TESTBIOTECH Background 7 – 3 – 2021

Testbiotech comment on EFSA’s assessment of genetically engineered maize MON 87427 x MON 87460 x MON 89034 x 1507 x MON 87411 x 59122 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2017-139) from Bayer/Monsanto

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

Christoph Then, Andreas Bauer-Panskus

Table of Contents

Introduction

The EFSA GMO panel assessed the six-stacked maize MON87427 x MON 87460 x MON 89034 x 1507 x MON 87411 x 59122 , which is derived from crossing genetically engineered maize events (EFSA, 2021a). The parental plants were assessed by EFSA in previous opinions. The maize contains genes conferring doubled resistance to glyphosate and produces three insecticides. It was also developed to be drought tolerant:

- MON 87427 expressing CP4 EPSPS protein for tolerance to glyphosate-containing herbicides;
- MON 87460 expressing the cold shock protein B (CspB) to confer drought tolerance and expressing the neomycin phosphotransferase II protein (NPTII) used as selectable marker to facilitate the selection process of transformed plant cells;
- MON 89034 expressing the insecticidal proteins Cry1A.105 (artificially synthesized) and Cry2Ab2;
- 1507 expressing the Cry1F protein to confer protection against certain lepidopteran pests and the PAT protein to confer tolerance to glufosinate-ammonium-containing herbicides;
- MON 87411 produces an insecticidal dsRNA (DvSnf7) as well as the EPSPS protein for tolerance to glyphosate-containing herbicides. In addition it also produces the insecticidal protein Cry3Bb1 (artificially synthesized).
- 59122 expressing the Cry34Ab1 and Cry35Ab1 protein to confer protection against certain coleopteran pests and the PAT protein.

In consequence, there are three traits combined in the stacked maize plants (in the following also addressed as ‘the Maize’): Herbicide resistance, insect toxicity and drought tolerance:

- The stacked GE maize has doubled resistance to glyphosate, making it tolerant to high dosages and repeated sprayings as applied in fields with herbicide-resistant weeds. It also has doubled resistance to glufosinate.
- Further, it produces three toxins against the larvae of *Lepidoptera* (butterflies) that feed on the plants (‘pest insects’) and three toxins against the larvea of Coleoptera (beetles) that feed below the gound. In addition, it produces an biological active molecule (dsRNA) that can negatively interfere with metabolism in larvae of *Coleoptera* (beetle).
- In addition, the cold shock protein is intended to confer drought tolerance.

In addition, there are additional genetic elements used during the process of transformation: The maize produces the PMI protein (phosphomannose isomerase) derived from *Escherichia coli*. Expression of PMI enables transformed maize cells to utilise mannose and therefore to survive on specific media used for selecting the maize plants after the process of genetic engineering (so called marker gene). Furthermore, it produces the NPTII protein conferring resistance to antibiotics. In addition, genetic elements from viruses or other plants were used as promoters to enhance gene activity of the inserted genes.

Implementing Regulation 503/2013 was applied in the EFSA risk assessment.

1. Molecular characterisation and gene expression

Annex II of Implementing Regulation 503/2013 requests that

"*The risk assessment of genetically modified food and feed containing stacked transformation events shall also include an assessment of the following aspects:*

> *(a) stability of the transformation events;*
> *(b) expression of the transformation events;*
> *(c) potential synergistic or antagonistic effects resulting from the combination of the transformation events shall be subject to an assessment in accordance with Sections 1.4 (Toxicology), 1.5 (Allergenicity) and 1.6 (Nutritional assessment).*" (Introduction)

"*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).*" (Scientific requirements 1.2.2.3)

"*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.*" (Scientific requirements 1.3.1)

"*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.*" (Scientific requirements 1.3.2.1)

Assessment of open reading frames

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 concerns; therefore, no detailed investigations were carried out in this regard.

Furthermore, other biologically active gene products such as unintended non coding small RNAs (ncsRNAs) from additional open reading frames were not assessed. Thus, uncertainties remain in regard to other biologically active substances arising from the method of genetic engineering and the newly introduced gene constructs.

Consequently, EFSA neither took into account all the relevant data requested by EU regulation nor potential synergistic or antagonistic effects resulting from the combination of the transformation events. This would however be needed to draw reliable conclusions on health safety, including the assessment of toxicity and impact on the immune system.

Impact of environmental factors, agricultural practice and genetic backgrounds

There are several reasons why the data presented do not meet the requirements of Implementing Regulation 503/2013: (1) the field trials were not conducted in all relevant regions where the Maize is to be be cultivated, and no extreme weather conditions were taken into account; (2) the field trials did not take current agricultural management practices into account; (3) only one transgenic variety was included in the field trials.

Data on environmental factors, stress conditions and their impact on gene expression

Environmental stress can cause unexpected patterns in expression of the newly introduced DNA (see, for example, Trtikova et al., 2015; Lohn et al., 2020). There is plenty of evidence showing that drought or heat can significantly impact the content of Bt in the plant tissue (Adamczyk & Meredith, 2004; Adamczyk et al., 2009; Chen et al., 2005; Dong & Li, 2006; Luo et al., 2008; Then & Lorch, 2008; Trtikova et al., 2015; Jiang et al., 2018; Girón-Calva et al., 2020). Therefore, to assess gene expression, the plants should have been grown under conditions of severe drought, with and without irrigation, with and without application of the complementary herbicide and in comparison to more moderately severe climate conditions. However, no such data were requested or used for detailed comparison to assess the genome x environment interactions.

More specifically, Fang et al. (2018) and Yang et al. (2017) show that stress responses can lead to unintended changes in plant metabolism inheriting additional EPSPS enzymes. In this context, there are strong indications that the EPSPS enzyme, which confers glyphosate tolerance, also interferes with the auxin metabolism in the plants (Fang et al., 2018). This plant hormone plays a key role in growth, fecundity and adaptation to environmental stressors. Thus, changes in the auxin content can also result in changes in plant composition that can raise safety concerns.

Several publications support these findings showing unintended effects in plants inheriting additional EPSPS genes (Beres, 2019; Beres et al., 2018; Wang et al., 2014). In addition, other authors show the need for further investigations (Vila-Aiub et al., 2009 and Vila-Aiub et al., 2019).

The EPSPS enzymes occur in the stacked Maize in higher concentration compared to the parental plants. Therefore, the Maize should have been subjected to a broad range of defined environmental conditions and stressors to gather reliable data on gene expression and functional genetic stability. This is especially relevant for the stacked Maize since these plants are the first in which expression of the epsps gene constructs are intended to be exposed to more extreme climate conditions.

Therefore, the Maize should have been tested in all major maize producing countries, such as North and South America. Furthermore, the Maize should have been subjected to defined drought conditions since it must be assumed it will be grown in accordance with its intended purpose. However, this was not the case: Only five sites suited in the US were used in this case. They are all relatively close and therefore only represent a very limited region of the maize-producing areas in the US; no other maize-producing country was included.

No extreme weather conditions were reported during the cultivation. In summary, the available publications strongly indicate that plants inheriting a combination of EPSPS and CSPB are likely to show gene expression responses when grown under stress conditions such as drought. In addition, Bt toxin expression in GE plants is known to be influenced by environmental conditions such as drought.

Thus, in the stacked Maize, there is a combination of a trait which is meant to expose the plant to extreme climate conditions and gene constructs (such as for the EPSPS enzyme and the production of Bt toxins) which are likely to show or to cause major changes in gene expression if exposed to environmental stressors such as drought. There are also no data on gene expression and the dsRNA DvSnf7 under stress conditions. Furthermore, data on the concentration of the biologically active molecules in the plants are missing.

Further, additional proteins are expressed which are absent in conventional maize: NPTII which confers antibiotic resistance (and should have been avoided as requested by EU Directive 2001/18) and PMI which was used for selecting process during plant production. These traits and genetic elements may synergise and interact with each other. The resulting effects are not unlikely to impact plant composition and biological characteristics crucial for the assessment of food and feed safety. However, no specific data were requested or used for detailed comparison to assess genome x environment interactions.

At the very least, the unintended effects of the EPSPS enzymes plausibly interfere with the activity of the other gene constructs, for example, via the auxin hormone (see above). This is especially relevant if the plants are exposed to stress conditions. Even if no such effects were observed in the parental plants (which, to our knowledge, were not tested in field trials under stressful conditions), these enzymes are produced in the stacked Maize at higher concentrations. Therefore, the likelihood of interaction between the gene constructs and gene expression is higher in the stacked compared to the parental plants.

It has to be expected that the stacked maize will be grown under drought conditions to an extent that most of the parental GE plants were not tested for in their previous risk assessment. Moreover, it is the first time that the combination of artificial gene constructs will be exposed to more extreme drought conditions. It is obvious that in the absence of adequate data, there can be no assessment of whether interactions will occur under stress conditions. Therefore, there is no scientifically sound way of arguing why much more specific data is not necessary. Consequently, the stacked plants should have been grown under conditions of severe drought, with and without irrigation, with and without application of the complementary herbicide and in comparison to more moderately severe climate conditions.

In summary, while the plants are expected to show improved performance under more extreme drought conditions, no such data have been reported from the field trials. No data are made available as requested by Implementing regulation 503/2013 to assess whether the expected environmental conditions under which the plants are likely to be cultivated will influence the expression of the studied endpoints. Furthermore, no such data are available from the parental plants or any subcombinations.

<u>These gaps in risks assessment are further emphasised in other meteorological data:</u>

- US weather data from 2014 show that, for example, there was more precipitation than normal at the trial sites in Illinois. This means that a relevant stressor that might influence gene expression, composition or phenotype of the Maize may not be covered by the field trial data.

- Trial sites such as in Illinois neither represent the climatic conditions in other maize growing regions of the US nor the conditions in other relevant maize producing countries such as Brazil. USDA data show relevant maize production in North Dakota, Minnesota, Mississippi, Louisiana and other states.
- According to climate data, precipitation in maize production regions such as Illinois is significantly different to that of other maize producing regions like South Dakota.[1] The same is true for lower average temperatures in northern maize growing states e.g. Minnesota in comparison to other maize growing regions in the US.[2]
- Even more striking is the difference between the climatic conditions in the major maize growing regions of the US and the conditions in maize growing regions in Brazil, another major producer of GE maize. Data show much more precipitation in maize producing states like Paraná or Mato Grosso compared to the US.[3] Furthermore, the average temperature in most Brazilian maize production regions is much higher compared to US regions, e.g. Iowa, Illinois or Ohio.[4]

Consequently, the Maize plants tested in field trials do not sufficiently represent the imported Maize. The data presented by the applicant are insufficient to conclude on the impact of environmental factors and stress conditions on gene expression, plant composition and the biological characteristics of the plant as requested by the EU Regulation 503/2013.

Data on herbicide application rates and their impact on gene expression

Due to increased weed pressure, it must be assumed that these plants will be exposed to high and also repeated dosages of glyphosate. Higher rates of herbicide application will not only lead to a higher burden of residues in the harvest, but can also influence the expression of the transgenes or other genome activities in the plants. These observations are evidenced by a large amount of data showing substantial changes in the composition of GE herbicide-resistant soybeans (Miyazaki et al., 2019) while more specific data on GE maize seem to be lacking.

It is likely that glyphosate was applied at a rate of 0.87 kg a.e./ha only. At the same time, industry recommendations suggest dosages of up to approx. 3,5 kg a.i./ha glyphosate post-emergence, 7 kg per season, and even higher rates, can be sprayed on herbicide-resistant maize.[5]

Fang et al. (2018) and Yang et al. (2017) show that stress responses can lead to unexpected changes in plant metabolism inheriting additional EPSPS enzymes. These findings showing unintended effects in plants inheriting additional EPSPS genes are supported in several other publications (Beres, 2019; Beres et al., 2018; Wang et al., 2014). In addition, other authors show the need for further investigations (Vila-Aiub et al., 2009 and Vila-Aiub et al., 2019).

---

[1] https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=0440000&regionid=us&nationalGraph=False&cntryid=USA&sel_year=2021&startRow=1&fctypeid=23&fcattributeid=1

[2] https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=0440000&regionid=us&nationalGraph=False&cntryid=USA&sel_year=2021&startRow=1&fctypeid=24&fcattributeid=1

[3] https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=0440000&regionid=br&nationalGraph=False&cntryid=BRA&sel_year=2021&startRow=1&fctypeid=23&fcattributeid=1

[4] https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=0440000&regionid=us&nationalGraph=False&cntryid=USA&sel_year=2021&startRow=1&fctypeid=24&fcattributeid=1

[5] www.greenbook.net/monsanto-company/roundup-weathermax; www.greenbook.net/monsanto-company/roundup-ultra

In this context, there are strong indications that the EPSPS enzyme also interferes with the auxin metabolism in the plants (Fang et al., 2018). Auxin has a role in multiple metabolic pathways in plants. Thus, changes in the auxin content can also result in changes in plant composition that can raise safety concerns.

The EPSPS enzymes occur in the stacked Maize in much higher concentrations compared to the parental plants. Therefore, it should be taken into account that unintended effects caused by high concentrations of EPSPS enzymes in combination with higher rates of spraying, may also affect gene expression and plant composition. When the Maize is exposed to higher rates of glyphosate application, the plants may experience stress conditions, impacting gene expression and plant composition as well as the biological characteristics of the Maize.

Consequently, the Maize plants tested in field trials do not sufficiently represent the Maize as imported. The data presented by the applicant are insufficient to conclude on the impact of the herbicide applications on gene expression, plant composition or biological characteristics of the plant as requested by the EU Regulation 503/2013.

Data on genetic backgrounds and their impact on gene expression

It is very well known that the genomic background of the variety can influence the expression of the inserted genes and / or the concentration of the additional proteins being present in the GE plants (see Trtikova et al., 2015). For example, Lohn et al, (2020), in a multigenerational study with Bt maize, show that the Bt content was generally lower in offspring if the maize was crossed with Brazilian varieties, but much higher than expected in offspring from South African plants.

Therefore, EFSA, should have requested additional data from several varieties, including those cultivated in South America. However, no such data were provided or requested. Furthermore, no such data are available for the parental plants or any subcombinations.

In conclusion, the Maize plants tested in field trials do not represent the Maize as imported. The data presented by the applicant are insufficient to conclude on the impact of genetic background on gene expression, plant composition and biological characteristics. However, reliable data are needed to demonstrate safety as requested by EU Regulation.

It also should have been taken into account that previous research indicates the 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. Several publications point out the unintended changes in the genome, the transcriptome, the proteome or the metabolome of transgenic maize. (see Agapito-Tenfen et al., 2013; Agapito-Tenfen et al., 2014; Benevenuto et al., 2017; Mesnage et al., 2016; Ben Ali et al., 2020).

Several of these transgenes are also present in the stacked Maize. In addition, four other proteins are expressed which are absent in conventional maize: NPTII which confers antibiotic resistance (which should have been avoided as requested by EU Directive 2001/18), PMI which was used for the selecting process during plant production, dsRNA DvSnf7 (insecticidal toxicity) and CSPB (for drought tolerance). These traits and genetic elements can synergise and interact with each other. These effects are also likely to impact plant composition and biological characteristics crucial for the assessment of food and feed safety. However, no specific data for a detailed comparison to assess the genome x environment interactions were presented by the applicant.

Robust data should have been requested to assess whether metabolic changes with relevance to food and feed safety occur in the stacked maize, and EFSA should have requested a much more detailed investigation into potential biologically active gene products and changes in metabolic pathways under more extreme climate conditions.

However, EFSA risk assessment has not yet taken these issues into account. Nor does EFSA use more sensitive methods, such transcriptomics, proteomics and metabolomics, to explore and assess unintended changes in the stacked Maize.

In summary, EFSA did not take into account all relevant data as requested by EU regulation to come to reliable conclusions regarding health safety, including the assessment of toxicity and impact on the immune system.

Consequently, the Maize plants tested in the field trials do not sufficiently represent the Maize as imported. The data presented by the applicant are insufficient to conclude on the impact of environmental factors, stress conditions, herbicide application rates, genetic backgrounds and stacking on gene expression, plant composition or the biological characteristics of the plant as requested by the EU Regulation 503/2013.

Conclusions on molecular characterisation and gene expression

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, herbicide spraying or environmental factors, such as stress conditions.

Therefore, the plants should have been exposed to a much broader range of defined environmental conditions and stressors to gather reliable data on gene expression and functional genetic stability. This is especially relevant in this case since the stacked Maize is meant to be cultivated under more extreme drought conditions, such as those caused by ongoing climate change. Whatever the case, it 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 which represent current agricultural practices with much higher rates of complementary herbicide applications on the plants, including repeated spraying. In addition, EFSA should have requested data for several varieties, including those cultivated in South America.

Plant material should have also been assessed by using -omics techniques to investigate changes in the gene activity of the transgene and the plant genome, as well as to investigate changes in metabolic pathways and the emergence of unintended biologically active gene products.

However, only 4 samples (each for grain and forage) from 5 closely located field trials were used to generate the data on gene expression. Furthermore, only one variety of the stacked Maize was used in the trials. The impact of environmental factors and agricultural practices was assessed without taking more extreme climate conditions into account, although cultivation in drought conditions is one of the intended traits in the stacked Maize. For example, according to weather data, in 2014 there was more precipitation in most field trial sites than normal. Herbicide applications in the field trials did not represent current agricultural practices, the rates were much lower. Relevant data on comparison of sprayed and unsprayed GE Maize plants are completely missing.

In summary, the Maize plants tested in field trials do not sufficiently represent the imported Maize. Consequently, the data presented by the applicant and accepted by EFSA are insufficient to conclude on the impact of the combination of traits and gene constructs (stacking), or the impact of environmental factors, herbicide applications and of genetic background on gene expression and plant metabolism. However, reliable data are needed to demonstrate safety as requested by EU Regulation.

Based on the available data, no final conclusions can be drawn on the safety of the plants. Consequently, the data do not fulfill the requirements of Implementing Regulation 503/2013 and Regulation 1829/2003.

2. Comparative assessment of plant composition and agronomic and phenotypic characteristics

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 provided by Monsanto 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: (2.2.1) 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; (2.2.2) the field trials did not take the current agricultural management practices into account; (2.2.3) only one transgenic stacked variety was included in the field trials.

Data on environmental factors and stress conditions and their impact on plant composition and agronomic and phenotypic characteristics

Field trials for the assessment of plant composition and agronomic and phenotypic characteristics of the stacked Maize were conducted in the US for one year only, but not in other relevant maize production areas such as Brazil and Argentina. As shown in the EFSA opinion (2021a), "*no exceptional weather conditions were reported at any of the selected field trial sites*".

Taking into account the purpose of the genetic engineering in this case, it is not acceptable that EFSA failed to require further studies:

- 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 resulting in more extreme droughts.
- No data were generated that represent the growing conditions in other relevant maize growing regions outside the US.

These gaps in risks assessment are further emphasised in other meteorological data:

- US weather data from 2014 show that, for example, there was more precipitation than normal at the trial sites in Illinois. This means that a relevant stressor that might influence gene expression, composition or phenotype of the Maize may not be covered by the field trial data.
- Trial sites such as in Illinois neither represent the climatic conditions in other maize growing regions of the US nor the conditions in other relevant maize producing countries such as Brazil. USDA data show relevant maize production in North Dakota, Minnesota, Mississippi, Louisiana and other states.
- According to climate data, precipitation in maize production regions such as Illinois is significantly different to that of other maize producing regions like South Dakota .[6] The same is true for lower average temperatures in northern maize growing states e.g. Minnesota in comparison to other maize growing regions in the US.[7]
- Even more striking is the difference between the climatic conditions in the major maize growing regions of the US and the conditions in maize growing regions in Brazil, another major producer of GE maize. Data show much more precipitation in maize producing states like Paraná or Mato Grosso compared to the US.[8] Furthermore, the average temperature in most Brazilian maize production regions is much higher compared to US regions, e.g. Iowa, Illinois or Ohio.[9]

Fang et al. (2018) as well as Yang et al. (2017) show that stress responses can lead to unintended changes in plant metabolism inheriting additional EPSPS enzymes. In this context, there are strong indications that the EPSPS enzyme, which confers glyphosate tolerance, also interferes with the auxin metabolism in the plants (Fang et al., 2018). This plant hormone plays a key role in growth, fecundity and adaptation to environmental stressors. Thus, changes in the auxin content can also result in changes in plant composition that can raise safety concerns.

Several publications support these findings showing unintended effects in plants inheriting additional EPSPS genes (Beres, 2019; Beres et al., 2018; Wang et al., 2014). Other authors also show the need for further investigations (Vila-Aiub et al., 2009 and Vila-Aiub et al., 2019).

The EPSPS enzymes occur in the stacked Maize in higher concentrations compared to the parental plants. Therefore, the Maize should have been subjected to a broad range of defined environmental

---

[6] https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=0440000&regionid=us&nationalGraph=False&cntryid=USA&sel_year=2021&startRow=1&fctypeid=23&fcattributeid=1

[7] https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=0440000&regionid=us&nationalGraph=False&cntryid=USA&sel_year=2021&startRow=1&fctypeid=24&fcattributeid=1

[8] https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=0440000&regionid=br&nationalGraph=False&cntryid=BRA&sel_year=2021&startRow=1&fctypeid=23&fcattributeid=1

[9] https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=0440000&regionid=us&nationalGraph=False&cntryid=USA&sel_year=2021&startRow=1&fctypeid=24&fcattributeid=1

conditions and stressors to gather reliable data on gene expression and functional genetic stability. This is especially relevant to the stacked Maize since EPSPS gene constructs will, for the first time, be expressed in these plants in more extreme climate conditions.

The stacked Maize carries a combination of a trait making the plants more tolerant to extreme climate conditions and gene constructs (such as for Bt toxins) likely to show or cause major changes in gene expression if exposed to environmental stressors, such as drought. In addition, two other proteins are expressed which are absent in conventional maize: NPTII which confers antibiotic resistance - and should have been avoided as requested by EU Directive 2001/18 - and PMI which was used in the selecting process during plant production. These traits and genetic elements can synergise and interact. The effects are not unlikely to impact plant composition and biological characteristics crucial for the assessment of food and feed safety. However, no specific data necessary for detailed comparison to assess the genome x environment interactions were presented by the applicant.

Whatever the case, it should have been tested in all the major maize producing countries such as North and South America. Furthermore, the Maize should have been subjected to defined drought conditions as there is an expectation that it will be grown in accordance with its intended purpose.

However, no experiments were requested to show to which extent specific environmental conditions will influence plant composition or agronomic and phenotypic characteristics. No data were made available as requested by Implementing regulation 503/2013 to assess whether the expected environmental conditions under which the plants are likely to be cultivated will influence the expression of the studied endpoints. Furthermore, no such data are available for the parental plants or any subcombinations.

It has to be assumed that the stacked maize will be grown under drought conditions to an extent that most of the parental GE plants were not tested for in their previous risk assessment. Moreover, it is the first time that the artificial gene constructs in combination will be exposed to more extreme drought conditions. It is obvious that in the absence of adequate data, it cannot be assessed whether interactions will occur under stress conditions. Therefore, there is no scientifically sound way of arguing why much more specific data is not necessary. For this reason, the stacked plants should have been grown under conditions of severe drought, with and without irrigation, with and without application of the complementary herbicide and in comparison to more moderately severe climate conditions.

Consequently, the Maize plants tested in field trials do not sufficiently represent the imported Maize. The data presented by the applicant are insufficient to conclude on the impact of environmental factors and stress conditions on gene expression, plant composition and the biological characteristics of the plant, as requested by the EU Regulation 503/2013.

Data on herbicide application rates and their impact on plant composition and agronomic and phenotypic characteristics

Due to high weed pressure in many maize growing regions, there is an expectation that these plants will be exposed to higher amounts and repeated dosages of glyphosate. It also has to be taken into account that the herbicides can be sprayed repeatedly and at high dosages. Such 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 applicant: glyphosate was only sprayed at an early stage of vegetation and at comparably low dosages (likely dosage: 0.87 kg a.e./ha). Industry recommendations suggest dosages to be sprayed on herbicide resistant maize of up to approx. 3,5 kg a.i./ha glyphosate post-emergence, 7 kg per season, and even higher rates.[10]

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, agronomic and phenotypic characteristics, for example, due to stress conditions caused by high amounts of complementary herbicides and unintended stress responses as described by Fang et al. (2018) and Yang et al., (2017).

Several publications report unintended effects in plants inheriting additional EPSPS genes (Beres, 2019; Beres et al., 2018; Wang et al., 2014). In addition, other authors show the need for further investigations (Vila-Aiub et al., 2009 and Vila-Aiub et al., 2019).

In this context, there are strong indications that the EPSPS enzyme, which confers glyphosate tolerance, also interferes with auxin metabolism in the plants (Fang et al., 2018). Auxin plays a role in multiple metabolic pathways in plants. Changes in the auxin content can result in changes in plant composition which can raise concerns about safety.

The EPSPS enzymes occur in the stacked Maize in much higher concentrations compared to the parental plants. Therefore, it should be taken into account that unintended effects caused by high concentrations of EPSPS enzymes in combination with higher rates of spraying, may also affect gene expression and plant composition. If the Maize is exposed to higher rates of glyphosate application, this may cause stress conditions, which can in turn impact gene expression and plant composition as well as the biological characteristics of the Maize.

EFSA should have requested the applicant to submit data from field trials; at a minimum, data on complementary herbicide application rates as recommended by industry, including repeated applications. However, no such data were provided or requested. Furthermore, no such data are available for the parental plants or any subcombinations.

Consequently, the Maize plants tested in the field trials do not sufficiently represent the imported Maize. The data presented by the applicant are insufficient to conclude on the impact of the herbicide applications on gene expression, plant composition and the biological characteristics of the plant as requested by the EU Regulation 503/2013.

Data on genetic backgrounds and their impact on plant composition and agronomic and phenotypic characteristics

It is very well known that the genomic background of the variety can influence the expression of the inserted genes and / or the concentration of the additional proteins present in the GE plants (see Trtikova et al., 2015). For example, Lohn et al. (2020), in a multigenerational study with Bt maize, show that the Bt content was generally lower in offspring if the maize was crossed with Brazilian varieties, but much higher than expected in offspring from South African plants.

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

---

[10] www.greenbook.net/monsanto-company/roundup-weathermax; www.greenbook.net/monsanto-company/roundup-ultra

background of the plants. This approach is supported by the analysis of gene expression given above, but was ignored in the EFSA risk assessment. However, no such data were provided or requested. Furthermore, no such data are available for the parental plants or any subcombinations.

In conclusion, the Maize plants tested in field trials do not represent the imported Maize. The data presented by the applicant are insufficient to conclude on the impact of genetic background on gene expression, plant composition and the biological characteristics. However, reliable data are needed to demonstrate safety as requested by EU Regulation.

Data from compositional analysis show the need for further investigation

Only data from a low number of agronomic parameters (12) were subjected to statistical analysis in accordance with EFSA guidance; of these 6 (no spraying) and 7 (spraying of the complementary herbicide) were found to be statistically and significantly different.

Compositional analysis in the grain revealed many (and partly major) statistically significant differences: Of 54 endpoints, 36 were statistically significantly different in plants spayed with the complementary herbicides, 27 in plants not sprayed with glyphosate (but other conventional herbicides).

Not only the number of changes but also the magnitude of differences where much greater in the Maize being sprayed with with complementary herbicides, compared not the non-sprayed Maize. This is a strong indication that stacking in combination with the application of the complementary herbicides are actually impact plant composition.

Even if changes taken as isolated data might not directly raise safety concerns, the overall number of significant effects in the group of the sprayed Maize has to be taken as a starting point for much more detailed investigations: more than half of the parameters measured for agronomic characteristics as well as for plant composition were significantly different.

Looking at these data, EFSA should have concluded that the plants are likely to be substantially different compared to their conventional counterparts. Therefore, more detailed analysis would have been necessary to investigate changes in the plant composition and phenotype caused by the stacking, by spraying with the complementary herbicide including investigation into potential unintended changes in metabolic pathways and the emergence of unintended biologically active gene products.

Furthermore, the data presented did not take into account cultivation of the stacked maize under more extreme drought conditions, even though these are the most relevant environmental conditions in which the plants will be cultivated. Under such environmental conditions, the range of differences and their significance are likely to be substantially increased.

As explained above, EFSA should have requested further tests, with repeated herbicide applications using higher dosages and with exposure to a much wider range of environmental conditions, taking more extreme drought conditions into account. Furthermore, the plant material should have been assessed by using -omics techniques to investigate changes in plant composition or agronomic and phenotypic 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 for evidence of potential harm. This approach turns the comparative approach into a trivial concept of assessing bits and pieces, and it ignores questions on the overall safety of the whole food and feed. A more in-depth investigation of unintended changes is not only necessary if there are findings indicating adverse effects, it is always needed to come to sufficiently robust conclusions to inform the next steps in risk assessment.

Previous research indicates 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. This research should have been taken into account. Several publications also report unintended changes in the genome, the transcriptome, the proteome or the metabolome of transgenic maize (see Agapito-Tenfen et al., 2013; Agapito-Tenfen et al., 2014; Benevenuto et al., 2017; Mesnage et al., 2016; Ben Ali et al., 2020).

Several of these transgenes are also present in the stacked Maize. In addition, four other proteins are expressed that are absent in conventional maize: NPTII which confers antibiotic resistance - and should have been avoided as requested by EU Directive 2001/18 - PMI which was used for selecting process during plant production, dsRNA DvSnf7 (insecticidal toxicity) and CSPB (for drought tolerance). These traits and genetic elements can synergise and interact. These effects are also likely to impact plant composition and biological characteristics crucial to the assessment of food and feed safety. However, no specific data needed for detailed comparison to assess the genome x environment interactions were presented by the applicant.

At a minimum, the unintended effects of the EPSPS enzymes will very plausibly interfere with the activity of the other gene constructs, for example, via the auxin hormone (see above). This is especially relevant if the plants are exposed to stress conditions. Even if no such effects were observed in the parental plants (which, to our knowledge, were not tested in field trials under stress conditions), these enzymes are now produced in the stacked Maize at higher concentrations. Therefore, the likelihood of interaction between the gene constructs and gene expression, plant composition as well as agronomic and phenotypic characteristics is higher in the stacked Maize compared to the parental plants.

Thus, robust data should have been presented to assess whether metabolic changes with relevance to food and feed safety occur in the stacked maize. EFSA should have requested a much more detailed investigation into potential biologically active gene products and changes in metabolic pathways under more extreme climate conditions.

However, these issues have not yet been taken into account in EFSA risk assessment. Neither do they use more sensitive methods, such as transcriptomics, proteomics and metabolomics to explore and assesses unintended changes in the stacked Maize.

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.

Conclusion on comparative assessment of plant composition and phenotypic and agronomic characteristics

The stacked Maize has a trait enabling cultivation of the plants under more extreme climate conditions. The trait is combined with gene constructs (the EPSPS enzyme and the production of Bt toxins) which are likely to cause or to show major changes in gene expression if exposed to environmental stressors, such as drought. In addition, three other proteins are expressed in the stacked Maize which are absent in conventional maize: NPTII which confers antibiotic resistance - which should have been avoided as requested by EU Directive 2001/18 - PMI which was used in the selecting process during plant production and dsRNA DvSnf7 (insecticidal toxicity). These traits and genetic elements can synergise and interact with each other. These effects are also likely to impact plant composition phenotype, especially if exposed to environmental stress conditions.

However, the data provided by the applicant and accepted by EFSA are insufficient to conclude on the impact of the combination of traits and gene constructs, of environmental factors, of herbicide applications and the genetic background on gene expression, plant metabolism, plant composition and the agronomic and phenotypic characteristics. Since the field trials did not represent the conditions under which the plants are to be grown in practice, the data from GE plants tested in field trials do not sufficiently represent the imported GE plants. Consequently, these data do not fulfill the requirements of Implementing Regulation 503/2013.

In summary, the Maize plants tested in field trials do not sufficiently represent the Maize as imported. Based on the available data, no final conclusions can be drawn on the safety of the plants. Consequently, the data do not fulfill the requirements of Implementing Regulation 503/2013 and Regulation 1829/2003.

3. Toxicity

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;"*

In addition, Implementing Regulation 503/2013 requests:

> *"For silencing approaches by RNAi expression, potential 'off target' genes should be searched by in silico analysis to assess if the genetic modification could affect the expression of other genes which raise safety concerns."*

Findings from molecular characterisation and comparative approach

As explained above, many significant changes were identified: more than half of the parameters measured for agronomic characteristics and plant composition were significantly different if sprayed with the complementary herbicides. Even if the 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 into their potential health impacts.

However, the data presented by the applicant did not take into account cultivation of the stacked maize under more extreme drought conditions, i.e. neither under realistic agricultural conditions nor considering all relevant countries of cultivation. Drought would have been especially relevant since this the Maize was developed to be grown in such conditions. The range of differences and their significance are likely to be substantially increased in these conditions. Thus, without more data, the true range of unintended effects cannot be determined and safety cannot be demonstrated as requested by EU regulation.

It is also shown that no reliable conclusions can be drawn on the content of insecticidal proteins and the other intended new proteins from the available data. For example, the overall concentration of the three insecticidal proteins is relevant for the assessment of overall toxicology as well as for the immune system. Thus, without more data, the true impact of intended effects on health at the stage of consumption cannot be determined and safety cannot be demonstrated as requested by EU regulation.

Despite these findings, and in awareness of the lack of more specific data and the resulting major uncertainties, no testing of the whole stacked plant (feeding study) was requested.

Toxicity of the Bt toxins

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. They are produced in the plants but their biological characteristics are altered and not identical to their natural templates (Hilbeck & Otto, 2015).

Several publications describe the effects of Bt toxins in mammals: some Cry toxins are known to bind to epithelial cells in the intestines 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. Further 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 to consider 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).

It has to be considered that the concentration of the insecticidal proteins is much higher in gluten meal produced from the maize, it can reach much higher concentrations than in 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 toxicity compared to genetically engineered plants producing just one Bt toxin.

Relevant findings show that the selectivity and efficacy of Bt toxins produced in GE plants can be influenced by many co-factors (see, for example, Then, 2010; Hilbeck & Otto, 2015). Higher toxicity can also cause lower selectivity (Then, 2010): if synergistic or additive effects occur that increase efficacy of the Bt toxin, its selectivity may be decreased and a wider range of non-target organisms may become susceptible.

One crucial impact factor in this context are protease inhibitors (PI), which show synergistic effects with Bt toxins, strongly enhancing their toxicity. It is likely that PI delay the degradation of Bt proteins and thereby also enhance their toxicity. In many of its comments on EFSA opinions, Testbiotech has highlighted these effects by referring, for example, to Pardo-López et al. (2009). However, EFSA has never provided a detailed response.

Testbiotech is aware of several publications confirming this gap in risk assessment that EFSA has constantly ignored or denied: as Monsanto already showed in the 1990s, maize, cotton and soybeans produce protease inhibitors (PI), which considerably enhance the toxicity of Bt proteins in plants. In the presence of PIs, Bt toxin will degrade much more slowly than in isolation. This results in a much higher toxicity of the Bt toxin (if it is taken up together with the plant tissue) compared to the isolated toxin (MacIntosh et al., 1990; Zhao et al., 1999; Zhang et al., 2000; Gujar et al., 2004; Zhu et al., 2007; Pardo-López et al., 2009; Ma et al., 2013; Mesén-Porras et al., 2020). The effects described indicate, for example, a 20-fold higher toxicity of Bt proteins if produced in the plants and taken up with PIs (MacIntosh et al., 1990). Differences in toxicity between toxins produced in isolation compared to those produced by the plants are also described for Vip3A efficacy in transgenic plants (Khan et al., 2020).

It also should be taken into account that the toxicity of Bt toxins can not only be enhanced through interaction with plant enzymes such as PI, but also by Bt toxins (Sharma et al., 2004; Sharma et al., 2010; Tabashnik et al., 2013; Bøhn et al., 2016; Bøhn, 2018), gut bacteria (Broderick et al., 2009), residues from spraying with herbicides (Bøhn et al., 2016; Bøhn, 2018) and other co-stressors (Kramarz et al., 2007; Kramarz et al., 2009; Khalique and Ahmed, 2005; Singh et al., 2007; Zhu et al., 2005; Mason et al., 2011; Reardon et al., 2004).

Therefore, any risk assessment that does not take synergistic effects caused by the combination of plant material or other stressors with the Bt toxin into account is not reliable and systematically underestimates the risks.

These issues are especially relevant for the stacked events since the overall concentration of Bt toxins is higher and combinatorial effects with other stressors (such as residues from spraying) more likely.

In summary, the evidence for enhanced toxicity of Bt proteins produced in maize, cotton and soybeans was published by Monsanto 30 years ago (MacIntosh et al., 1990) and has since then been confirmed in multiple studies. Crucially, EFSA has never assessed this aspect in any of its opinions.

Instead, the toxicity of the Bt toxins was assessed on the basis of feeding studies, using only isolated Bt proteins produced by bacteria for gavage experiments in mice **(MSL0028115)**. The data from these experiments were then used to calculate NOAEL (No-Observed-Adverse-Effect Level) and to assess the impact of exposure at the stage of consumption. Therefore, considering the above findings, the basic data for toxicity assessment of the stacked Maize are neither valid nor reliable. The lack of toxicity studies was also criticised by Member States experts (EFSA, 2021b). In addition, incorrect assumptions were made on the degradation of the Bt toxins at the stage of consumption and similarity to known toxins (see below). Therefore, the Monsanto risk assessment depends entirely on incorrect assumptions in regard to toxicity and exposure.

Immunogenicity of the Bt toxins

There are several studies indicating that immune responses in mammals can be triggered by Bt toxins and have to be considered in this context. Studies with the Cry1Ac toxin (Moreno-Fierros et al., 2000; Vázquez-Padron 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). Since Cry1Ac is also used as adjuvance in vaccines, its risks for food consumption, which can be fostered by synergistic effects need to be addressed and carefully examined.

The synergistic effects described by MacIntosh et al. (1990), Zhao et al. (1999), Zhang et al. (2000), Gujar et al. (2004), Zhu et al. (2007), Pardo-López et al. (2009), Ma et al. (2013), or Mesén-Porras et al. (2020) causing higher toxicity of the Bt toxins are also relevant in risk assessment in regard to the immune system: combination with protease inhibitors is likely to be associated with a delay in the degradation of the Bt toxins after consumption. This delay in degradation extends the exposure of the intestinal immune system to Bt toxins and may trigger or enhance chronic inflammation and other immune response. For example, a study testing corn with a combination of Bt toxins (Cry1Ab and Cry34Ab1) indicates inflammatory effects in rats (Zdziarski et al., 2018).

In this context, it is relevant that Bt toxins produced by plants can survive digestion to a much higher degree than has been assumed by EFSA and shown by the data of the applicant. Chowdhury et al. (2003) and Walsh et al. (2011) showed that when pigs were fed with Bt maize, Cry1A proteins could frequently and successfully still be found in the colon of pigs at the end of the digestion process. This means that Bt toxins are not degraded quickly in the gut and can persist in larger amounts until digestion is completed; therefore, there is enough time for interaction between various food compounds.

These issues are especially relevant for the stacked events since the overall concentration of Bt toxins is higher compared to the parental plants.

Not only is the concentration of Bt toxins higher in the stacked Maize, there is also a higher likelihood of combinatorial effects with other stressors (such as residues from spraying). However, neither EFSA nor the applicant considered the potential enhancement of toxic or immunogenic effects caused by interaction with plant components such as PI. In this context, potential impacts on the microbiome also have to be taken into account (see below).

Effects of complementary herbicide residues specific to GE plants and mixed toxicity

The residues from spraying with complementary herbicides 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 pattern of sprayings, 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 (2015a, 2015b and 2018a) explicitly stated that no conclusion can be drawn on the safety of residues from spraying with glyphosate 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. (…)."*[11]

The stacked Maize combines several EPSPS enzymes conferring enhanced resistance to glyphosate; therefore, a higher burden of the residues from spraying might be expected compared to the parental plants. 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.

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 needs to be tested in regard to its mixed toxicity (EFSA, 2019b).

Glyphosate is particularly problematic in regard to chronic exposure when it is consumed in food. This is because glyphosate is known to cause shifts in the microbial composition and associated microbiomes of plants and animals: glyphosate (Roundup) has been shown to cause shifts not only in soil organisms (van Bruggen et al., 2018) but also in the composition of the intestinal flora in cattle (Reuter et al., 2007), poultry (Shehata et al., 2013; Ruuskanen et al, 2020) and rodents (Mao et al., 2018; Mesnage et al., 2021; Tang et al., 2020) as well as honey bees (Motta et al., 2020) and *Daphnia* (Suppa et al., 2020). Therefore, antibiotic effects caused by chronic exposure to food and feed derived from glyphosate-resistant GE plants, including the stacked Maize, is not unlikely to trigger significant changes in intestinal bacteria.

Alarmingly, similar effects are reported from the exposure to glufosinate, showing that glufosinate can severely impact the microbiome (Dong et al., 2020). Therefore, the mixed toxicity of the stacked event needs to be assessed much more carefully compared to the parental plants.

---

[11] www.testbiotech.org/content/eu-commission-request-consider-impact-glyphosate-residues-feed-animal-health-february-2016

In general, the microbiome can be seen as a common network of life, circumventing and closely interacting with plants, animals and humans. Microbial networks are thought to have co-evolved with their hosts and have developed a mutualistic relationship that benefit both the host and microorganisms. They act at the interphase and communicate between an organism and its wider environment while at the same time being part of the closer environment of an organism. Microbiomes are considered key for the health of higher organisms, i.e. humans, animal and plants.

Just recently, a document published by EFSA (EFSA, 2020), called attention to the role of the microbiome in environmental risk assessment and food and feed safety. In regard to food and feed safety, EFSA (2020) considers microbiomes to be highly relevant to the health status of their hosts. Therefore, it is desirable to understand the importance of their role in risk assessment. EFSA expects that gut microbiome research (not only in the case of GE plants) will play a relevant role in regulatory science with potential implications for future risk assessments and predictive risk models. As EFSA states: "*considering that the gut microbiome is a biological component directly and indirectly involved in the metabolism of food/feed components and chemicals and in the protection of the host against adverse environmental exposure, it would be useful to establish criteria on how to evaluate the potential adverse impacts of perturbators on this defensive barrier, and consequently, on human/animal health.*"

In 2019, EFSA commissioned a study on the adjuvanticity / immunogenicity assessment of proteins, including the role of the microbiome. Parenti et al. (2019) state that "*one of the most important drivers of immune response is the gut microbiota and other microbial constituent of the human body which are able to regulate host-pathogen balance and to produce systemic pro-inflammatory stimuli. The lifelong antigenic load represented by foods and bacteria/bacterial products leads to a profound remodeling of the gut microbiota and these changes are emerging as a driving force of the functional homeostasis of the immune system. As a matter of fact, a perturbation of the gut microbiota homeostasis due to irregular lifestyles, stress and age may lead to gut microbiota dysbiosis. This condition may predispose the host to metabolic disorders and inflammation*."

This finding is highly relevant for the risk assessment of the stacked Maize. Several EPSPS enzymes are combined in the stacked Maize for enhanced resistance to glyphosate, therefore a higher burden of residues from spraying can be expected compared to the parental plants. These residues my cause a perturbation of the gut microbiome. At the same time, stacking in the Maize results in a combination of several Bt toxins that are likely to show immunogenicity. It is plausible that this combination of Bt toxins, together with residues from spraying, can trigger effects on the immune system either directly or via the microbiome. This hypothesis needs to be tested before any conclusion can be drawn on the health safety of food and feed.

However, no attempts have been made to integrate the microbiome into the risk assessment of food and feed derived from the stacked Maize. This is contrary to Regulation 1829/2003 which requests "*genetically modified food and feed should only be authorised for placing on the Community market after a scientific evaluation of the highest possible standard, to be undertaken under the responsibility of the European Food Safety Authority (Authority), of any risks which they present for human and animal health and, as the case may be, for the environment.*" (Recital 9).

Toxicity of ncsRNA DvSnf7

A report commissioned and published by EFSA in 2019 (Davalos et al., 2019) considers the role of ncsRNA in the risk assessment of GE plants. Davalos et al. summarise current findings on ncsRNAs produced by plants; they discuss to which extent they can be taken up via food or feed consumption and show cross kingdom activity due to unintentional interaction with human or animal gene regulation. The findings of Davalos et al. (2019) and Nawaz et al. (2019) were not mentioned by EFSA. Therefore, these publications have to be regarded as new information and also as new evidence since they go beyond the EFSA assessment.

- Potential off target genes in mammals

As Davalos et al. (2019) show, there are many matches between the ncsRNA produced in food and medical plants and regulatory pathways in human and animals. There is no doubt that in cases where relevant plant molecules are transmitted into the cells of humans and animals, RNAi effects, such as gene silencing, can occur and, for example, genes in animals can be downregulated by plant nscRNA.

Therapeutic effects from the uptake of ncsRNA from the gut have been evidenced in several publications. Some of the research shows that biological effects can be achieved with very low dosages (for references see Davalos et al., 2019).

- Stability of ncsRNA

It appears that some findings depend on the specific type of ncsRNA. For example, naked synthetic ncsRNA used by some researchers, is degraded very quickly compared to ncsRNA produced by plants (for references see Davalos et al., 2019).

The Davalos et al. (2019) study found strong indications that plant miRNAs are more stable than previously anticipated. This is due to structural properties influencing their stability and turnover (EFSA, 2019a): *"However, when assessing the stability of plant ncRNAs outside the plant, compelling evidence exists that plant miRNAs are highly stable under different conditions including food storage, processing, cooking, or simulated digestion. Moreover, they seem to survive after long incubation in serum, or are detected in the gastric content of mice, suggesting that plant miRNAs are more resistant to degradation than synthetic or animal miRNAs."*

- ncsRNA uptake from the gut

Contrary to assumptions made in the assessment of the parental plants (EFSA, 2018a) and the stacked event (EFSA, 2021a), research by Davalos et al. (2019) shows that the uptake of ncsRNA from plants and microorganisms via the gut into the cells of humans and animals is an established fact.

It is known that there are many barriers between the intestine, the blood stream, the cells and the cell nuclei, which lower the likelihood of such RNAi effects occurring. However, according to Davalos et al. (2019), there are mechanisms that can allow the molecules to pass through these barriers: plant ncsRNA is protected against degradation by methylation, it can be excreted and taken up in vesicles (such as exosomes); nano-particles are also produced by plants which can serve as transport elements.

The ncsRNA molecules originating from plants can reportedly be found in many bodily fluids of humans and animals, including blood and milk. Similar findings have been reported by Nawaz et al., (2019): “*Strong evidence suggested that plant-food-miRNAs can survive digestion, enter the body and affect gene expression patterns.*”

In this context, Davalos et al. (2019) see the need for further research to explore the uptake and biological effects of ncsRNA: *“Exogenous plant-derived ncRNAs have been found in exosomes or macrovesicles. How they reach these types of structures in biological fluids is unknown. In summary, supporting and contradicting evidence concerning the existence of systemic effects of dietary plant-derived exogenous ncRNAs is heavily debated. Important aspects such as the precise mechanism/s of transport of plant ncRNAs from food into the systemic circulation, the amount of exogenous ncRNAs reaching tissues or the molecular mechanisms of cellular uptake need to be determined.”*

- Interactions on the level of the microbiome

There is strong evidence that ncsRNAs originating in the host (e.g. produced by intestinal epithelial cells) are taken up by the gut microbiota and can manipulate its gene regulation. The same evidence is available for ncsRNA produced in the gut microbiome: it can be taken up by the host and enact RNAi in its cells, demonstrating the existence of bidirectional ncRNAs based host-microbial interactions (for details see Davalos et al., 2019).

In this context, Davalos et al. (2019) show that plant-derived ncsRNA does not necessarily have to be taken up from the intestine to exert its effects. Instead, interaction with the intestinal microbiome can emerge which, in a next step, may impact the health of the animal or human host.

This is of specific interest in the case of DvSnf7: the Snf7 gene which is targeted by the dsRNA produced in maize MON 87441, is involved in important biological processes in insects as well as in yeast. The essential role of the Snf7 as part of the ESCRT pathway is well described (see www.yeastgenome.org/locus/S000004015).

Thus, there is a plausible hypothesis on how the additional dsRNA might affect the gut microbiome community after ingestion, and further research is needed to understand the impact of exogenous dsRNA in mammalian host microbiota composition and identify microbial targets along with their effect on physiological conditions.

There is broad consensus on the role of the gut microbiome in human and animal health. For example, in 2019, in a study commissioned by EFSA, Parenti et al. (2019) states that “*one of the most important drivers of immune response is the gut microbiota and other microbial constituent of the human body which are able to regulate host-pathogen balance and to produce systemic pro-inflammatory stimuli. The lifelong antigenic load represented by foods and bacteria/bacterial products leads to a profound remodeling of the gut microbiota and these changes are emerging as a driving force of the functional homeostasis of the immune system. As a matter of fact, a perturbation of the gut microbiota homeostasis due to irregular lifestyles, stress and age may lead to gut microbiota dysbiosis. This condition may predispose the host to metabolic disorders and inflammation.*”

- Conclusions on toxicity assessment of ncsRNA DvSnf7

Therefore, the interaction between the ncsRNAs produced by GE plants and the microbiome of humans or animals has to be considered in food and feed safety assessment. In this context, the barrier for ncsRNA to pass from plants to gut microorganisms seems to be much lower compared to those identified in the human or animal body.

In summary, it is clear that interference with gene regulation following the absorption and processing of dsRNAs to siRNA within humans and animals after ingestion of RNAi-based *GM* *crops is both feasible and plausible. As Nawaz et al. (2019) conclude: "Based on the currently available evidence, off-target effects from the ingestion of novel siRNA present in foods derived from either GM crops or foliar insecticidal or anti-viral spray application, cannot be ignored and thus should form an integral part of the risk assessment of these products."*

As shown by Davalos et al., (2019) and Nawaz et al. (2019), the uptake of ncsRNA from plants via ingestion in sufficient amounts to exert effects on gene regulation in mammalian cells must be seen as a certainty. Further, the impact on the host via its microbiome is another way in which human or animal health could be affected.

Therefore, further risk assessment has to be performed

- to trace the fate of the artificial ncsRNA after ingestion
- to identify the potential target site in the microbial community in the gut and mammalian cells
- to assess the magnitude of potential effects if identified.

Additional questions have arisen from risk assessment in respect to the mixed toxicity of the stacked Maize. These questions are highly relevant for demonstrating the safety of the plants because other newly expressed proteins, residues from spraying or plant constituents, can influence the impact on the microbiome in the gut or the uptake from the gut.

Overall conclusions on toxicity

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. In this context, it is relevant to consider that the outcome of the feeding studies with the parental plants raised several questions concerning their results, methodology and reliability.

Testbiotech is also aware that feeding studies with similar stacked maize indicated potential health impacts such as inflammatory responses in the stomach (Zdziarski et al., 2018). Inflammatory responses are an alarm signal typical of many chronic diseases and therefore require close attention. While the applicant provided some data in regard to celiac disease, other diseases associated with symptoms of chronic inflammation were not considered at all.

EFSA should have requested the applicant to submit data from field trials which represent the environmental and agricultural conditions under which the Maize will be grown. Drought and high rates of complementary herbicide applications should have been taken into account. The material derived from the plants should have been assessed, especially in regard to long-term organ toxicity, immune responses and impact on the gut microbiome, also taking combinatorial effects and mixed toxicity into account.

4. 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;*"

Potential allergenicity

EFSA does not mention that Cry1Ac is thought to be allergenic (Santos-Vigil et al., 2018)[12]. According to Santos-Vigil et al. (2018), the Bt toxin Cry1Ac can act as an allergen if ingested. This publication is 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 Cry1Acis thought to be an allergen, the source of Cry1A.105 has to be verified as allergenic and therefore investigated in detail.

The EU Commission also 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. In response, 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 deficiencies. 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 et al. (2018) findings are invalid or irrelevant (for more details see Moreno-Fierros et al., 2018). Consequently, the Santos-Vigil et al. (2018) publication has to be considered valid but not properly assessed by EFSA. Certainly, EFSA should have requested testing of the hypothesis that the consumption of products derived from the maize can trigger allergic reactions.

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.

Potential adjuvanticity

The synergistic effects between PI and Bt toxins as described above are also relevant for risk assessment in regard to adjuvanticity: the combination with protease inhibitors is likely to be associated with a delay in the degradation of the Bt toxins after consumption. This delay in degradation extends the exposure of the intestinal immune system to Bt toxins and may trigger or enhance relevant effects.

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-Padron et al. 1999; Legorreta-Herrera et al., 2010; Jarillo-

---

[12] see also: www.testbiotech.org/en/press-release/can-bt-toxins-cause-allergies

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). Since Cry1Ac is also used as an adjuvant in vaccines, the risks to food consumption can be promoted through synergistic effects, this needs to be addressed and carefully examined. The need for more detailed investigations in regard to potential immunogenic effects is also pointed out in the minority opinion in another EFSA opinion (Annex II of EFSA, 2018c).

Given the fact that potential effects of Bt toxins on the immune system have meanwhile been discussed for many years (for overview see, for example, Then & Bauer-Panskus, 2017), and already 45 GE crop events producing Bt toxins have been approved for the EU market, this explanatory excuse cannot be accepted. In accordance with EU Regulation 1829/2003, safety of whole food and feed has to be demonstrated before approval for import can be issued. Since this is not the case with the stacked Maize, the risk assessment is not conclusive and no market authorisation can be granted.

These issues are especially relevant for stacked events since the overall concentration of Bt toxins is higher and combinatorial effects with other stressors (such as residues from spraying) are more likely. This is also relevant for immune responses exerted via the microbiome (see above). However, neither EFSA nor the applicant considered the potential enhancement of toxic or immunogenic effects caused by interaction with plant components.

Conclusion on allergenicity and adjuvanticity

Considering these uncertainties, EFSA should have requested empirical testing of allergenic or adjuvant effects. In conclusion, allergenicity and adjuvanticity were not assessed to sufficiently demonstrate that the food and feed from the stacked Maize has no adverse effects on human and animal health.

5. Others

(1) 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.

(2) 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.

6. Environmental risk assessment

The appearance of teosinte in Spain and France (see Testbiotech, 2016; Trtikova et al., 2017) has to be considered in more detail. 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 biological characteristics of maize MON87460 and the findings of Fang et al. (2018), the stacked maize needs to be examined in detail for next generation effects, volunteer potential (persistence) and gene flow. In doing so, the hypothesis that the maize and its offspring will show a higher fitness compared to conventional maize, is evident. This might also concern adaption to colder climate due to the biological characteristics of the cold shock protein (CSPB). Under these circumstances, even a rare single outcrossing event that goes unnoticed can have a huge long-term impact on agro-ecosystems.

Likelihood of gene flow

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.

There are new publications confirming that the risks from crossings of GE maize and teosinte cannot be predicted from the data assessed by EFSA (2021a): as already shown by Trtikova et al. (2017), another publication, Le Corre et al. (2020), confirms that European teosinte plants from Spain and France in fact, integrated larger genomic parts from European maize varieties.

As Le Corre et al. (2020) show, teosinte has changed its biological characteristics in ways that will facilitate further genetic exchange with maize plants. Similarly, Diaz et al. (2019) show that these new weeds seem to have a complex origin. Therefore, the likelihood of hybridisation with the GE maize has strongly increased. For example, teosinte has now altered its flowering time. Furthermore, teosinte has already acquired herbicide-resistance from conventional European maize varieties. In awareness of their findings, the authors emphasise that their results show that risks of crop-wild introgression should not be underestimated in forecasting the risk of invasiveness. They show that crop-wild introgression can be a two-way street, enhancing gene flow to both partners, maize and teosinte. The scientists have therefore explicitly warned that the risk of the plants becoming invasive should not be underestimated.

Enhanced fitness

If the known characteristics of the stacked Maize were to be transferred to teosinte this would render them herbicide resistant, toxic for pest insects and drought tolerant.

In addition, a higher overall fitness of hybrid offspring can result from the additional EPSPS enzymes. Fang et al. (2018) showed that higher fitness does occur in GE glyphosate-resistant *Arabidopsis thaliana* in a glyphosate-free environment as an unintended effect. According to this research, the expressed enzyme EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) not only makes the plants resistant to glyphosate, it also interferes with metabolic processes associated with their growth and fecundity. As a consequence, plant offspring can produce more seeds and be more resistant (tolerant) to environmental stressors such as drought and heat. The authors stated that the observed effects are likely to be caused by increased production of the hormone auxin in the transgenic plants. This plant hormone plays a key role in growth, fecundity and adaptation to environmental stressors. The general findings regarding enhanced fitness of plants inheriting additional EPSPS genes are supported by several other publications (Beres, 2019; Beres et al., 2018; Wang et al., 2014; Yang et al., 2017). In addition, other authors show the need for further investigations (Vila-Aiub et al., 2009 and Vila-Aiub et al., 2019). Thus if gene flow occurs, this effect which was completely ignored by EFSA and the applicant, can strongly enhance the spread of teosinte in the fields and possibly also beyond the fields.

Next generation effects

Unlike maize, teosinte can overwinter in the fields and pass new genetic information to offspring - from where it has the potential to spread and become a new European super-weed. These risks are not only a concern for farmers, they could also seriously damage the environment and protected species.

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; for overview see Bauer-Panskus et al., 2020). This issue is relevant for gene flow from maize to teosinte as well from teosinte to maize.

Therefore, the characteristics of potential hybrids and next generations have to be investigated and cannot be predicted simply from the data of the original event, as suggested by EFSA.

Conclusion on environmental risk assessment

Without detailed consideration and examination 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.

This is especially relevant for the stacked Maize which combines several traits which are likely to result in higher fitness of hybrid offspring.

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.

The risk assessment as performed does not fulfill EU regulations which require the examination of the direct and indirect as well as the immediate and delayed effects of the GMO on human health and the environment.

7. Conclusions

The EFSA risk assessment cannot be accepted. Given the findings, there are multiple violations of EU Regulations requesting that the safety of the GE plants is demonstrated:

1. The plants were not exposed to bioclimatic conditions that sufficiently represent the regions in which these plants will be cultivated. Consequently, expression data and the assessment of plant composition as well as of agronomic and phenotypic characteristics are not sufficiently reliable to inform the next steps in risk assessment. This is especially relevant since (i) the plants are intended for cultivation in drought conditions, (ii) the EPSPS enzymes are known to show unintended effects under stress conditions (such as drought) and (iii) the Bt content is known to be influenced by environmental factors (such as drought).

2. The plants were not exposed to agricultural practices which sufficiently represent the conditions under which these plants will be cultivated. Consequently, expression data and the assessment of plant composition as well as agronomic and phenotypic characteristics are not sufficiently reliable to inform the next steps in risk assessment.

3. No detailed examinations were requested despite data from other events and previous applications indicating that environmental stress factors, herbicide applications rates, genetic backgrounds and stacking all impact gene expression and plant metabolism.

4. Risks which indicate toxicological health impacts, potentially enhanced by combinatorial effects caused by the stacking, were not assessed against relevant, reasoned and plausible hypotheses. For example, it was not examined to which extent the toxicity of the selectivity of the Bt toxins are changed by the mixed toxicity of whole food and feed. This is especially relevant in this case since it is known that enzymes (protease inhibitors) produced in the plants can multiply the toxicity of the Bt toxins and prolong exposure to the toxins in the gut after ingestion.

5. Risks which indicate immunological health impacts, potentially enhanced by the combinatorial effects of stacking, were not assessed against relevant, reasoned and plausible hypotheses. For example, it was not examined to which extent changes in the microbiome caused by the consumption of the Maize will impact its immunogenic properties. This is especially relevant since it is known that enzymes (protease inhibitors) produced in the plants can prolong exposure to the toxins in the gut after ingestion. Furthermore, residues from spraying with glyphosate (Roundup) are known to impact the composition of the microbiome.

6. The interaction between the ncsRNAs produced in the GE plants and the microbiome of humans or animals was not assessed, despite new findings showing that these interactions are likely to occur, and adverse impacts on human and animal health are a plausible consequence. That a combination of DvSnf7 dsRNA, Bt toxins and residues from spraying can trigger effects on the

immune system or other adverse health effects, either directly or via the microbiome, even if these were absent in the parental plants, has to be considered a plausible hypothesis. Therefore, the stacked Maize needs to be tested for synergistic effects due to other plant constituents or additional factors, which may enhance stability or uptake of DvSnf7 dsRNA from the gut.

7. The environmental risk assessment and the biological characteristics of the hybrid offspring of teosinte and the Maize were not assessed against relevant, reasoned and plausible hypotheses. This is especially relevant since the offspring may show invasive characteristics due to the unintended effects caused by the EPSPS enzymes, higher resistance to damage by insects and potentially higher tolerance to drought conditions.

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 Res, 18: 143-149. https://doi.org/10.1007/s11248-008-9198-z

Agapito-Tenfen, S.Z., Guerra, M. P., Wikmark, O.G., Nodari, R.O. (2013) Comparative proteomic analysis of genetically modified maize grown under different agroecosystems conditions in Brazil. Proteome Science, 11(1): 46. https://doi.org/10.1186/1477-5956-11-46

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: 1-19. https://doi.org/10.1186/s12870-014-0346-8

Bauer-Panskus, A., Miyazaki, J., Kawall, K., Then, C. (2020) Risk assessment of genetically engineered plants that can persist and propagate in the environment. Environ Sci Eur, 32(1): 1-15. https://doi.org/10.1186/s12302-020-00301-0

Ben Ali, S.E., Draxler, A., Poelzl, D., Agapito-Tenfen, S., Hochegger, R., Haslberger, A.G., Brandes, C. (2020) Analysis of transcriptomic differences between NK603 maize and near-isogenic varieties using RNA sequencing and RT-qPCR. Environ Sci Eur, 32(1): 1-23. https://doi.org/10.1186/s12302-020-00412-8

Benevenuto, R.F., Agapito-Tenfen, S.Z., Vilperte, V., Wikmark, O.-G., van Rensburg, P.J., Nodari, R.O. (2017) Molecular responses of genetically modified maize to abiotic stresses as determined through proteomic and metabolomic analyses. PLoS ONE, 12(2): e0173069. https://doi.org/10.1371/journal.pone.0173069

Beres, Z.T. (2019) Ecological and evolutionary implications of glyphosate resistance in Conyza canadensis and *Arabidopsis thaliana*. Dissertation presented in partial fulfillment of the requirements for the degree Doctor of Philosophy in the graduate school of the Ohio State University. http://rave.ohiolink.edu/etdc/view?acc_num=osu1555600547328876

Beres, Z.T., Yang, X., Jin, L., Zhao, W., Mackey, D.M., Snow, A.A. (2018) Overexpression of a native gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) may enhance Fecundity in *Arabidopsis thaliana* in the absence of glyphosate. Int J Plant Sci, 179(5):390–401. https://doi.org/10.1086/696701

Bøhn, T. (2018) Criticism of EFSA's scientific opinion on combinatorial effects of ‘stacked’GM plants. Food Chem. Toxicol, 111: 268-274. https://doi.org/10.1016/j.fct.2017.11.023

Bøhn, T., Rover, C.M., Semenchuk, P.R. (2016) *Daphnia magna* negatively affected by chronic exposure to purified Cry-toxins. Food Chem. Toxicol. 91: 130-140. https://doi.org/10.1016/j.fct.2016.03.009

Bollinedi, H., Gopala Krishnan, S., 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: e67079. https://doi.org/10.1371/journal.pone.0067079

Broderick, N.A., Robinson, C.J., McMahon, M.D., Holt, J., Handelsman, J., Raffa, K.F. (2009) Contributions of gut bacteria to *Bacillus thuringiensis*-induced mortality vary across a range of Lepidoptera. BMC Biol, 7: 11. https://doi.org/10.1186/1741-7007-7-11

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(12): 1138-1148. https://doi.org/10.1111/j.1744-7909.2009.00877.x

Chen, D., Ye, G., Yang, C., Chen Y., Wu, Y. (2005) The effect of high temperature on the insecticidal properties of Bt Cotton. Environ Exp Bot, 53: 333-342. https://doi.org/10.1016/j.envexpbot.2004.04.004

Chowdhury, E.H., Kuribara, H., Hino, A., Sultana, P., Mikami, O., Shimada, N., Guruge, K.S., Saito, S., 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. J Anim Sci, 81(10): 2546-2551. https://doi.org/10.2527/2003.81102546x

Dávalos, A., Henriques, R., Latasa, M.J., Laparra, M., Coca, M. (2019) Literature review of baseline information on non-coding RNA (ncRNA) to support the risk assessment of ncRNA-based genetically modified plants for food and feed. EFSA Supporting Publication, 16(8): EN-1688. https://doi.org/10.2903/sp.efsa.2019.EN-1688

de Souza Freire, I., Miranda-Vilela, A.L., Barbosa, L.C.P., Martins, E.S., Monnerat, R.G., Grisolia, C.K. (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://doi.org/10.3390/toxins6102872

Díaz, A., Taberner, A., Vilaplana, L. (2020) The emergence of a new weed in maize plantations: characterization and genetic structure using microsatellite markers. Genet Resour Crop Evol, 67: 225-239. https://doi.org/10.1007/s10722-019-00828-z

Dong, H.Z., & Li, W.J. (2006) Variability of endotoxin expression in Bt transgenic cotton. J Agron Crop Sci , 193: 21-29. https://doi.org/10.1111/j.1439-037X.2006.00240.x

Dong, T., Guan, Q., Hu, W., Zhang, M., Zhang, Y., Chen, M., ... & Xia, Y. (2020) Prenatal exposure to glufosinate ammonium disturbs gut microbiome and induces behavioral abnormalities in mice. J Hazard Mater, 389: 122152. https://doi.org/10.1016/j.jhazmat.2020.122152

EFSA (2015a) Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate. EFSA J, 13 (11): 4302. http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2015.4302/full

EFSA (2015b) Statement of EFSA on the request for the evaluation of the toxicological assessment of the coformulant POE-tallowamine. EFSA J, 13(11): 4303. https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/j.efsa.2015.4303

EFSA (2018a) Review of the existing maximum residue levels for glyphosate according to Article 12 of Regulation (EC) No 396/2005. EFSA J, 16(5): e05263. 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, 15(11): EN-1504. https://doi.org/10.2903/sp.efsa.2019.EN-1504

EFSA (2018c) 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 J, 16(7): e05309. https://doi.org/10.2903/j.efsa.2018.5309

EFSA (2019a) Scientific Opinion on the assessment of genetically modified maize MON 87427 x MON 89034 x MIR162 x MON 87411 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2017-144). EFSA J, 17 (11): 5848. https://doi.org/10.2903/j.efsa.2019.5848

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

EFSA (2020) Editorial: Exploring the need to include microbiomes into EFSA's scientific assessments. EFSA J, 18(6): e18061. https://doi.org/10.2903/j.efsa.2020.e18061

EFSA (2021a) Scientific Opinion on the assessment of genetically modified maize MON 87427 × MON 87460 × MON 89034 × 1507 × MON 87411 × 59122 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2017-139). EFSA J, 19(1): 6351,. https://doi.org/10.2903/j.efsa.2021.6351

EFSA (2021b) Application , 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. Front Plant Sci, 9: 233. https://doi.org/10.3389/fpls.2018.00233

Girón-Calva, P.S., Twyman, R.M., Albajes, R., Gatehouse, A. M., Christou, P. (2020) The impact of environmental stress on Bt crop performance. Trends Plant Sci, 25(3), 264-278. https://doi.org/10.1016/j.tplants.2019.12.019

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(2-4): 382-388. https://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. Mol Immunol, 41(12): 1177-1183. https://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. https://doi.org/10.1111/j.1365-3083.2007.01992.x

Gujar, T., Kalia, V., Kumari, A., Prasad, T.V. (2004) Potentiation of insecticidal activity of *Bacillus thuringiensis* subsp. kurstaki HD-1 by proteinase inhibitors in the American bollworm, *Helicoverpa armigera* (Hübner). Indian J Exp Biol, 42: 157-163. http://hdl.handle.net/123456789/23352

Hilbeck, A. & Otto, M. (2015) Specificity and combinatorial effects of *Bacillus thuringiensis* Cry toxins in the context of GMO risk assessment. Front Environ Sci, 3: 71. https://doi.org/10.3389/fenvs.2015.00071

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. PNAS USA, 101: 10995-11000. https://doi.org/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 Immunology, 36(6): 266-270. https://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. J Biol Chem, 279: 21282-21286. https://doi.org/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(1): 31-38. https://doi.org/10.1111/j.1365-3024.2007.00999.x

Jiang, Y., Ling, L., Zhng, L., Wang, K., Li, X., Cai, M., ... & Cao, C. (2018) Comparison of transgenic Bt rice and their non-Bt counterpart in yield and physiological response to drought stress. Field Crops Res, 217: 45-52. https://doi.org/10.1016/j.fcr.2017.12.007

Kawata, M., Murakami, K., Ishikawa, T. (2009) Dispersal and persistence of genetically modified oilseed rape around Japanese harbors. Environ Sci Pollut Res 16(2):120–126. https://doi.org/10.1007/s11356-008-0074-4

Khalique, F. & Ahmed, K. (2005) Compatibility of bio-insecticide with chemical insecticide for management of *Helicoverpa armigera* Huebner. Pak J Biol Sci, 8: 475-478. https://dx.doi.org/10.3923/pjbs.2005.475.478

Khan, M.H., Jander, G., Mukhtar, Z., Arshad, M., Sarwar, M., Asad, S. (2020). Comparison of in vitro and in planta toxicity of Vip3A for lepidopteran herbivores. J Econ Entomol, 113(6): 2959-2971. https://doi.org/10.1093/jee/toaa211

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 Manag Sci, 67(10):1193-1210. https://doi.org/10.1002/ps.2128

Kramarz, P., de Vaufleury, A., Gimbert, F., Cortet, J., Tabone, E., Andersen, M.N., Krogh, P.H. (2009) Effects of Bt-maize material on the life cycle of the land snail *Cantareus aspersus*. Appl Soil Ecol, 42: 236-242. https://doi.org/10.1016/j.apsoil.2009.04.007

Kramarz, P.E., de Vaufleury, A., Zygmunt, P.M.S., Verdun, C. (2007) Increased response to cadmium and *Bacillus thuringiensis* maize toxicity in the snail *Helix aspersa* infected by the nematode *Phasmarhabditis hermaphrodita.* Environ Toxicol Chem, 26: 73-79. https://doi.org/10.1897/06-095R.1

Le Corre, V., Siol, M., Vigouroux, Y., Tenaillon, M.I., Délye, C. (2020) Adaptive introgression from maize has facilitated the establishment of teosinte as a noxious weed in Europe. PNAS USA, 117(41): 25618-25627. https://doi.org/10.1073/pnas.2006633117

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: 198921. https://doi.org/10.1155/2010/198921

Lohn, A.F., Trtikova, M., Chapela, I., Van den Berg, J., Du Plessis, H., Hilbeck, A. (2020) Transgene behavior in *Zea mays* L. crosses across different genetic backgrounds: Segregation patterns, cry1Ab transgene expression, insecticidal protein concentration and bioactivity against insect pests. PLoS ONE, 15(9): e0238523. https://doi.org/10.1371/journal.pone.0238523

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

Luo, Z., Dong, H., Li, W., Ming, Z., Zhu, Y. (2008) Individual and combined effects of salinity and waterlogging on Cry1Ac expression and insecticidal efficacy of Bt cotton. Crop Protection, 27(12): 1485-1490. https://doi.org/10.1016/j.cropro.2008.06.006

Ma, Y., Zhang, Y., Chen, R.-R., Ren, X.-L., Wan, P.-J., Mu, L.-L., Li, G.-Q. (2013) Combined effects of three crystalline toxins from *Bacillus thuringiensis* with seven proteinase inhibitors on beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae). Pestic Biochem Physiol, 105: 169-176. https://doi.org/10.1016/j.pestbp.2013.01.007

MacIntosh, S.C., Kishore, G.M., Perlak, F.J., Marrone, P.G., Stone, T.B., Sims, S.R., Fuchs, R.L. (1990) Potentiation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitors. J Agric Food Chem, 38: 1145-1152. https://doi.org/10.1021/jf00094a051

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

Mason, K.L., Stepien, T.A., Blum, J.E., Holt, J.F., Labbe, N.H., Rush, J.S., Raffa, K.F., Handelsman, J. (2011) From commensal to pathogen: translocation of *Enterococcus faecalis* from the midgut to the hemocoel of *Manduca sexta*. mBio 2: e00065-00011. https://doi.org/10.1128/mBio.00065-11

Mesén-Porras, E., Dahdouh-Cabia, S., Jimenez-Quiros, C., Mora-Castro, R., Rodríguez, C., Pinto-Tomás, A. (2020) Soybean protease inhibitors increase *Bacillus thuringiensis* subs. israelensis toxicity against *Hypothenemus hampei*. Agronomía Mesoamericana, 31: 461-478. https://doi.org/10.15517/am.v31i2.36573

Mesnage, R., Clair, E., Gress, S., Then, C., Székács, A., Séralini, G.-E. (2013) Cytotoxicity on human cells of Cry1Ab and Cry1Ac Bt insecticidal toxins alone or with a glyphosate-based herbicide. J Appl Toxicol, 33: 695-699. https://doi.org/10.1002/jat.2712

Mesnage, R., Agapito-Tenfen, S.Z., Vilperte, V., Renney, G., Ward, M., Séralini, G.E., Nodari, R.O., Antoniou, M.N. (2016) An integrated multi-omics analysis of the NK603 Roundup-tolerant GM maize reveals metabolism disturbances caused by the transformation process. Sci Rep, 6: 37855. https://doi.org/10.1038/srep37855

Mesnage, R., Teixeira, M., Mandrioli, D., Falcioni, L., Ducarmon, Q.R., Zwittink, R.D., Mazzacuva, F., Caldwell, A., Halket, J., Amiel, C., Panoff, J.-M., Belpoggi, F. Antoniou, M.N. (2021) Use of shotgun metagenomics and metabolomics to evaluate the impact of glyphosate or Roundup MON 52276 on the gut microbiota and serum metabolome of Sprague-Dawley rats. Environ Health Perspect, 129(1): 017005. https://doi.org/10.1289/EHP6990

Mezzomo, B.P. (2013) Hematotoxicity of Bacillus thuringiensis as spore-crystal strains Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa in Swiss albino mice. J Hematol Thromb Dis, 1(1): 1-9. http://repositorio.unb.br/handle/10482/18532

Miyazaki, J., Bauer-Panskus, A., Bøhn, T., Reichenbecher, W., Then, C. (2019) Insufficient risk assessment of herbicide-tolerant genetically engineered soybeans intended for import into the EU. Environ Sci Eur, 31(1): 92. https://doi.org/10.1186/s12302-019-0274-1

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 and Infection, 2(8): 885-890. https://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. https://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 by European Food Safety Authority (EFSA). https://www.testbiotech.org/sites/default/files/Response%202018-EFSA_Supporting_Publications_Santos_Vigill_1.pdf

Motta, E.V., Mak, M., De Jong, T.K., Powell, J.E., O'Donnell, A., Suhr, K.J., Riddington, I.M., Moran, N.A. (2020) Oral and topical exposure to glyphosate in herbicide formulation impact the gut microbiota and survival rates of honey bees. Appl Environ Microbiol, 6: e01150-20. https://doi.org/10.1128/AEM.01150-20

Nawaz, M.A, Mesnage, R., Tsatsakis, A.M., Golokhvast, K.S., Yang, S.H., Antoniou, M.N., Chung, G. (2019) Addressing concerns over the fate of DNA derived from genetically modified food in the human body: a review. Food Chem Toxicol, 124: 423-430. https://doi.org/10.1016/j.fct.2018.12.030

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

Parenti, M.D., Santoro, A., Del Rio, A., Franceschi, C. (2019) Literature review in support of adjuvanticity/immunogenicity assessment of proteins. EFSA Supporting Publications, 16(1): 1551E. https://doi.org/10.2903/sp.efsa.2019.EN-1551

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

Reardon, B.J., Hellmich, R.L., Sumerford, D.V., Lewis, L.C. (2004) Growth, Development, and Survival of Nosema pyrausta -Infected European Corn Borers (Lepidoptera: Crambidae) Reared on Meridic Diet and Cry1Ab. J Econ Entomol, 97: 1198-1201. https://doi.org/10.1093/jee/97.4.1198

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. J Sci Food Agric, 87(15): 2837–2843. https://doi.org/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. J Appl Toxicol, 36(5): 630-648. https://doi.org/10.1002/jat.3252

Rubio-Infante, N., Ilhuicatzi-Alvarado, D., Torres-Martínez, M., Reyes-Grajeda, J.P., Nava-Acosta, R., González-González, E., Moreno-Fierros, L. (2018) The macrophage activation induced by Bacillus thuringiensis Cry1Ac protoxin involves ERK1/2 and p38 pathways and the interaction with cell-surface-HSP70. J Cell Biochem, 119(1): 580-598. https://doi.org/10.1002/jcb.26216

Ruuskanen, S., Rainio, M.J., Gomez-Gallego, C., Selenius, O., Salminen, S., Collado, M.C., Saikkonena, K., Saloniemia, I., Helander, M. (2020) Glyphosate-based herbicides influence antioxidants, reproductive hormones and gut microbiome but not reproduction: A long-term experiment in an avian model. Environ Pollut, 266(1): 115108. https://doi.org/10.1016/j.envpol.2020.115108

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. Int Immunopharmacol, 61: 185-196. https://doi.org/10.1016/j.intimp.018.05.029

Sharma, H.C., Sharma, K.K., Crouch, J.H. (2004) Genetic transformation of crops for insect resistance: potential and limitations. Crit Rev Plant Sci, 23(1): 47-72. https://doi.org/10.1080/07352680490273400

Sharma, P., Nain, V., Lakhanpaul. S, Kumar, P.A. (2010) Synergistic activity between *Bacillus thuringiensis* Cry1Ab and Cry1Ac toxins against maize stem borer (*Chilo partellus* Swinhoe). Lett Appl Microbiol, 51(1):42-47. https://doi.org/10.1111/j.1472-765X.2010.02856.x

Shehata, A.A., Schrödl, W., Aldin, A.A., Hafez, H.M., Krüger., M. (2013) The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. Curr Microbiol, 66(4): 350-358. https://doi.org/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 Veterinary Sci, 65: 187-191. https://doi.org/10.1292/jvms.65.187

Singh, G., Rup, P.J., Koul, O. (2007) Acute, sublethal and combination effects of azadirachtin and Bacillus thuringiensis toxins on *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae. Bull Entomol Res, 97: 351-357. https://doi.org/10.1017/S0007485307005019

Suppa, A., Kvist, J., Li, X., Dhandapani, V., Almulla, H., Tian, A.Y., Kissane, S., Zhou, J., Perotti, A., Mangelson, H., Langford, K., Rossi, V., BrownJ.B., Orsini, L. (2020) Roundup causes embryonic development failure and alters metabolic pathways and gut microbiota functionality in non-target species. Microbiome, 8(1): 1-15. https://doi.org/10.1186/s40168-020-00943-5

Tabashnik, B.E., Fabrick, J.A., Unnithan, G.C., Yelich, A.J., Masson, L., Zhang, J., Bravo, A., Soberón, M. (2013) Efficacy of genetically modified Bt toxins alone and in combinations against pink bollworm resistant to Cry1Ac and Cry2Ab. PLOS ONE, 8(11): e80496. https://doi.org/10.1371/journal.pone.0080496

Tang, Q., Tang, J., Ren, X., Li, C. (2020) Glyphosate exposure induces inflammatory responses in the small intestine and alters gut microbial composition in rats. Environ Poll, 261: 114129. https://doi.org/10.1016/j.envpol.2020.114129

Testbiotech (2016) Cultivation of genetically engineered maize: Risks not under control. Testbiotech Background, 24 - 11 - 2016, www.testbiotech.org/node/1759

Then, C. (2010) Risk assessment of toxins derived from *Bacillus thuringiensis*: synergism, efficacy, and selectivity. Environ Sci Pollut Res Int, 17: 791-797. https://doi.org/10.1007/s11356-009-0208-3

Then, C. & Lorch, A. (2008) A simple question in a complex environment: How much Bt toxin do genetically engineered MON810 maize plants actually produce? In: Breckling, B., Reuter, H. & Verhoeven, R. (2008) Implications of GM-Crop Cultivation at Large Spatial Scales. Theorie in der Ökologie 14. Frankfurt, Peter Lang: 17-21. http://www.mapserver.uni-vechta.de/generisk/gmls2008/beitraege/Then.pdf

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. Environ Sci Eur, 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. J Cell Sci, 60(1): 181-197. https://jcs.biologists.org/content/60/1/181.short

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. https://doi.org/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. Sci Rep, 7: 1560. https://doi.org/10.1038/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. Sci Total Environ 616–617:255–268. https://doi.org/10.1016/j.scitotenv.2017.10.309

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: 1897-1912. https://doi.org/10.1016/S0024-3205(99)00136-8

Vázquez-Padrón, R.I., Gonzáles-Cabrera, J., García-Tovar, C., Neri-Bazan, L., Lopéz-Revillac, L., Hernández, M., Moreno-Fierro, 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 Biophys Res Commun, 271(1): 54-58. https://doi.org/10.1006/bbrc.2000.2584

Vila-Aiub, M., Yu, Q., Powles, S. (2019) Do plants pay a fitness cost to be resistant to glyphosate? New Phytol, 223(2): 532-547. https://doi.org/10.1111/nph.15733

Vila-Aiub, M.M., Neve, P., Powles, S.B. (2009) Fitness costs associated with evolved herbicide resistance alleles in plants. New Phytol, 184(4): 751-767. https://doi.org/10.1111/j.1469-8137.2009.03055.x

Walsh, M.C., Buzoianu, S.G., Gardiner, G.E., Rea, M.C., Gelencsér, E., Jánosi, A., Jánosi, A., Epstein, M.M., 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. https://doi.org/10.1371/journal.pone.0027177

Wang, W., Xia, H., Yang, X., Xu, T., Si, H.J., Cai, X.X., Wang, F., Su, J., Snow, A.A., Lu, B.-R. (2014) A novel 5-enolpyruvoylshikimate-3-phosphate (EPSP) synthase transgene for glyphosate resistance stimulates growth and fecundity in weedy rice (*Oryza sativa*) without herbicide. New Phytol, 202(2): 679-688. https://doi.org/10.1111/nph.12428

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(1): 1-12. https://doi.org/10.1038/s41598-017-07089-9

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

Zhang, J., Wang, C., Qin, J. (2000) The interactions between soybean trypsin inhibitor and δ-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera* larva. J Invertebr Pathol, 74(5): 259-266. https://doi.org/10.1006/jipa.2000.4936

Zhao, J.Z., Fan, Y.L., Fan, X.L., Shi, X.P., Lu, M.G. (1999) Evaluation of transgenic tobacco expressing two insecticidal genes to delay resistance development of *Helicoverpa armigera*. Chin Sci Bull, 44: 1871-1874. https://doi.org/10.1007/BF02886343

Zhu, Y.C., Abel, C.A., Chen, M.S. (2007) Interaction of Cry1Ac toxin (*Bacillus thuringiensis*) and proteinase inhibitors on the growth, development, and midgut proteinase activities of the bollworm, *Helicoverpa zea*. Pestic Biochem Physiol, 87(1): 39-46. https://doi.org/10.1016/j.pestbp.2006.05.004

Zhu, Y.C., Adamczyk, J.J., West, S. (2005) Avidin, a potential biopesticide and synergist to *Bacillus thuringiensis* toxins against field crop insects. J Econ Entomol, 98: 1566-1571. https://doi.org/10.1093/jee/98.5.1566