TESTBIOTECH Background 08- 08 - 2019

Testbiotech comment on EFSA's assessment of genetically engineered maize MON 87427 x MON 89034 x MIR162 x NK603 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2016-131) by Bayer/Monsanto

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Introduction

The EFSA GMO panel assessed the four-stacked maize MON 87427 x MON 89034 x MIR162 x NK603, which is derived from crossing genetically engineered maize events. This maize was assessed previously (EFSA, 2019a). The maize contains genes conferring triple resistance to glyphosate and produces three insecticides:

- MON87427 expressing CP4 EPSPS protein for tolerance to glyphosate-containing herbicides;
- MON 89034 expressing the insecticidal proteins Cry1A.105 (artificially synthesized) and Cry2Ab2,
- MIR162 expressing the insecticidal protein Vip3Aa20 and phosphomannose isomerase (PMI) which is a selectable marker;
- NK603 expressing two variants of CP4 EPSPS protein for tolerance to glyphosate-containing herbicides.

Consequently, the stacked GE maize has triple resistance to glyphosate, making it tolerant to high dosages and repeated sprayings as applied in fields with herbicide-resistant weeds. Further, it produces three toxins against the larvae of *Lepidoptera* (butterflies) that feed on the plants ('pest insects'). Implementing Regulation 503/2003 has been applied in this case.

1. Molecular characterisation

The process of genetic engineering involved several deletions and insertions in the parental GE 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; therefore, no detailed investigations were carried out in this regard. Furthermore, other gene products such as dsRNA 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 has indicated that expression of Cry1A.105, Cry2Ab2 and EPSPS proteins in genetically engineered maize can induce changes in the overall proteome of the respective GE maize line, with impacts on associated endogenous metabolic pathways (Agapito-Tenfen et al. 2014). Similar transgenes are also present in the stacked maize. Thus, robust data should have been

presented to assess whether metabolic changes with relevance to biosafety occur in the stacked maize. Further, Mesnage et al. (2016) demonstrated alteration in stress-related metabolic pathways for NK603, which were, amongst others, accompanied by increased levels of polyamines. The authors stated that polyamines can provoke toxicological effects on their own or potentiate adverse effects of histamine.

Therefore, EFSA should have requested much more detailed investigation into potential biologically active gene products and changes in metabolic pathways.

In regard to the expression of the additionally inserted genes, Implementing Regulation 503/2013 requests “Protein expression data, including the raw data, obtained from field trials and related to the conditions in which the crop is grown” (in regard to the newly expressed proteins).”

However, there are three reasons why the data presented do not represent the conditions in which the plants are grown: (1.1) the field trials were not conducted in all relevant regions where the maize will be cultivated, and no extreme weather conditions were taken into account; (1.2) the field trials did not take current agricultural management practices into account; (1.3.) only one transgenic variety was included in the field trials.

1.1.
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 responses can lead to unexpected changes in plant metabolism inheriting additional EPSPS enzymes. However, the expression of the additional enzymes was only measured under field conditions in the US for one year. 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. Whatever the case, they should have been tested in the maize producing countries in South America.

1.2.
Due to increased weed pressure, it has to be expected that these plants will be exposed to high and also repeated dosages of glyphosate. Higher applications of the herbicide 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 EFSA risk assessment. EFSA should have requested the applicant to submit data from field trials with the highest dosage of glyphosate that can be tolerated by the plants, including repeated spraying.

1.3.
It is known that the genomic background of the variety can influence the expression of the inserted genes (see, for example, Trtikova et al., 2015). Therefore, EFSA, should have requested additional data from several varieties, including those cultivated in South America.

1.4.
The findings on flaws in risk assessment are supported by analysis data from previous applications with the same parental events. Data presented in Table 1 show widely differing gene expression and content of Vip3Aa20.

Table 1: Gene expression and content of Vip3Aa20 present in maize MIR162 in grain (µg/g dry weight, mean values)

Application (EFSA opinion)	Details from field trials	Content of Vip3Aa20
MON 87427 x MON 89034 x MIR162 x NK603 (EFSA, 2019a)	Field trials at five locations in the USA in 2013 (sprayed with glyphosate)	59
Bt11 x MIR162 x MIR604 x 1507 x 5307 x GA21 (EFSA, 2019b)	Field trials at three locations in the US in 2012 (not sprayed with complementary herbicides)	100
Bt11 x MIR162 x 1507 x GA21 (EFSA, 2018a)	Field trials at one single location in the US 2008 (sprayed?)	28
Bt11 x MIR162 x MIR604 x GA21 (EFSA, 2015a)	Single location in the US in 2006 (sprayed?)	140
MIR162 (EFSA, 2012)	Bloomington, Illinois 2005, Hybrid A	46
	York, Nebraska, 2005, Hybrid B	41
	Bloomington, Illinois, 2006, Hybrid A	124
	Bloomington, Illinois, 2006, Hybrid B	84
	Brazil, Ituiutaba, 2007	62
	Brazil, Uberlandia , 2007	59

These data show a range of mean values between 28 µg/g and 140 µg/g for Vip3Aa20 in the grain; this is evidence of highly variable gene expression, with the actual content of the additional protein being unpredictable.

These findings are supported and strengthened by the range of values found in the each of the field trials and in the different plant tissues. For example, in the data provided on MON 87427 x MON 89034 x MIR162 x NK603 for grain, the content of Vip3Aa20 showed a range from 18 µg/g (parental event MIR162) to 95 µg/g (in the stacked) (EFSA, 2019a), while in other cases even 166 µg/g were measured as maximum range in the grain (EFSA, 2012). The factors influencing the content might seem variable. As EFSA (2012) stated in previous opinions (2012), "*a year-to-year and site-to-site variation is evident*". However, genetic backgrounds of different varieties and effects from stacking seem to be relevant as well.

There are other findings in regard to gene expression that show the need for much more detailed investigation: for example, a comparison of MON87427 data provided in a previous application (EFSA, 2017) with the data from the current application (EFSA, 2019a) shows a clear trend towards higher gene expression of the EPSPS protein. Other observations can be made by comparing the gene expression in MON89034: data on Cry1A.105 in leaves (V2-V4) and whole plants show a much higher level in the EFSA 2019a data compared to EFSA 2017 data; for kernels it is the other way round.

Furthermore, the available data (EFSA, 2015b) indicate that spraying glyphosate has a strong impact on gene expression in some parts of the plants of MON87427: for forage, the CP4 EPSPS level (mean value) was 75 µg/g (dry weight), if sprayed on material it was 140 µg/g (dry weight). Compared to data on sprayed material from MON87427 forage given in EFSA (2019a), these show

a higher level 160 µg/g (dry weight), but there are no data provided on forage without spraying.

Conclusion on molecular characterisation
We conclude that the available data strongly indicate gene expression of several of the additional genes is likely to depend on, or be influenced by, stacking, varietal background, the spraying of the herbicide or environmental conditions.

Therefore, 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. In any case, they should have been tested in the maize producing countries in South America. Furthermore, EFSA should have requested the applicant to submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, including repeated spraying. In addition, EFSA should have requested data from several varieties, including those cultivated in South America.

The material derived from the plants should have been assessed by using omics techniques to investigate changes in the gene activity of the transgene and the plants genome, as well as changes in metabolic pathways and the emergence of unintended biological active gene products. Such in-depth investigations should not depend on findings indicating potential adverse effects, they should always be necessary to come to sufficiently robust conclusions to inform the next steps in risk assessment.

2. Comparative analysis (for compositional analysis and agronomic traits and GM phenotype)

Implementing Regulation 503/2013 requests:

> *"In the case of herbicide tolerant genetically modified plants and in order to assess whether the expected agricultural practices influence the expression of the studied endpoints, three test materials shall be compared: the genetically modified plant exposed to the intended herbicide; the conventional counterpart treated with conventional herbicide management regimes; and the genetically modified plant treated with the same conventional herbicide management regimes."*
>
> *"The different sites selected for the field trials shall reflect the different meteorological and agronomic conditions under which the crop is to be grown; the choice shall be explicitly justified. The choice of non-genetically modified reference varieties shall be appropriate for the chosen sites and shall be justified explicitly."*

However, the data presented do not represent expected agricultural practices or the different meteorological and agronomic conditions under which the crop is to be grown. There are three reasons: (2.1.) the field trials were not conducted in all relevant regions where the maize will be cultivated, and no extreme weather conditions were taken into account; (2.2.) the field trials did not take the current agricultural management practices into account; (2.3.) only one transgenic variety was included in the field trials.

2.1.
Field trials for compositional and agronomic assessment of the stacked maize were conducted in the US for only one year and not in other relevant maize production areas, such as Brazil and Argentina. As shown in the EFSA opinion (2019a), "*An exceptional weather condition was reported at one of the selected site*." This weather condition was early frost before the harvest,

which is not representative for extreme weather conditions at an earlier stage of cultivation that would be much more relevant.

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. However, as our analysis on gene expression shows, specific site by site and year by year effects have to be expected.
- No data were generated representing more extreme environmental conditions, such as those caused by climate change.

More specifically, Fang et al (2018) showed that stress responses can lead to unexpected changes in plant metabolism inheriting additional EPSPS enzymes. However, no experiments were requested to show to which extent specific environmental conditions will influence plant composition and agronomic characteristics. In any case, the plants should have been subjected to a much broader range of defined environmental conditions and stressors to gather reliable data. This necessity is underlined in our analysis of gene expression shown above.

2.2.
Due to high weed pressure in many maize growing regions, it has to be expected that these plants will be exposed to higher amounts and repeated dosages of glyphosate. It has to be taken into account that the herbicides can be sprayed with high dosages and repeated sprayings. These agricultural practices have to be taken into account to assess whether the expected agricultural practices will influence the expression of the studied endpoints. However, this requirement was mostly ignored by EFSA and the company: glyphosate was only sprayed at an early stage of vegetation and at comparably low dosages.

Industry recommendations suggest dosages to be sprayed on herbicide resistant maize of up to approx. 3,5 kg a.i./ha glyphosate post-emergence, 9 kg per season, and even higher rates (www.greenbook.net/monsanto-company/roundup-weathermax; www.greenbook.net/monsanto-company/roundup-ultra). From the available data, 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, which is supported by the analysis of the gene expression provided above, was ignored in the EFSA risk assessment.

EFSA should have requested the company to submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, including repeated spraying with each active ingredient individually as well as in combination. Taking into account the specific characteristics of the stacked maize, only the application of high and repeated dosages of glyphosate should have been regarded as representative for expected agricultural practices.

2.3.
It is known that the genomic background of the variety can influence the expression of the inserted genes (see, for example, Trtikova et al., 2015). Therefore, EFSA should have requested additional data from several varieties, including those cultivated in South America, to examine how the gene constructs interact with the genetic background of the plants. This approach is supported by the analysis of the gene expression provided above but was ignored in the EFSA risk assessment.

2.4.
Only data from a low number of agronomic parameters (13) were subjected to statistical analysis in accordance with EFSA guidance, 4 of these were found to be statistically and significantly different in plants not sprayed with the complementary herbicides.

Compositional analysis of 54 endpoints in the grains revealed many (and partly major) statistically significant differences: 27 endpoints were statistically significantly different.

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.

As explained above, EFSA should have requested further tests (toxicological data, repeated spraying with higher herbicide dosages or exposure to a wider range of environmental conditions). Furthermore, the plant material should have been assessed by using omics techniques to investigate changes in plant composition or agronomic characteristics in more detail.

However, instead of assessing the overall pattern of changes in plant components, their causes and possible impacts in more detail, EFSA only assessed the observed changes in isolation in regard to evidence of potential harm. This approach turns the comparative approach into a trivial concept of assessing bits and pieces, and it ignores questions concerning the overall safety of the whole food and feed. However, more in-depth investigations should not depend on findings indicating adverse effects, they should always be necessary to come to sufficiently robust conclusions to inform the next steps in risk assessment.

Based on the available data, no final conclusions can be drawn on the safety of the plants. The data do not fulfill the requirements of Implementing Regulation 503/2013.

Toxicology
Implementing Regulation 503/2013 requests:

> *"Toxicological assessment shall be performed in order to:*
> *(a) demonstrate that the intended effect(s) of the genetic modification has no adverse effects on human and animal health;*
> *(b) demonstrate that unintended effect(s) of the genetic modification(s) identified or assumed to have occurred based on the preceding comparative molecular, compositional or phenotypic analyses, have no adverse effects on human and animal health;"*
>
> *"In accordance with the requirements of Articles 4 and 16 of Regulation (EC) No 1829/2003, the applicant shall ensure that the final risk characterisation clearly demonstrates that:*
> *(a) the genetically modified food and feed has no adverse effects on human and animal health;"*

There were many significant changes especially in the composition of the plants, but no testing of the whole stacked plant (feeding study) was requested. Even if changes taken as isolated data might not directly raise safety concerns, the overall number of effects should have been considered as a starting point for much more detailed investigation of their potential health impacts.
Furthermore, our findings on gene expression show that no reliable conclusion on the content of insecticidal proteins can be derived from the available data. It should be taken into account that in

processed products, such as maize gluten, the toxins can even show a much higher concentration. These higher overall concentrations of the three insecticidal proteins is relevant for the assessment of overall toxicology as well as for the immune system; nevertheless, there were no empirical investigations. This is especially relevant for Vip3Aa20, which was never subjected to more detailed analysis regarding immunological or other toxicological effects, and that can be present in comparably high concentrations in the grain. The safety of Cry1A.105 (artificially synthesized) and Cry2Ab2 is an issue since these can trigger health effects (see below).

In regard to toxicology and potential synergistic or other combinatorial effects, the 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; therefore, they are not identical to their natural templates (Hilbeck & Otto, 2015). It should not be overlooked that the mode of action of Vip3Aa20 is described as similar to Bt toxins. This has, however, not so far been assessed in detail.

In is known that not all modes of action of the insecticidal proteins produced in the plants depend on the specific mechanisms that only occur in the target insect species. Only very few Bt toxins (especially Cry1Ab, for overview see, 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 become 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. (2013) 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 after high concentrations and tend to become stronger after several days. Such observations call for the study of effects after long-term exposure to various dosages, including in combination with material sprayed with the complementary herbicides. In this context, it is important that the stacked maize is also resistant to the herbicide glyphosate, and the resulting residues should be seen as potential co-stressors at the stage of consumption (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.

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 (2015c and 2018b) 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, 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 tallowamine 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/content/eu-commission-request-consider-impact-glyphosate-residues-feed-animal-health-february-2016)

Consequently, EFSA should have requested the company to submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, including repeated spraying. 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 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). 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.

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.

EU legal provisions such as Regulation 1829/2003 (as well as Implementing Regulation 503/2013) state that "*any risks which they present for human and animal health and, as the case may be, for the environment*" have to be avoided. Therefore, potential adverse effects that result from combinatorial exposure of various potential stressors need specification, and their assessment needs to be prioritised. We conclude that the health risk assessment currently performed by EFSA for the stacked maize is unacceptable. We propose testing these plants following the whole mixture approach, considering them to be "*insufficiently chemically defined to apply a component-based approach"* (EFSA, 2019c).

Despite all these open questions regarding potential health impacts, we are not aware of a single sub-chronic or chronic feeding study performed with whole food and feed derived from the stacked maize. This observation is supported by the literature review carried out by the company which did yield any peer reviewed publication. Testbiotech is also aware that feeding studies with similar stacked maize indicated potential health impacts such as inflammatory reactions in the stomach (Zdziarski et al., 2018). Inflammatory responses are an alarm signal typical of many chronic diseases which therefore require close attention.

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.

For this purpose, EFSA should have requested the company to submit data from field trials with the highest dosage of complementary herbicides that can be tolerated by the plants, including repeated spraying. The material derived from the plants should have been assessed in regard to organ toxicity, immune responses and reproductive toxicity, also taking combinatorial effects with other plants components into account.

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

Allergenicity

Implementing Regulation 503/2013 requests:

> *"In cases when known functional aspects of the newly expressed protein or structural similarity to known strong adjuvants may indicate possible adjuvant activity, the applicant shall assess the possible role of these proteins as adjuvants. As for allergens, interactions with other constituents of the food matrix and/or processing may alter the structure and bioavailability of an adjuvant and thus modify its biological activity."*
>
> *"In accordance with the requirements of Articles 4 and 16 of Regulation (EC) No 1829/2003, the applicant shall ensure that the final risk characterisation clearly demonstrates that:*
> *(a) the genetically modified food and feed has no adverse effects on human and animal health;"*

However, EFSA did not request the applicant to provide data to verify whether the source of the transgene is allergenic. According to Santos-Vigil et al (2018), the Bt toxin Cry1Ac can act as an allergen if ingested. This publication is highly relevant: the Bt toxin Cry1Ac was used as a source for the synthesis of Cry1A.105 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, 2018c) came to the conclusion that the Santos-Vigil et al. (2018) publication does not provide any new information and suffers from methodological flaws. However, this EFSA opinion 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 valid and not properly assessed by EFSA (Moreno-Fierros et al., 2018). In awareness of the high concentrations of insecticidal proteins 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-Padrón et al. 1999; Legorreta-Herrera et al., 2010; Jarillo-Luna et al. 2008; González-González et al., 2015; Ibarra-Moreno et al., 2014; Guerrero et al. 2007; Guerrero et al., 2004; Moreno-Fierros et al. 2013; Rubio-Infante et al. 2018) are especially relevant (for review also see Rubio-Infante et al. 2016).

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 insecticidal proteins is much higher in gluten meal produced from the maize, and that it can reach a much higher concentrations 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 system responses caused by the allergens in the soybeans might be considerably enhanced by the adjuvant effects of the Bt toxins.

Our findings on gene expression show that no reliable conclusion on the content of insecticidal proteins can be derived from the available data. Furthermore, in processed products, such as maize gluten, the toxins can even show a much higher concentration. These higher overall concentrations of the three insecticidal proteins is relevant for the assessment of overall toxicology as well as for the immune system; nevertheless, there were no empirical investigations. This is especially relevant for Vip3Aa20, which so far was not subjected to more detailed analysis regarding immunological or other toxicological effects, and that can be present in comparably high concentrations in the grain.

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 on 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 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 further underlined in the minority opinion in another EFSA opinion (Annex II of EFSA, 2018a).

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

Others

For monitoring and methods to identify the specific event, Implementing Regulation 503/2013 requests:

> *"The method(s) shall be specific to the transformation event (hereafter referred to as 'event-specific') and thus shall only be functional with the genetically modified organism or genetically modified based product considered and shall not be functional if applied to other transformation events already authorised; otherwise the method cannot be applied for unequivocal detection/identification/quantification. This shall be demonstrated with a selection of non-target transgenic authorised transformation events and conventional counterparts. This testing shall include closely related transformation events."*

However, no such method for identification was made available. Based on the information available, it will not be possible to distinguish the stacked event from a mixture of single parental events or stacked events that overlap with the actual stack.

If approval for import is given, the applicant has to ensure that post-market monitoring (PMM) is developed to collect reliable information on the detection of indications showing whether any (adverse) effects on health may be related to GM food or feed consumption. Thus, the monitoring report should at very least contain detailed information on:

- i) actual volumes of the GE products imported into the EU,
- ii) the ports and silos where shipments of the GE products were unloaded,
- iii) the processing plants where the GE products was transferred to,
- iv) the amount of the GE products used on farms for feed, and
- v) transport routes of the GE products.

Environmental monitoring should be run in regions where viable material of the GE products such as kernels are transported, stored, packaged, processed or used for food/feed. In case of losses and spread of viable material (such as kernels) all receiving environments need to be monitored. Furthermore, environmental exposure through organic waste material, by-products, sewage or faeces containing GE products during or after the production process, and during or after human or animal consumption should be part of the monitoring procedure (see also comments from experts of Member States, EFSA, 2019d).

We agree with comments made by experts from member states (EFSA, 2019d), that the applicant should be asked to provide a detailed analysis of the fate of the Bt proteins in the environment and a quantitative estimate of subsequent exposure of non-target organisms.

Besides methods of detection, other methods for quantifying exposure to the insecticidal proteins 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 which 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 human 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.

Finally, in regard to the literature research, we do not agree with the way it was carried out. The review should take into account all publications on the parental plants and provide all relevant information regarding gene expression, findings from field trials and feeding studies. Further, monitoring data should be provided on imports of parental plants into the EU.

Environmental risk assessment

Monsanto completely ignored the appearance of teosinte in Spain and France (see Testbiotech, 2016; Trtikova et al, 2017). In its assessment of the volunteer potential, the information provided by Monsanto is largely outdated. As Pascher et al (2016) show, the volunteer potential of maize is higher than assumed by Monsanto. 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.

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.

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., et al. (2014) Effect of stacking insecticidal cry and herbicide tolerance epsps transgenes on transgenic maize proteome. BMC Plant Biology 14: 346. doi: 10.1186/s12870-014-0346-8

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. doi: 10.1371/journal.pone.0067079

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. Journal of Integrative Plant Biology, 51(12):1138-1148. doi: 10.1111/j.1744-7909.2009.00877.x

Chowdhury, E. H., Kuribara, H., Hino, A., Sultana, P., et al. (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., Barbosa, L.C.P., et al. (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(10):2872–2885. doi: 10.3390/toxins6102872

EFSA (2012) Scientific Opinion on application (EFSA-GMO-DE-2010-82) for the placing on the market of insect-resistant genetically modified maize MIR162 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Syngenta. EFSA Journal 10(6): 2756. doi: 10.2903/j.efsa.2012.2756

EFSA (2015a) Scientific Opinion on an application by Syngenta (EFSA-GMO-DE-2009-66) for placing on the market of herbicide tolerant and insect resistant maize Bt11 × MIR162 × MIR604 × GA21 and subcombinations independently of their origin for food and feed uses, import and processing under Regulation (EC) No 1829/2003. EFSA Journal 13(12): 4297. doi: 10.2903/j.efsa.2015.4297

EFSA (2015b) Scientific Opinion on the application (EFSA-GMO-BE-2012-110) for the placing on the market of tissue-selective herbicide-tolerant genetically modified maize MON 87427 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Monsanto. EFSA Journal 13(6):4130. doi: 10.2903/j.efsa.2015.4130

EFSA (2015c) Request for the evaluation of the toxicological assessment of the co-formulant POE-tallowamine. EFSA Journal, 13(11): 4303. doi: 10.2903/j.efsa.2015.4303

EFSA (2017) Scientific Opinion on application EFSA-GMO-BE-2013-118 for authorisation of genetically modified maize MON 87427 × MON 89034 × 1507 × MON 88017 × 59122 and subcombinations independently of their origin, for food and feed uses, import and processing submitted under Regulation (EC) No 1829/2003 by Monsanto Company. EFSA Journal 15(8): e04921. doi: 10.2903/j.efsa.2017.4921

EFSA (2018a) 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 16(7): e05309. doi: 10.2903/j.efsa.2018.5309

EFSA (2018b) 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. https://doi.org/10.2903/j.efsa.2018.5263)

EFSA (2018c) 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. https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/sp.efsa.2019.EN-1504

EFSA (2019a) Assessment of genetically modified maize MON 87427 × MON 89034 × MIR162 × NK603 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2016-131). EFSA Journal 17(7): e05734. doi: 10.2903/j.efsa.2019.5734

EFSA (2019b) Assessment of genetically modified maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-DE-2011-103). EFSA Journal 17(4): e05635. doi: 10.2903/j.efsa.2019.5635

EFSA (2019c) Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. EFSA Journal, 17(3):5634. https://doi.org/10.2903/j.efsa.2019.5634

EFSA (2019d) Application application EFSA-GMO-NL-2016-131, Comments and opinions submitted by Member States during the three-month consultation period, Register of Questions, http://registerofquestions.efsa.europa.eu/roqFrontend/ListOfQuestionsNoLogin?0&panel=ALL

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. Veterinary Microbiology, 175: 382-388. http://dx.doi.org/10.1016/j.vetmic.2014.11.021

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(12): 1177-1183. http://dx.doi.org/10.1016/j.molimm.2004.06.026

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(6): 610-618. http://dx.doi.org/10.1111/j.1365-3083.2007.01992.x

Hilbeck, A., & Otto, M. (2015) Specificity and combinatorial effects of *Bacillus thuringiensis* cCry toxins in the context of GMO environmental risk assessment. Frontiers in Environmental Science, 3: 71. doi: 10.3389/fenvs.2015.00071

Huffman, D.L., Abrami, L., Sasik, R., et al. (2004) Mitogen-activated protein kinase pathways defend against bacterial pore-forming toxins. Proceedings of the National Academy of Sciences, 101(30): 10995–11000. doi: 10.1073/pnas.0404073101

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 Immunolology, 36(6): 266-270. http://dx.doi.org/10.1111/pim.12103

Ito, A., Sasaguri, Y., Kitada, S., et al. (2004) A *Bacillus thuringiensis* crystal protein with selective cytocidal action to human cells. Journal of Biological Chemistry, 279(20): 21282–21286. doi: 10.1074/jbc.M401881200

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 Immunology, 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. doi: 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 Management Sciences, 67(10): 1193-1210. doi: 10.1002/ps.2128

Kraemer, L. (2016) Teosinte plants in the European environment and its implication for market authorisation of genetically engineered maize - legal analysis commissioned by Testbiotech, https://www.testbiotech.org/sites/default/files/Teosinte_legal%20dossier.pdf

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. BioMed Research International. 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

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., et al. (2013) 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. doi: 10.1002/jat.2712

Mesnage, R., Agapito-Tenfen, S.Z., Vilperte, V., et al. (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. doi: 10.1038/srep37855

Mezzomo, B.P. (2013) Hematotoxicity of Bacillus thuringiensis as spore-crystal strains Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa in Swiss albino mice. Journal of Hematology & Thromboembolic Diseases, 1:104.

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 and infection, 2(8): 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. International immunopharmacology, 17(4):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

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):28-30. https://link.springer.com/article/10.1186/s12302-016-0098-1

Reuter, T., Alexander, T.W., Martínez, 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. Journal of the Science of Food and Agriculture, 87(15): 2837-2843. doi: 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

Rubio-Infante, N., Ilhuicatzi-Alvarado, D., Torres-Martínez, M., et al. (2018) The Macrophage Activation Induced by Bacillus thuringiensis Cry1Ac Protoxin Involves ERK1/2 and p38 Pathways and the Interaction with Cell-Surface-HSP70: pCry1Ac AND csHSP70. Journal of Cellular Biochemistry 119: 580-598. doi: 10.1002/jcb.26216

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., et al. (2013) The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. Current Microbiology, 66(4):350-358. doi: 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. Journal of Veterinary Medical Science, 65(2): 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. 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. doi: 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., Wikmark, O.G., Zemp, N., et al. (2015) Transgene expression and Bt protein content in transgenic Bt maize (MON810) under optimal and stressful environmental conditions. PLoS ONE 10(4): e0123011. doi: 10.1371/journal.pone.0123011

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

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(4), 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

Vázquez-Padrón, R.I., Moreno-Fierros, L., Neri-Bazán, L., et al. (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. doi: 10.1016/S0024-3205(99)00136-8

Vázquez-Padrón, R.I., Gonzáles-Cabrera, J., García-Tovar, C., et al. (2000) Cry1Ac protoxin from *Bacillus thuringiensis* sp. kurstaki HD73 binds to surface proteins in the mouse small intestine. Biochemical and Biophysical Research Communications, 271(1): 54-58. doi: 10.1006/bbrc.2000.2584

Walsh, M.C., Buzoianu, S.G., Gardiner, G.E., Rea, M.C., et al. (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., et al. (2017) Genetically engineered rice endogenous 5-enolpyruvoylshikimate-3-phosphate synthase (epsps) transgene alters phenology and fitness of crop-wild hybrid offspring. Scientific Reports 7. doi: 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