stress reaction can lead to unexpected changes in the plants metabolism, inheriting additional EPSPS enzymes. However, the expression of the additional enzymes was only measured under field conditions in the US for one year. It is unclear, to which extent specific environmental conditions will influence the overall concentration of the enzymes in the plants. The plants should have been subjected to a much broader range of defined environmental conditions and stressors to gather reliable data on gene expression and functional genetic stability.

Due to increased weed pressure, it has to be expected that these plants can and will be exposed to high and also repeated dosages of glyphosate alone and / or in combination with the other complementary herbicides. Higher applications of herbicides will not only lead to a higher burden of residues in the harvest, but may also influence the expression of the transgenes or other genome activities in the plants. This aspect was completely ignored in the risk assessment even though Hungarian experts raised specific questions on differences between untreated and sprayed plants that showed higher gene expression (see comments from Member States).

Industry in its own recommendations, suggests dosages on herbicide resistant maize up to

- approx. 90 g a.i./ha quizalofop (postemergence)[1],
- approx. 0,7 kg a.i./ha glufosinate postemergence, approx. 1,5 kg per season[2],
- approx. 2,7 kg a.i. /ha 2,4-D postemergence, approx.4 kg per season[3],
- approx. 2,5 kg a.i./ha glyphosate postemergence, approx. 3,5 kg per season in pesticide mix product containing glyphosate and 2,4-D (Enlist Duo)[4];
- approx. 3,5 kg a.i./ha glyphosate postemergence, 9 kg per season[5], and even higher doses[6] in glyphosate single formulations.

EFSA should have requested that Dow submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, also including repeated spraying and the application of each of the relevant herbicides alone and in combination. The material derived from those plants should have been assessed by using omics techniques to investigate changes in the gene activity of the transgene, as well as the natural genome of the plants.

[1] https://www.greenbook.net/dupont-crop-protection/dupont-assure-ii
[2] https://www.greenbook.net/bayer-cropscience/liberty-280-sl
[3] https://www.greenbook.net/dow-agrosciences/enlist-duo
[4] https://www.greenbook.net/dow-agrosciences/enlist-duo
[5] https://www.greenbook.net/monsanto-company/roundup-ultra
[6] https://www.greenbook.net/monsanto-company/roundup-weathermax

2. Comparative analysis (for compositional analysis and agronomic traits and GM phenotype)
Field trials for compositional and agronomic assessment of the stacked maize were conducted in the US for only one year (2010) and not in other relevant maize production areas, such as Brazil or Argentina.

Only data from a low number of agronomic parameters (11), were subjected to statistical analysis in accordance with EFSA guidance, 5 of these were found to be statistically different within categories I and II. There were many significant differences even in this small data set, and therefore EFSA should have requested more data.

Compositional analysis revealed many (and major) statistically significant differences:

- Statistically significant differences between the five-event stack maize (untreated) and the non-GM comparator were identified for 22 out of 65 endpoints, with several endpoints in category III / IV.
- Statistically significant differences between the five-event stacked maize (treated with complementary herbicides) and the non-GM comparator were identified for 35 of 65 endpoints, with several endpoints in category III / IV.

The most relevant differences that were identified concern protein, glutamic acid, glycine, leucine, lysine, threonine, magnesium and manganese in grain.

Since the maize treated with the complementary herbicides shows many more significant differences compared to maize that was not treated, it is likely that this has an impact on plant composition. However, EFSA did not request any further tests (toxicological data, repeated spraying with higher herbicide dosages or exposure to a wider range of environmental conditions). Instead EFSA simply concluded:

> *"Protein, glutamic acid, glycine, leucine, lysine, threonine, magnesium and manganese in grain were significantly different in the five-event stack maize when compared to its comparator and showed lack of equivalence with the set of non-GM reference varieties. Taking into account the known biological role of these compounds, these differences are considered of no toxicological concern by the GMO Panel."*

Consequently, instead of assessing the overall pattern of changes in plant components as well as their causes and possible impacts, EFSA only assessed each of the compounds in isolation (!!). This approach turns the comparative approach into a trivial concept of assessing bits and pieces and ignores questions concerning the overall safety of the whole food and feed.

It has to be assumed that this event is essentially different from its comparator in regard to many compositions and biological characteristics, especially if sprayed with the complementary herbicide. Even if changes taken as isolated data might not directly raise safety concerns, the overall number of effects and their clear significance has to be taken as a starting point for much more detailed investigations. It is not acceptable that EFSA failed to require further studies e.g.

- No field trials were conducted that lasted more than one season. Thus, based on current data, it is hardly possible to assess site-specific effects.
- Further, no data were generated representing more extreme environmental conditions, such as those caused by climate change.

Due to high weed pressure in many maize growing regions, it has to be expected that these plants can and will be exposed to higher amounts and also repeated dosages of the herbicides. Industry in

its own recommendations suggests dosages on herbicide resistant maize up to:

- approx. 90 g a.i./ha quizalofop (postemergence)[7],
- approx. 0,7 kg a.i./ha glufosinate (postemergence), approx. 1,5 kg per season[8],
- approx. 2,7 kg a.i. /ha 2,4-D (postemergence), approx.4 kg per season[9],
- approx. 2,5 kg a.i./ha glyphosate (postemergence), approx. 3,5 kg per season in pesticide mix product containing glyphosate and 2,4-D (Enlist Duo)[10];
- approx. 3,5 kg a.i./ha glyphosate postemergence, 9 kg per season[11], and even higher rates[12] in glyphosate single formulations.

From the data that is available, it has to be assumed that the specific patterns of complementary herbicide applications will not only lead to a higher burden of residues in the harvest, but may also influence the composition of the plants and agronomic characteristics. This aspect was ignored in the risk assessment. EFSA should have requested that Dow submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, also including repeated spraying with each active ingredient in isolation as well as in combination. In addition, more varieties carrying the transgenes should have been included in the field trials to see how the gene constructs interact with the genetic background of the plants.

The material derived from those plants should have been assessed by using omics techniques to investigate changes in plant composition or agronomic characteristics. Further more powerful statistical analysis, such as multidimensional analysis, was not applied to the data.

Based on the available data, no final conclusions can be drawn on the safety of the plants.

3. Toxicology

Despite many highly significant changes in the composition of the plants and agronomic characteristics, no testing of the whole plant (feeding study) was requested. It has to be assumed that this event is essentially different from its comparator in regard to many compositions and biological characteristics. Even if changes taken as isolated data might not directly raise safety concerns, the overall number of effects and their clear significance has to be taken as a starting point for much more detailed investigation of their potential health impacts. In addition, as mentioned, a higher number of applications of the complementary herbicide is not likely to just lead to a higher burden of residues in the harvest, but may also influence the expression of the transgenes or other genome activities in the plants due to interaction with the additionally inserted gene constructs.

Furthermore, the stacked maize differs from the parental lines in regard to the overall amount of toxin produced, which is much higher than in the parental lines. In processed products, such as maize gluten, the toxins can even show a more than tenfold higher concentration. These higher concentrations are relevant for the assessment of overall toxicology as well as for the immune system; nevertheless there were no empirical investigations. This was not considered by EFSA which only – and in absence of any data – tried to conclude on the concentration of Bt toxins in the kernels:

[7] https://www.greenbook.net/dupont-crop-protection/dupont-assure-ii
[8] https://www.greenbook.net/bayer-cropscience/liberty-280-sl
[9] https://www.greenbook.net/dow-agrosciences/enlist-duo
[10] https://www.greenbook.net/dow-agrosciences/enlist-duo
[11] https://www.greenbook.net/monsanto-company/roundup-ultra
[12] https://www.greenbook.net/monsanto-company/roundup-weathermax

"From the limited evidence available, the GMO Panel did not find indications that the presence of the Cry proteins at the levels expressed in the five-event stack maize might act as adjuvants with the potential to enhance a specific immunoglobulin E (IgE) response and to favour the development of an allergic reaction."

In regard to toxicology and potential synergistic or other combinatorial effects, negative impacts of Bt toxins on human and animal health cannot be excluded a priori. Bt toxins have several modes of action and are altered in their biological quality; and are therefore not identical to their natural templates (Hilbeck & Otto, 2015). These facts were completely ignored by EFSA in their opinion which states:

"The Cry1A.105, Cry2Ab2, Cry3Bb1, Cry3Bb1, Cry34Ab1 and Cry1F proteins are delta endotoxins with highly specific insecticidal properties acting through cellular receptors found in target insect species. It is reported that the gastrointestinal tract of mammals, including humans, lacks receptors with high affinity to Cry proteins (...)."

Despite what is claimed by EFSA, not all modes of actions are dependent on the specific mechanisms that only occur in the target insect species. Only very few Bt toxins (especially Cry1Ab, for overview see, for example, Then, 2010) were investigated in more detail in regard to their exact mode of action; and there is no data on the Bt toxins produced in the maize. Further, no data were presented to show that the toxins produced in the plants are only activated and effective in insects. On the other hand, several publications exist showing the effects of Bt toxins in mammals: some Cry toxins are known to bind to epithelial cells in the intestine of mice (Vázquez-Padrón et al., 1999, Vásquez-Padrón et al., 2000). As far as potential effects on health are concerned, Thomas and Ellar (1983), Shimada et al. (2003) Huffmann et al. (2004), Ito et al. (2004), Mesnage et al. (2012) and Bondzio et al. (2013) show that Cry proteins could potentially have an impact on the health of mammals. Two recent publications (de Souza Freire et al., 2014; Mezzomo et al., 2014) confirm hematotoxicity of several Cry toxins, including those being used in genetically engineered plants, such as Cry 1Ab and Cry1Ac. These effects seem to occur with high concentrations and tend to become stronger after several days. Such observations make clear the need for studies on effects after long-term exposure to various dosages, also in combination with material that was sprayed with the complementary herbicides. In this context it is important that the stacked maize is also resistant to the herbicides glyphosate, glufosinate 2-4D and quizalofop, which should be seen as potential co-stressors (see also Then & Bauer-Panskus, 2017).

Moreover, it is evident that Bt toxins can survive digestion to a much higher degree than has been assumed by EFSA: Chowdhury et al., (2003) as well as Walsh et al. (2011) have found that Cry1A proteins can frequently and successfully still be found in the colon of pigs at the end of digestion when they were fed with Bt maize. The Cry1A proteins can show much higher stability at least in monogastric species than predicted by current in vitro digestion experiments. This shows that Bt toxins are not degraded quickly in the gut and can persist in larger amounts until digestion is completed, and there is enough time for interaction between various food compounds. Consequently, there is substantiated concern that especially the stacked event can trigger immune system responses and have adverse health effects.

Notable in this respect are the comments made by Austrian experts (see comments from the Member States) in their summary of findings from feeding studies with Bt-producing plants:

"Some feeding studies in mammals have been performed with GM Bt crops and have found adverse effects, such as:
• toxic effects or signs of toxicity in the small intestine, liver, kidney, spleen, pancreas,
• disturbances in the functioning of the digestive system,

• increased or decreased weight gain compared with controls,
• male reproductive organ damage,
• blood biochemistry disturbances, and
• immune system disturbances.

As Pardo-López at al. and Pigott and Ellar demonstrated, synthetically derived and modified Bt toxins can show higher toxicity than native proteins. Even small changes in the structure of the proteins can cause massive changes in toxicity (Pigott and Ellar 2007; Pardo-López et al. 2009).

Mezzomo et al. evaluated, in Swiss albino mice, the haematotoxicity and genotoxicity of four Bt spore-crystals genetically modified to express individually Cry proteins administered alone by gavage with a single dose of 27 mg/kg, 136 mg/kg or 270 mg/kg, 24 h, 72 h or 7 days before euthanasia. Their results showed that the Bt spore-crystals genetically modified to express individually Cry proteins can cause some haematological risks to vertebrates, increasing their toxic effects with long-term exposure. Taking into account the increased risk of human and animal exposures to significant levels of these toxins, especially through diet, the authors argue that their results suggest that further studies are required to clarify the mechanism involved in the haematotoxicity found in mice, and to establish the toxicological risks to non-target organisms, especially mammals, before concluding that these microbiological control agents are safe for mammals (Mezzomo et al. 2013).”

In addition, French experts are concerned about the safety of the parental plant MON89034:

“In 2007, during the assessment of event MON89034, the agency had requested that additional information be provided regarding the difference in the onset of bladder stones (bladder urinary calculi) between the historical date (0,49%) and the 10% incidence (based on 20 animals) observed in the female of the group that had ingested the highest dose of MON89034 in the 90 days sub chronic toxicity. Even if the historical data from 70 studies run between 1999 and 2006 on rats of the same strain had been provided by the applicants, the Anses considered in 2012 that they were not sufficient to conclude on the absence of connection between the oral administration of MON89034 and the onset of bladder stones observed in the female rats fed with high doses of MON89034.”

Beyond that, the residues from spraying were considered to be outside the remit of the GMO panel. However, without detailed assessment of these residues, no conclusion can be drawn on the safety of the imported products: due to specific agricultural practices in the cultivation of these herbicide resistant plants, there are, for example, specific patterns of applications, exposure, occurrence of specific metabolites and emergence of combinatorial effects that require special attention (see also Kleter et al., 2011).

More detailed assessment is also in accordance with pesticide regulation that requires specific risk assessment of imported plants if the usage of pesticides is different in the exporting countries compared to the usage in the EU. In this regard, it should be taken into account that EFSA (2018a) explicitly stated that no conclusion can be derived on the safety of residues from spraying with glyphosate occurring in genetically engineered plants resistant to this herbicide. Further, in the case of 2,4-D, there are publications suggesting that carcinogenic metabolites are produced in genetically modified plants (Lurquin, 2016), but these were not assessed by EFSA. Further, as stated by experts from member states (see comments from the Member States), the metabolism of quizalofop in quizalofop-resistant plants was not assessed in quizalofop risk assessment (EFSA 2008). Since, in addition, glufosinate is classified as showing reproductive toxicity

(http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN) EFSA should have at least requested data on the combined toxicity of the residues from spraying with the complementary herbicides.

Further, there is a common understanding that commercially traded formulations of glyphosate, such as Roundup, can be more toxic than glyphosate itself. Therefore, the EU has already taken measures to remove problematic additives known as POE tallowmine from the market. Problematic additives are still allowed in those countries where the genetically engineered plants are cultivated. The EU Commission has confirmed the respective gaps in risk assessment:

> *"A significant amount of food and feed is imported into the EU from third countries. This includes food and feed produced from glyphosate-tolerant crops. Uses of glyphosate-based plant protection products in third countries are evaluated by the competent authorities in those countries against the locally prevailing regulatory framework, but not against the criteria of Regulation (EC) No. 1107/2009. (…)." (www.testbiotech.org/node/1637)*

Consequently, EFSA should have requested that Dow submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, also including repeated spraying. It should further be taken into account that not always a mixture of all complementary herbicides will be used in the fields where the maize is cultivated; in some cases just one of them will be used. This might lead to an increase in dosages of the respective complementary herbicides. The choice of herbicide will depend on the price of the herbicide formulations, the respective weed problem and regional agricultural practices. For example, it can be expected that in Argentina, Brazil and the US, there will be different prices, different herbicide formulations and varying regimes of herbicide applications under which the maize is cultivated. None of these specific agronomic practices were considered in the design of the field trials or in EFSA risk assessment.

The material derived from those plants should have been assessed in regard to organ toxicity, immune system responses and reproductive toxicity, also taking combinatorial effects with other plant components and the Bt toxins into account.

There are further relevant issues: for example, the potential impact on the intestinal microbiome also has to be considered. Such effects might be caused by the residues from spraying since glyphosate has been shown to have negative effects on the composition of the intestinal flora of cattle (Reuter et al., 2007), poultry (Shehata et al., 2013) and rodents (Mao et al., 2018). Such effects might be also be caused by the residues from spraying with glufosinate since glufosinate interferes with bacterial growth, and in certain circumstances acts as an antimicrobial agent causing shifts in bacterial community structures (Ahmad and Malloch 1995; Hsiao et al. 2007; Pampulha et al. 2007; Kopcáková et al. 2015; see also comments from Member States). In general, antibiotic effects and other adverse health effects might occur from exposure to a diet containing these plants which were not assessed under pesticide regulation. Further, Bremmer and Leist (1997) examined the possible conversion of NAG to glufosinate in rats. Up to 10% deacetylation occurred at a low dose of 3 mg/kg bw as shown by the occurrence of glufosinate in the faeces. The authors concluded that most of the conversion was caused by bacteria in the colon and rectum, although toxicity findings indicate partial bioavailability (Bremmer & Leist, 1997).

In general, antibiotic effects and other adverse health effects might occur from exposure to a diet containing these plants that were not assessed under pesticide regulation. These adverse effects on health might be triggered by the residues from spraying with the complementary herbicide (see also

van Bruggen et al., 2017). Further attention should be paid to the specific toxicity of the metabolites of the pesticide active ingredients that might occur specifically in the stacked event.

Whatever the case, both the EU pesticide regulation and the GMO regulation require a high level of protection for health and the environment. Thus, in regard to herbicide-resistant plants, specific assessment of residues from spraying with complementary herbicides must be considered to be a prerequisite for granting authorisation.

In addition, cumulative effects have to be investigated if a plant contains or produces other compounds of potential toxicity. It should be acknowledged, that no new methodology is needed to assess the health risks emerging from the combinatorial application of the herbicides and their potential interaction with the other plant constituents. Suitable methodology to assess combinatorial effects that emerge from *simultaneous exposure* to a *fixed combination* of potential stressors via a *defined route of exposure* (as is the case with food and feed products derived from genetically engineered plants that are resistant to several herbicides) is available and widely used. For example, chronic feeding or multigenerational studies are a well-established method to generate the relevant data.

Despite all these open questions regarding potential health impacts, we are not aware of a single sub-chronic or chronic feeding study being performed with whole food and feed derived from the stacked maize. There is feeding study (Zdziarski et al., 2018) with a similar stacked maize which indicates significant health effects and should have caused EFSA to request further studies.

In conclusion, the EFSA opinion on the application for authorisation of the stacked maize (EFSA 2019a) cannot be said to fulfil the requirements for assessment of potential synergistic or antagonistic effects resulting from the combination of the transformation events in regard to toxicology and allergenicity. The hypothesis which should have been used as a starting point is that there will be synergistic effects between the various Bt toxins and between the various Bt toxins and other stressors, such as residues from spraying. Therefore, the effects of the Bt toxins in regard to mammalian cell systems and intestinal microbiomes should have been tested in combination with other stressors. Furthermore, combinatorial (adjuvant) effects triggered by Bt toxins occurring in high concentrations in the stacked maize and especially in gluten prepared from the maize, have to be tested in interaction with known allergens, such as the one occurring in soybeans. For this purpose, EFSA should have requested that Dow submit data from field trials with the highest dosage of glyphosate that can be tolerated by the plants, also including repeated spraying. The material derived from those plants should have been assessed in regard to organ toxicity, immune responses and reproductive toxicity, also taking combinatorial effects with other plants components and the Bt toxins into account.

As a result, the toxicological assessment carried out by EFSA is not acceptable.

4. Allergenicity

According to Santos-Vigil et al (2018), the Bt toxin Cry1Ac can act as an allergen if ingested. This publication highly relevant: the Bt toxin Cry1Ac was used as a source for the synthesis of Cry1A.105 as expressed in the stacked maize. Therefore, the synthetically derived Cry1A.105 toxin produced in the maize has structural similarity with Cry1Ac. If Cry1Ac is suspected of being an allergen, the source of Cry1A.105 has to be verified as allergenic and therefore investigated in detail.

The EU Commission initially noted that the Santos-Vigil et al (2018) publication was relevant for the risk assessment of genetically engineered plants producing Bt toxins, and therefore requested the European Food Safety Authority (EFSA) for an assessment. However, EFSA (EFSA, 2018b) came to the conclusion that the Santos-Vigil et al. (2018) publication does not provide any new information and suffers from methodological flaws. This EFSA opinion, however, is based on a rather biased interpretation of existing publications and it does not provide any evidence that the Santos-Vigil (2018) findings are invalid or irrelevant (Moreno-Fierros et al., 2018).

In conclusion, the EFSA assessment of the stacked maize cannot be said to fulfil the requirements for assessing allergenicity of the source of the transgene. The Santos-Vigil et al (2018) publication has to be considered to be both valid, and not properly assessed by EFSA (Moreno-Fierros et al., 2018). In awareness of the high concentrations of Bt toxins produced in the stacked maize and products derived thereof, EFSA should have started with the hypothesis that the consumption of products derived from the maize can trigger allergic reactions – and should therefore have requested empirical investigations.

Furthermore, there are several studies indicating that immune responses such as adjuvanticity in mammals are triggered by Bt toxins and have to be considered in this context. Studies with the Cry1Ac toxin (Moreno-Fierros et al., 2000; Vázquez et al. 1999; Legorreta-Herrera et al., 2010; Jarillo-Luna et al. 2008; E. González-González et al., 2015; Ibarra-Moreno et al., 2014; Guerrero et al. 2007; Guerrero et al., 2004; Moreno-Fierros et al. 2013) are especially relevant (for review also see Rubio-Infante et al. 2016).

As mentioned, the Bt toxin Cry1Ac was used as a source for the synthesis of Cry1A.105 expressed in the maize.[13] Therefore, the synthetically derived Cry1A.105 toxin produced in the maize has structural similarity with Cry1Ac. If Cry1Ac is immunogenic, Cry1A.105 is also likely to be immunogenic.

All the responses described in the above publications are likely to be dependent on the dosage to which the mammals were exposed. In this regard and again as mentioned above, the investigation of potential immune responses triggered by the maize is highly relevant, it has to be considered that the concentration of the Bt toxins is much higher in gluten meal produced from the maize and can reach a more than tenfold higher concentration compared to the kernels. Therefore, the food and feed products derived from the stacked maize need to be much more carefully risk assessed in regard to their impact on the immune system and potential adjuvanticity compared to those genetically engineered plants producing just one Bt toxin.

In its risk assessment, EFSA did not consider that under real conditions and contrary to what is suggested by the findings of in-vitro studies, Bt toxins will not be degraded quickly in the gut but are likely to occur in substantial concentrations in the large intestine and faeces (Chowdhury et al., 2003; Walsh et al., 2011).

In regard to the degradation of the Bt toxins during ingestion, there is specific cause for concern that the maize or gluten is likely to be fed together with soybeans that naturally produce enzymes which can substantially delay the degradation of Bt toxins in the gut (Pardo-López et al., 2009). In addition, soybeans are known to produce many food allergens. Therefore, the immune responses caused by the allergens in the soybeans might be considerably enhanced by the adjuvant effects of the Bt toxins. Such effects are likely to lead to detrimental effects on health.

[13] See US patent application Patent 6,326,169

Furthermore, it also has to be taken into account that so far only very few Bt toxins produced in genetically engineered plants have been investigated in regard to their potential impact upon the immune system. As yet, only two Bt toxins (Cry1Ac and Cry1Ab) have been tested for their possible effects on the immune system; none of the toxins produced in the maize were investigated in this regard in any empirical research. The effects caused by a combination of these toxins also remain untested. The need for more detailed investigations in regard to potential immunogenic effects is also underlined in the minority opinion in another EFSA opinion (Annex II of EFSA, 2018c).

In conclusion, the EFSA assessment of the stacked maize cannot be said to fulfill the requirements for assessing risks to the immune system.

5. Others

We support the statement of the German experts (BfN) on monitoring (EFSA, 2019b):

> *"To our under-standing present studies are not sufficient to conclude that exposure of the environment and thus effects on non-target organisms will be negligible. Instead, further experiments are necessary to conclude on the exposure and subsequent effects and risks for non-target organisms from the exposure to Bt proteins via manure or sewage. We therefore suggest that EFSA initiates respective research or asks applicants to provide studies suitable to i) quantify exposure, and ii) in the case of exposure provide chronic and subchronic studies on the hazard for soil and water organisms.*
>
> *The monitoring plan has to be elaborated in more detail in order to meet the following requirements:*
> *• Provision of a fully specified list of monitoring parameters,*
> *• Application of standardised sampling methodologies: A basic prerequisite for comparing GMO monitoring data is the use of appropriate standard detection or analytical methods. Several standards specific for GMO monitoring are provided by the Association of German Engineers (VDI). They are available under http://www.vdi.eu/engineering/vdi-standards/,*
> *• Elaboration of a sampling concept,*
> *• In case of monitoring data being collected by external persons or institutions other than the applicant, binding agreements/contracts with third parties are requested which clearly determine what data are provided and how these data are made available,*
> *• Elaboration of the methods of data analysis including the statistical methods,*
> *• Application of the concept of adverse effects and environmental damages: Adverse environmental effects can only be determined if they are related to certain relevant subjects of protection (Bartz et al. 2009). The subject of protection is damaged if it is significantly adversely affected. The identification of a significant adverse effect should consider both its intensity (e.g. extent of loss) and the value of the impaired subject of protection (e.g. high value of protected species).*
>
> *The monitoring should be run in regions, where viable MON89034 x 1507 x MON88017 x 59122 x DAS40278-9 maize will be transported, stored, packaged, processed or used for food/feed. In case of substantial losses and spread of MON89034 x 1507 x MON88017 x 59122 x DAS40278-9 maize all receiving environments need to be monitored.*
>
> *The time period of monitoring needs to be sufficient to detect delayed or long-term adverse effects. Therefore, it may be necessary to extend the monitoring regarding certain parameters beyond the period of consent.*

Since traders may commingle MON89034 x 1507 x MON88017 x 59122 x DAS40278-9 maize with other commercial GM maize imported, processed or used for food/feed, the applicant is requested to explain how the monitoring will be designed to distinguish between potential adverse effects caused by MON89034 x 1507 x MON88017 x 59122 x DAS40278-9 maize and those caused by other GM maize."

Besides the methods of detection, other methods for quantifying exposure to Bt toxins need to be made publicly available in order to facilitate monitoring. Food and feed producers, farmers as well as experts dealing with environmental exposure (for example, via waste material, spillage and manure) have to be able to gather independent information on their exposure to the toxins via independent laboratories. As yet, these methods are regarded as confidential business information and are not made available upon request by EFSA. Thus, the Commission should ensure that the relevant data are both publicly available and also reliable.

As existing evidence shows (Székács et al., 2011; Shu et al., 2018), the methods need to be carefully evaluated to ensure that the results are reliable, comparable and reproducible. Therefore, fully evaluated methods have to be published that allow the Bt concentration in the maize to be measured by independent scientists as is the case for other plant protection compounds used in food and feed production. This is necessary to make sure that the environment as well as humans and animals coming into contact with the material (for example, via dust, consumption or manure) are not exposed to higher quantities of Bt toxins than described in the application.

6. Environmental risk assessment

Dow completely ignored the appearance of teosinte in Spain and France (see Testbiotech, 2016; Trtikova et al, 2017). Thus, the statement that no wild relatives of maize would occur in Europe is simply wrong. In its assessment of the volunteer potential, the information provided by Dow is largely outdated. As Pascher et al (2016) show, the volunteer potential of maize is higher than assumed by Dow. Further, in awareness of the findings of Fang et al. (2018), the glyphosate-resistant maize needs to be examined in detail regarding next generation effects, volunteer potential (persistence) and gene flow. There are substantial reasons for following a hypothesis that the maize can show higher fitness compared to conventional maize.

In its opinion, EFSA (2019a) was aware of the occurrence of teosinte in the EU and tried to assess the risks of gene flow. However, EFSA (2019a) is wrong for several reasons:

- Without more data on the teosinte species growing in the EU, the likelihood of gene flow from the maize to teosinte cannot be assessed (Trtikova et al, 2017). The same is true for gene flow from teosinte to genetically engineered plants.
- Furthermore, the characteristics of potential hybrids and next generations have to be investigated and cannot be predicted simply from the data of the original event. It is well known that there can be next generation effects and interference from genetic background that cannot be predicted from the assessment of the original event (Kawata et al., 2009; Cao et al., 2009; Yang et al., 2017; Bollinedi et al., 2017; Lu and Yang, 2009; Vacher et al., 2004; Adamczyk & Meredith, 2004; Adamczyk et al., 2009). This issue is relevant for gene flow from maize to as well from teosinte to maize.
- Finally, it is well established under EU regulation that it is the applicant who has to present data sufficient to show that the respective event is safe before the application can be considered to be valid (see Kraemer, 2016). Thus, an application with incorrect or missing

information on crucial aspects of environmental risk assessment cannot be accepted as a starting point for EFSA risk assessment.

As the German experts (BfN) summarise (EFSA, 2019b):

> *"The potential for gene flow between teosinte and maize is high (Ellstrand et al. 2007, Chavez et al. 2012). Chavez et al. concluded that biosafety regulators in regions where teosinte occurs should not only consider outcrossing from maize to teosinte but also the possibility of teosinte acting as a genetic bridge back to maize. Teosinte grains are very difficult to control. The kernels have got a high duration in the seedbank and long dormancy. Teosinte flowers earlier and longer than maize and pollen of both species can spread over long distances. Teosinte is considered an agricultural pest which needs management."*

EFSA should have requested data from the applicant to show that no adverse effects can occur through gene flow from the maize to teosinte and / or from teosinte to the maize volunteers. In the absence of such data, the risk assessment and the authorisation have to be regarded as not valid.

Without detailed consideration of the hazards associated with the potential gene flow from maize to teosinte and from teosinte to maize, no conclusion can be drawn on the environmental risks of spillage from the stacked maize.

Consequently, environmental risk assessment carried out by EFSA is not acceptable.

7. Conclusions and recommendations

The EFSA risk assessment cannot be accepted.

References

Adamczyk Jr, J.J., & Meredith Jr, W.R. (2004) Genetic basis for variability of Cry1Ac expression among commercial transgenic Bacillus thuringiensis (Bt) cotton cultivars in the United States. Journal of Cotton Science, 8(1): 433-440. http://www.cotton.org/journal/2004-08/1/17.cfm

Adamczyk, J.J., Perera, O., Meredith, W.R. (2009) Production of mRNA from the cry1Ac transgene differs among Bollgard® lines which correlates to the level of subsequent protein. Transgenic Research, 18: 143-149. https://doi.org/10.1007/s11248-008-9198-z

Agapito-Tenfen, S.Z., Vilperte, V., Benevenuto, R.F., Rover, C.M., Traavik, T.I., Nodari, R.O. (2014) Effect of stacking insecticidal cry and herbicide tolerance epsps transgenes on transgenic maize proteome. BMC plant biology 14: 346.

Ahmad, I., Malloch, D. (1995) Interaction of soil microflora with the bioherbicide phosphinothricin. Agriculture, Ecosystems and Environment 54(3): 165-174.

Bollinedi, H., S. G.K, Prabhu, K.V., Singh, N.K., Mishra, S., Khurana, J.P., Singh, A.K. (2017) Molecular and Functional Characterization of GR2-R1 Event Based Backcross Derived Lines of Golden Rice in the Genetic Background of a Mega Rice Variety Swarna. PLoS ONE 12(1): e0169600. https://doi.org/10.1371/journal.pone.0169600

Bondzio, A., Lodemann, U., Weise, C., Einspanier, R. (2013) Cry1Ab treatment has no effects on viability of cultured porcine intestinal cells, but triggers hsp70 expression. Plos One, 8(7): e67079. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0067079

Bremmer, J.N. & Leist, K.-H. (1997) Disodium-N-acetyl-L-glufosinate; AE F099730 – Hazard evaluation of Lglufosinate produced intestinally from N-acetyl-L-glufosinate. Hoechst Schering AgrEvo GmbH, Safety Evaluation Frankfurt. TOX97/014. A58659. Unpublished.

Cao, Q.-J., Xia, H., Yang, X., Lu, B.-R. (2009) Performance of Hybrids between Weedy Rice and Insect-resistant Transgenic Rice under Field Experiments: Implication for Environmental Biosafety Assessment. J. Integr. Plant Biol. 51: 1138-1148. https://doi.org/10.1111/j.1744-7909.2009.00877.x

Chavez, N.B., Flores, J.J., Martin, J., Ellstrand, N.C., Guadagnuolo, R., Heredia, S., Welles, S.R. (2012) Maize x teosinte hybrid cobs do not prevent crop gene introgression. Economic Botany, 66(2), 132-137.

Chowdhury, E. H., Kuribara, H., Hino, A., Sultana, P., Mikami, O., Shimada, N., ... & Nakajima, Y. (2003) Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. Journal of Animal Science, 81(10): 2546-2551. https://academic.oup.com/jas/article-abstract/81/10/2546/4789819

de Souza Freire, I., Miranda-Vilela, A.l., Pereira Barbosa, L.C., Soares Martins, E., Gomes Monnerat, R., Koppe Grisolia, C. (2014) Evaluation of cytotoxicity, genotoxicity and hematotoxicity of the recombinant spore-crystal complexes Cry1Ia, Cry10Aa and Cry1Ba6 from Bacillus thuringiensis in Swiss mice. Toxins, 6: 2872-2885. https://www.mdpi.com/2072-6651/6/10/2872/htm

EFSA (2008) Conclusion regarding the peer review of the pesticide risk assessment of the active substance quizalofop-P (considered variants quizalofop-P-ethyl and quizalofop-P-tefuryl). EFSA Scientific Report, 205: 1-216.

EFSA (2018a) Reasoned Opinion on the review of the existing maximum residue levels for glyphosate according to Article 12 of Regulation (EC) No 396/2005. EFSA Journal 2018;16(5):5263, 230 pp. https://doi.org/10.2903/j.efsa.2018.5263)

EFSA (2018b) Relevance of new scientific information (Santos-Vigil et al., 2018) in relation to the risk assessment of genetically modified crops with Cry1Ac. EFSA supporting publication 2018:EN-1504. 13 pp. doi:10.2903/sp.efsa.2018.EN-1504. https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/sp.efsa.2019.EN-1504

EFSA (2018c) Scientific opinion on the assessment of genetically modified maize Bt11 x MIR162 x 1507 x GA21 and three subcombinations independently of their origin, for food and feed uses under Regulation (EC) No 1829/2003 (application EFSA-GMO-DE-2010-86). EFSA Journal 2018;16(7):5309, 35 pp. https://doi.org/10.2903/j.efsa.2018.5309

EFSA (2019a) Scientific Opinion on the assessment of genetically modified maize MON 89034 x 1507 x MON 88017 x 59122 x DAS-40278-9 and subcombinations independently of their origin for food and feed uses, import and processing under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2013-113). EFSA Journal 2019;17(1):5521, 30 pp. https://doi.org/10.2903/j.efsa.2019.5521

EFSA (2019b) Comments from the experts of Member States on the scientific opinion on the assessment of genetically modified maize MON 89034 x 1507 x MON 88017 x 59122 x DAS-40278-9 and subcombinations (application EFSA-GMO-NL-2013-113). Accessed via the register of EFSA, http://registerofquestions.efsa.europa.eu/roqFrontend/login?0

Ellstrand, N.C., Garner, L.C., Hegde, S., Guadagnuolo, R., Blancas, L. (2007) Spontaneous hybridization between maize and teosinte. Journal of Heredity, 98(2): 183-187.

Fang, J., Nan, P., Gu, Z., Ge, X., Feng, Y.-Q., Lu, B.-R. (2018) Overexpressing Exogenous 5-Enolpyruvylshikimate-3-Phosphate Synthase (EPSPS) Genes Increases Fecundity and Auxin Content of Transgenic Arabidopsis Plants. Frontiers in Plant Sciences, 9: 233. https://doi.org/10.3389/fpls.2018.00233

González-González, E., García-Hernández A.L., Flores-Mejía, R., López-Santiago, R., Moreno-Fierros L. (2015) The protoxin Cry1Ac of Bacillus thuringiensis improves the protection conferred by intranasal immunization with Brucella abortus RB51 in a mouse model. Vet. Microbiol. 175: 382–388. http://dx.doi.org/10.1016/j.vetmic.2014.11.021

Guerrero, G.G. & Moreno-Fierros L. (2007) Carrier potential properties of Bacillus thuringiensis Cry1A toxins for a diphtheria toxin epitope, Scandinavian Journal of Immunology, 66: 610–618. http://dx.doi.org/10.1111/j.1365-3083.2007.01992.x

Guerrero, G.G., Dean, D.H., Moreno-Fierros, L. (2004) Structural implication of the induced immune response by Bacillus thuringiensis cry proteins: role of the N-terminal region. Molecular Immunology, 41: 1177-1183. http://dx.doi.org/10.1016/j.molimm.2004.06.026

Hilbeck, A. & Otto, M. (2015) Specificity and combinatorial effects of Bacillus thuringiensis Cry toxins in the context of GMO risk assessment. Frontiers Environmental Science, 3: 71.

Hsiao, C.L., Young, C.C., Wang C.Y.W. (2007) Screening and identification of glufosinate-degrading bacteria from glufosinate-treated soils. Weed science, 55(6), 631-637.

Huffmann, D.L., Abrami, L., Sasik, R., Corbeil, J., van der Goot, G., Aroian, R.V. (2004) Mitogenactivated protein kinase pathways defend against bacterial pore-forming toxins. Proceedings of the National Academy of Sciences, 101(30): 10995-11000. http://www.pnas.org/content/101/30/10995.short

Ibarra-Moreno, S., García-Hernández, A.L., Moreno-Fierros L. (2014) Coadministration of protoxin Cry1Ac from Bacillus thuringiensis with metacestode extract confers protective immunity to murine cysticercosis. Parasite Immunol. 36: 266-270. http://dx.doi.org/10.1111/pim.12103

Ito, A., Sasaguri, Y., Kitada, S., Kusaka, Y., Kuwano, K., Masutomi, K., Mizuki, E., Akao, T., Ohba, M. (2004) Bacillus thuringiensis crystal protein with selective cytocidal action on human cells. Journal of Biological Chemistry, 279: 21282-21286. http://www.jbc.org/content/279/20/21282.short

Jarillo-Luna, A., Moreno-Fierros L., Campos-Rodríguez R., Rodríguez-Monroy, M.A., Lara-Padilla, E., Rojas-Hernández, S. (2008) Intranasal immunization with Naegleria fowleri lysates and Cry1Ac induces metaplasia in the olfactory epithelium and increases IgA secretion. Parasite Immunol., 30: 31-38. http://dx.doi.org/10.1111/j.1365-3024.2007.00999.x

Kawata, M., Murakami, K., Ishikawa, T. (2009) Dispersal and persistence of genetically modified oilseed rape around Japanese harbors. Environmental Science and Pollution Research, 16(2): 120-126. https://link.springer.com/article/10.1007/s11356-008-0074-4

Kleter, G.A., Unsworth, J.B., Harris, C.A. (2011) The impact of altered herbicide residues in transgenic herbicide-resistant crops on standard setting for herbicide residues. Pest Managment Science, 67(10): 1193-1210. https://onlinelibrary.wiley.com/doi/abs/10.1002/ps.2128

Kopcáková, A., Legáth, J., Pristaš, P., Javorský, P. (2015) Already a short-term soils exposure to the field-rate glufosinate concentration significantly influences soil bacterial communities. Soil and Water Research 10(4): 271-277.

Kraemer, L. (2016) Teosinte plants in the European environment and its implication for market authorisation of genetically engineered maize Legal analysis commissioned by Testbiotech, http://www.testbiotech.org/node/1773

Legorreta-Herrera, M., Oviedo Meza, R., Moreno-Fierros L. (2010) Pretreatment with Cry1Ac protoxin modulates the immune response, and increases the survival of plasmodium -infected CBA/Ca mice. J Biomed Biotechnol, http://dx.doi.org/10.1155/2010/198921

Lu, B.-R., Yang, C. (2009) Gene flow from genetically modified rice to its wild relatives: Assessing potential ecological consequences. Biotechnology Advances, 27(6): 1083-1091. https://doi.org/10.1016/j.biotechadv.2009.05.018

Lurquin, P.F. (2016) Production of a toxic metabolite in 2, 4-D-resistant GM crop plants. 3 Biotech, 6(1): 1-4. https://link.springer.com/article/10.1007/s13205-016-0387-9#CR25

Mao, Q., Manservisi, F., Panzacchi, S., Mandrioli, D., Menghetti, I., Vornoli, A., Bua, L., Falcioni, L., Lesseur, C., Chen, J., Belpoggi, F., Hu, J. (2018) The Ramazzini Institute 13-week pilot study on glyphosate and Roundup administered at human-equivalent dose to Sprague Dawley rats: effects on the microbiome, Environmental Health, 17: 50. https://doi.org/10.1186/s12940-018-0394-x

Mesnage, R., Clair, E., Gress, S., Then, C., Székács, A., Séralini, G.-E. (2012) Cytotoxicity on human cells of Cry1Ab and Cry1Ac Bt insecticidal toxins alone or with a glyphosate-based herbicide. Journal of Applied Toxicology, 33(7): 695–699. https://onlinelibrary.wiley.com/doi/abs/10.1002/jat.2712

Mesnage, R., Agapito-Tenfen, S. Z., Vilperte, V., Renney, G., Ward, M., Séralini, G. E., ... & Antoniou, M. N. (2016) An integrated multi-omics analysis of the NK603 Roundup-tolerant GM maize reveals metabolism disturbances caused by the transformation process. Scientific Reports, 6: 37855.

Mezzomo, B.P., Miranda-Vilela, A.L., de Souza Freire, I., Pereira Barbosa, L.C., Portilho, F.A., Marques Lacava, Z.G., Koppe Grisolia, C. (2013) Hematotoxicity of Bacillus thuringiensis as Spore-crystal Strains Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa in Swiss Albino Mice. J Hematol Thromb Dis, 1:1.

Moreno-Fierros, L., García N., Gutiérrez, R., López-Revilla, R., Vázquez-Padrón, R.I., (2000) Intranasal, rectal and intraperitoneal immunization with protoxin Cry1Ac from Bacillus thuringiensis induces compartmentalized serum, intestinal, vaginal and pulmonary immune responses in Balb/c mice. Microbes Infect., 2: 885–890. http://dx.doi.org/10.1016/S1286-4579(00)00398-1

Moreno-Fierros, L., García-Hernández, A.L., Ilhuicatzi-Alvarado, D., Rivera-Santiago, L., Torres-Martínez, M., Rubio-Infante N., Legorreta-Herrera, M. (2013) Cry1Ac protoxin from Bacillus thuringiensis promotes macrophage activation by upregulating CD80 and CD86 and by inducing IL-6, MCP-1 and TNF-α cytokines, Int. Immunopharmacol. 17: 1051-1066. http://dx.doi.org/10.1016/j.intimp.2013.10.005

Moreno-Fierros, L., Santos-Vigil, K., Ilhicatzi-Alvarado, D. (2018) Response to assessment of the Relevance of new scientific information (Santos-Vigil et al., 2018) in relation to the risk assessment of genetically modified crops with Cry1Ac of European Food Safety Authority (EFSA). www.testbiotech.org/node/2304

Pampulha, M.E., Ferreira, M.A.S.S., Oliveira, A. (2007) Effects of a phosphinothricin based herbicide on selected groups of soil microorganisms. J Basic Microbiol 47(4): 325-331. https://onlinelibrary.wiley.com/doi/abs/10.1002/jobm.200610274

Pardo-López, L., Muñoz-Garay, C., Porta, H., Rodríguez-Almazán, C., Soberón, M., Bravo, A. (2009) Strategies to improve the insecticidal activity of Cry toxins from Bacillus thuringiensis. Peptides 30(3): 589–595. https://www.sciencedirect.com/science/article/pii/S0196978108003264

Pascher, K. (2016) Spread of volunteer and feral maize plants in Central Europe: recent data from Austria. Environmental Sciences Europe, 28(1): 30. https://link.springer.com/article/10.1186/s12302-016-0098-1

Pigott, C.R., Ellar, D.J. (2007) Role of receptors in Bacillus thuringiensis crystal toxin activity. Microbiol Mol Biol Rev 71(2): 255-281.

Reuter, T., Alexander, T.W., Martinez, T.F., McAllister, T.A. (2007) The effect of glyphosate on digestion and horizontal gene transfer during in vitro ruminal fermentation of genetically modified canola. J Sci Food Agric 87: 2837-2843. https://onlinelibrary.wiley.com/doi/abs/10.1002/jsfa.3038

Rubio Infante, N., & Moreno-Fierros, L. (2016) An overview of the safety and biological effects of Bacillus thuringiensis Cry toxins in mammals. Journal of Applied Toxicology, 36(5): 630-648. http://onlinelibrary.wiley.com/doi/10.1002/jat.3252/full

Santos-Vigil, K.I., Ilhuicatzi-Alvarado, D., García-Hernández, A.L., Herrera-García, J.S., Moreno-Fierros, L. (2018) Study of the allergenic potential of Bacillus thuringiensis Cry1Ac toxin following intra-gastric administration in a murine model of food-allergy. International immunopharmacology, 61: 185-196. https://www.sciencedirect.com/science/article/pii/S1567576918302467

Shehata, A.A., Schrödl, W., Aldin, A.A., Hafez, H.M., Krüger, M. (2012) The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. Curr Microbiol 6(4): 350-358. https://link.springer.com/article/10.1007/s00284-012-0277-2

Shimada, N., Kim, Y.S., Miyamoto, K., Yoshioka, M., Murata, H. (2003) Effects of Bacillus thuringiensis Cry1Ab toxin on mammalian cells. J Vet Med Sci, 65: 187-191. https://www.jstage.jst.go.jp/article/jvms/65/2/65_2_187/_article/-char/ja/

Shu, Y., Romeis, J., Meissle, M. (2018) No interactions of stacked Bt maize with the non-target aphid Rhopalosiphum padi and the spider mite Tetranychus urticae. Frontiers in Plant Science, 9: 39. https://www.frontiersin.org/articles/10.3389/fpls.2018.00039

Székács, A., Weiss, G., Quist, D., Takács, E., Darvas, B., Meier, M., Swain, T., Hilbeck, A. (2011) Interlaboratory comparison of Cry1Ab toxin quantification in MON 810 maize by ezyme-immunoassay. Food and Agricultural Immunology, 23(2): 99-121. www.tandfonline.com/doi/abs/10.1080/09540105.2011.604773

Testbiotech (2016) Cultivation of genetically engineered maize: Risks not under control - Overview: Why the EU should not allow the cultivation of transgenic maize engineered to produce insecticidal toxins. Testbiotech Background, 24 - 11 - 2016. https://www.testbiotech.org/node/1759

Then, C. (2010) Risk assessment of toxins derived from Bacillus thuringiensis: synergism, efficacy, and selectivity. Environmental Science and Pollution Research, 17(3): 791-797. https://link.springer.com/article/10.1007/s11356-009-0208-3

Then, C., & Bauer-Panskus, A. (2017) Possible health impacts of Bt toxins and residues from spraying with complementary herbicides in genetically engineered soybeans and risk assessment as performed by the European Food Safety Authority EFSA. Environmental Sciences Europe, 29(1): 1. https://enveurope.springeropen.com/articles/10.1186/s12302-016-0099-0

Thomas, W.E. & Ellar, D.J. (1983) Bacillus thuringiensis var israelensis crystal delta-endotoxin: effects on insect and mammalian cells in vitro and in vivo. Journal of Cell Science, 60(1): 181-197. http://jcs.biologists.org/content/60/1/181.short

Trtikova, M., Lohn, A., Binimelis, R., Chapela, I., Oehen, B., Zemp, N., Widmer, A., Hilbeck, A. (2017) Teosinte in Europe - Searching for the Origin of a Novel Weed. Scientific Reports, 7: 1560. https://www.nature.com/articles/s41598-017-01478-w

Trtikova, M., Wikmark, O.G., Zemp, N., Widmer, A., Hilbeck, A. (2015) Transgene expression and Bt protein content in transgenic Bt maize (MON810) under optimal and stressful environmental conditions. PloS one, 10(4): e0123011. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0123011

Vacher, C., Weis, A.E., Hermann, D., Kossler, T., Young, C., Hochberg, M.E. (2004) Impact of ecological factors on the initial invasion of Bt transgenes into wild populations of birdseed rape (Brassica rapa). Theor. Appl. Genet. 109: 806-814. https://doi.org/10.1007/s00122-004-1696-7

Van Bruggen, A.H.C., He, M.M., Shin, K., Mai, V., Jeong, K. C., Finckh, M.R., Morris, J.G. (2018) Environmental and health effects of the herbicide glyphosate. Science of The Total Environment, 616: 255-268. https://www.sciencedirect.com/science/article/pii/S0048969717330279

Vásquez-Padrón. R.I., Gonzáles-Cabrera. J., Garcia-Tovar. C., Neri-Bazan. L., Lopéz-Revilla. R., Hernández. M., Morena-Fierros. L., de la Riva, G.A. (2000) Cry1Ac Protoxin from Bacillus thuringiensis sp. kurstaki HD73 binds to surface proteins in the mouse small intestine. Biochem and Biophys Research Comm, 271: 54-58.
www.sciencedirect.com/science/article/pii/S0006291X00925841

Vázquez-Padrón, R.I., Moreno-Fierros, L., Neri-Bazán, L., de la Riva, G.A., López-Revilla, R. (1999) Intragastric and intraperitoneal administration of Cry1Ac protoxin from Bacillus thuringiensis induces systemic and mucosal antibody responses in mice. Life Sciences, 64(21): 1897-1912.
www.academia.edu/download/43177637/Intragastric_and_intraperitoneal_adminis20160228-1573-204m62.pdf

Walsh, M. C., Buzoianu, S. G., Gardiner, G. E., Rea, M. C., Gelencsér, E., Jánosi, A., ... & Lawlor, P. G. (2011) Fate of transgenic DNA from orally administered Bt MON810 maize and effects on immune response and growth in pigs. PLoS One, 6(11): e27177.
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0027177

Yang, X., Li, L., Jiang, X., Wang, W., Cai, X., Su, J., Wang, F., Lu, B.-R. (2017) Genetically engineered rice endogenous 5-enolpyruvoylshikimate-3-phosphate synthase (epsps) transgene alters phenology and fitness of crop-wild hybrid offspring. Sci. Rep. 7: 6834.
https://doi.org/10.1038/s41598-017-07089-9

Zdziarski, I.M., Carman, J.A. and Edwards, J.W. (2018) Histopathological investigation of the stomach of rats fed a 60% genetically modified corn diet. Food and Nutrition Sciences, 9: 763-796.
https://doi.org/10.4236/fns.2018.96058