TESTBIOTECH Background 1 - 6 - 2021

Testbiotech comment on EFSA's assessment of genetically engineered maize 1507 x MIR162 x MON810 x NK603 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2015-127) by Pioneer Hi-Bred International

TEST BIOTECH

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Table of Contents

Introduction

Genetically engineered (GE) maize 1507 x MIR162 x MON810 x NK603 (four-event stack maize) combines four single events:

- 1507 (expressing the Cry1F and PAT proteins),
- MON810 (expressing the Cry1Ab protein),
- MIR162 (expressing the Vip3Aa20 and PMI proteins) and
- NK603 (expressing two versions of EPSPS proteins).

According to EFSA, the four single events and six subcombinations have been assessed previously by the authority. Implementing Regulation 503/2013 was applied in the EFSA risk assessment (EFSA, 2020a). In the following, the stacked maize is also addressed as 'the Maize'.

1. Literature review

The literature review carried out by the applicant found only seven scientific papers relevant for risk assessment of maize 1507 x MIR162 x MON810 x NK603 for the period between 2005 and 2020. However, during this period of time, much more studies relevant for risk assessment of the four-event stack maize (including the parental plants) were published. It is unacceptable that EFSA accepted this literature review.

2. Molecular characterisation

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 biological active molecules that might emerge from these DNA sequences would raise no safety concerns; therefore, no detailed investigations were carried out in this regard.

Only in the case of MON810, more detailed bioinformatic information was presented showing interruption of the maize genome, going along with a deletion or rearrangement at the site of insertion. Therefore, EFSA requested the applicant to provide additional information to investigate this further and to analyse the safety consequences of the potential genomic deletion or rearrangement. As EFSA states

"The additional information provided by the applicant, including published proteomic and transcriptomic analyses of MON810, showed no significant differences in the expression of most of the genes located in this region compared to non-GM maize comparators …".

This statement indicates that at least some of the genes were significantly changed in its expression. It should be overlooked that already previous publications showed a high magnitude of changes in the gene expression in MON810 (Batista et al., 2008) which never have been assessed and qualified in detail.

Furthermore, other biologically active gene products such as unintended non coding small RNAs (ncsRNAs) from additional open reading frames should have been assessed. Thus, uncertainties remain in regard to potential 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 on 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

Gene expression data were only provided from four locations in the US, with low number of samples per site.

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It is known that 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 for example 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 for example 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.

In addition, 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).

Therefore, the Maize should have been tested in all major maize producing countries, including South America. Furthermore, the Maize should have been subjected to defined stress conditions which for example go along with climate change. This demand is valid for the stacked Maize as well as the parental plants. However, no extreme weather conditions were reported during the cultivation.

In addition, there are striking differences 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.[1] Furthermore, the average temperature in most Brazilian maize production regions is much higher compared to US regions, e.g. Iowa, Illinois or Ohio.[2]

In summary, the available publications strongly indicate that plants inheriting a combination of EPSPS and Bt toxins are likely to show gene expression responses, especially when grown under stress conditions. In its response to comments from experts of Member States (EFSA 2021b), EFSA claims that relevant criteria were not applied due to a transitional periods as foreseen in a specific guidance:

> *"Application EFSA-GMO-NL-2015-127 was submitted during the transitional period of the GMO Panel guidance. Therefore, the requirements of the guidance document were not fully applicable for this application."*

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[1] 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

[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

However, EFSA can not be allowed to introduce any additional transition period to fulfil EU Regulation criteria 503/2013. This means that EFSA is undermining the regulation, which is legally binding for all filed GE plant applications for import since December 2013.

As the comments from experts of Member States show (EFSA, 2021b), there are indications for changes in gene expression of the Vip3Aa20 protein. Furthermore, some experts of Member States in detail explain that there are specific indications for upregulation of stress-response pathways in 1507xMON810xMIR162xNK603 maize, which was most obvious at intended herbicide treatment. These findings clearly show the need for more detailed risk assessment:

> *"The reasons for this alteration need to be identified. In this respect the impact of genetic modification on plant metabolism should be elaborated. Most of the identified stress response pathways interact with the shikimate pathway, which includes the genetically modified cp4EPSPS. Upregulation of shikimate-pathway could be induced either due to the increased concentrations of total EPSPS (i.e. natural EPSPS plus transgenic cp4EPSPS) or due to increased enzymatic activity of cp4EPSPS compared to natural EPSPS. The shikimate-pathway corresponds with the phenylpropanoid-pathway. Literature reports demonstrate an alteration in phenylpropanoid-pathway in glyphosate-resistent soybean (Zonetti et al., 2011) and Bt cotton (Li et al., 2015), indicating that phenylpropanoid pathway is susceptible for alterations due to genetic engineering. Bt protein expression was found to correspond with upregulation of oxylipin and phenylpropanoid pathways both in Bt rice and Bt maize MON810, indicating a link between Bt expression and internally induced chemical defense systems (Liu et al., 2012; Feng et al., 2007). In particular, the interaction between Bt expression, jasmonic acid (as part of the oxylipin pathway) and direct defense proteins (synthesized via phenylpropanoid-pathway) was analyzed for MON810 (Feng et al. 2007). In addition alterations in amino acid composition have been demonstrated for Bt rice (Liu et al. 2012). The link between Bt-expression and stress response should be further investigated in 1507xMON810xMIR162xNK603 maize."*

In summary, the data as provided are not sufficient to fulfill the criteria as requested by Implementing regulation 503/2013. Furthermore, no such data are available from the parental plants or any subcombinations.

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.[3]

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

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).

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.

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.

The concerns regarding changes in gene expression (and consequences for plant composition) are also backed by comments from experts of Member States (EFSA, 2020b). As they explain,

> *"results of nutrient composition analysis indicate impact of intended herbicide application (i.e. mixture of glyphosate and glufosinate) on 1507xMON810xMIR162xNK603 plant metabolism. Alteration in nutrient composition increased at intended herbicide treatment (IHT) compared to conventional herbicide treatment (CHT) for all analytes corresponding with stress response pathways (i.e. all amino acids, ADF, NDF, pherulic acid, coumaric acid, linolenic acid, phytic acid) except vitamins. This is in line with literature reports where glyphosate was found to alter physiological processes in glyphosate resistant plants, including photosynthesis, mineral nutrition and oxidative events (reviewed by Gomes et al. 2014). In addition, glyphosate alters lignin and amino acid content (Zobiole et al. 2010a) as well as fatty acid composition of seeds (Zobiole et al. 2010b)."*

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.

These findings are backed by the fact that maize 1507 x MON810 x MIR162 x NK603 and the comparator, both were selected with a comparative relative maturity (CRM) of 107, which are considered appropriate for growing in environments across North America, where the comparative

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www.greenbook.net/monsanto-company/roundup-ultra

field trials were conducted. However, in other growing regions, also varieties with a CRM more than 111 are cultivated which are missing completely in the field trials. In result, a significant part of possible climatic regions for maize cultivation is not included in the data as presented by the applicant.

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.

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, including 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 which especially are occurring under climate change conditions. Whatever the case, the stacked maize should have been tested also 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 4 location from one year (2012) and only from the US 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. Herbicide applications in the field trials did not represent current agricultural practices, the rates were much lower.

In addition, EFSA failed to use more sensitive methods, such transcriptomics, proteomics and metabolomics (which some of them were used in the parental plant MON810), to explore and assess unintended changes in the stacked Maize.

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. The same problem concerns for data from the parental plants or any subcombinations.

3. 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 North America for one year (2012) only (for agronomic characteristics, data from 2012 and 2015 were used), but not in other relevant maize production areas such as Brazil and Argentina.

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.
- No data were generated that represent the growing conditions in other relevant maize growing regions outside North America.

For example, there are striking differences 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.[4] Furthermore, the average temperature in most Brazilian maize production regions is much higher compared to US regions, e.g. Iowa, Illinois or Ohio.[5]

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).

Furthermore, as some experts of Member States explain in detail (EFSA 2020b), there are specific indications for upregulation of stress-response pathways in 1507xMON810xMIR162xNK603 maize, which was most obvious at intended herbicide treatment. These findings clearly show the need for more detailed risk assessment:

> *"The reasons for this alteration need to be identified. In this respect the impact of genetic modification on plant metabolism should be elaborated. Most of the identified stress response pathways interact with the shikimate pathway, which includes the genetically modified cp4EPSPS. Upregulation of shikimate-pathway could be induced either due to the increased concentrations of total EPSPS (i.e. natural EPSPS plus transgenic cp4EPSPS) or due to increased enzymatic activity of cp4EPSPS compared to natural EPSPS. The shikimate-pathway corresponds with the phenylpropanoid-pathway. Literature reports demonstrate an alteration in phenylpropanoid-pathway in glyphosate-resistent soybean (Zonetti et al., 2011) and Bt cotton (Li et al., 2015), indicating that phenylpropanoid pathway is susceptible for alterations due to genetic engineering. Bt protein expression was found to correspond with upregulation of oxylipin and phenylpropanoid pathways both in Bt rice and Bt maize MON810, indicating a link between Bt expression and internally induced chemical defense systems (Liu et al., 2012; Feng et al., 2007). In particular, the interaction between Bt expression, jasmonic acid (as part of the oxylipin pathway) and direct defense proteins (synthesized via phenylpropanoid-pathway) was analyzed for MON810 (Feng et al. 2007). In addition alterations in amino acid composition have been demonstrated for Bt rice (Liu et al. 2012). The link between Bt-expression and stress response should be further investigated in 1507xMON810xMIR162xNK603 maize."*

Furthermore, a statistically significant reduction was observed between mean germination rates of maize 1507 × MON810 × MIR162 × NK603 and control maize only under warm growing conditions which was not subjected to any further investigations.

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[4]   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

[5]   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

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. 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 stress conditions as they have to be expected under ongoing climate change.

However, no experiments were requested to show 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.

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.[6]

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.

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

The concerns regarding changes in plant composition are also backed by comments from experts of Member States (EFSA 2020b). As they explain,

> "*results of nutrient composition analysis indicate impact of intended herbicide application (i.e. mixture of glyphosate and glufosinate) on 1507xMON810xMIR162xNK603 plant metabolism. Alteration in nutrient composition increased at intended herbicide treatment (IHT) compared to conventional herbicide treatment (CHT) for all analytes corresponding with stress response pathways (i.e. all amino acids, ADF, NDF, pherulic acid, coumaric acid, linolenic acid, phytic acid) except vitamins. This is in line with literature reports where glyphosate was found to alter physiological processes in glyphosate resistant plants, including photosynthesis, mineral nutrition and oxidative events (reviewed by Gomes et al. 2014). In addition, glyphosate alters lignin and amino acid content (Zobiole et al. 2010a) as well as fatty acid composition of seeds (Zobiole et al. 2010b).*"

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.

However, in case of maize 1507 x MON810 x MIR162 x NK603 and its comparator, both were selected with a comparative relative maturity (CRM) of 107, which are considered appropriate for growing in environments across North America, where the comparative field trials were conducted. However, in other growing regions, also varieties with a CRM more than 111 are cultivated which are missing completely in the field trials. In result, a significant part of possible climatic regions for maize cultivation is not included in the data as presented by the applicant.

Therefore, EFSA should have requested additional data from several varieties, including those cultivated in South America, to examine how the gene constructs interact with the genetic background of the plants and thereby may impact plant composition and other biological characteristics. 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.

11

Data from compositional analysis show the need for further investigation

Only data from a low number of agronomic parameters (8) were subjected to statistical analysis in accordance with EFSA guidance; of these 4 were found to be statistically and significantly different, some of them falling under category III (if sprayed with complementary herbicides).

Furthermore, a statistically significant reduction was observed between mean germination rates of maize 1507 × MON810 × MIR162 × NK603 and control maize only under warm growing conditions which was not subjected to any further investigations.

Compositional analysis of 62 endpoints in the grains revealed many (and partly major) statistically significant differences: 38 endpoints were statistically significantly different (37 in plants sprayed with the complementary herbicides).

Even if changes taken as isolated data might not directly raise safety concerns, the overall number of significant effects has to be taken as a starting point for much more detailed investigations: more than half of the parameters measured 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, including investigation into potential unintended changes in metabolic pathways and the emergence of unintended biologically active gene products.

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.

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 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 its response to comments from experts of Member States (EFSA, 2020b), EFSA claims that relevant criteria were not applied due to a transitional periods as foreseen in a specific guidance:
> *"Application EFSA-GMO-NL-2015-127 was submitted during the transitional period of the GMO Panel guidance. Therefore, the requirements of the guidance document were not fully applicable for this application."*

However, EFSA can not be allowed to introduce any additional transition period to fulfil EU Regulation criteria 503/2013. This means that EFSA is undermining the regulation, which is legally binding for all filed GE plant applications for import since December 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. The same problem concerns for data from the parental plants or any subcombinations.

4. 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 plant composition were significantly different. 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 stress conditions, i.e. neither under realistic agricultural conditions nor considering all relevant countries of cultivation. 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). It also should not be overlooked that the Bt toxins, as produced in the plants were never assessed in accordance with EU pesticide risk assessment.

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, Vázquez-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.

14

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, but for example 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. 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. 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 substantially suffers from 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 (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; for review also see Rubio-Infante et al., 2016).

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), 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. Similar findings result from feeding trials with cows (Gruber et al., 2011; Gürtler et al. 2010; Paul et al., 2010). Paul et al., (2010) observed that 44% of the immunoreactive Cry1Ab from MON810 present in feed was transferred to the feces while 34% of the Cry1Ab protein levels in feed could be detected in liquid manure (Gruber et al. 2011).

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.

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.

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.

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 2018) 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. (…)."[7]*

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, 2019).

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.

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[7] 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. Due to increased pressure by herbicide resistant weeds, a constantly burden of residues from spraying with the complementary herbicides can be expected. 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.

Further, as the experts from Member States (EFSA 2020b) point out,
> "*the toxicological assessment by the applicant is mainly based on the expression of the new proteins, but not on potential unintended effects deriving from genetic transformation. Potential alterations of metabolic pathways were not considered although results of nutrient analysis indicate upregulation of stress defense pathways in 1507xMON810xMIR162xNK603 maize. Such alterations could result in accumulation of secondary plant products, which are either toxic or endocrine disruptive.*"

Overall conclusions on toxicity

Despite all these open questions regarding potential health impacts, no sub-chronic or chronic feeding study performed with whole food and feed derived from the stacked Maize was requested by EFSA. This observation is supported by the literature review carried out by the company. In this context, it is relevant to consider that some of the outcome of 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. Defined environmental stress conditions 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.

In conclusion, toxicity assessment as performed by EFSA 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).

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 the Maize producing several Bt toxins as well as additional EPSPS enzymes 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. Under these circumstances, even a rare single outcrossing event that goes unnoticed can have a huge long-term impact on agro-ecosystems.

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, Díaz et al. (2020) show that these new weeds seem to have a complex origin. Therefore, the likelihood of hybridisation with the GE

19

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.

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.

In addition to geneflow to teosinte, also the environmental exposure to Bt toxins via manure deserves more attention. As mentioned by experts of the Member States (EFSA 2020b),

> *"for Bt proteins an exposure route via manure from cattle fed with Bt maize has been demonstrated (Gruber et al. 2011; Gürtler et al. 2010). Paul et al. (2010) observed that 44% of the immunoreactive Cry1Ab from MON810 present in feed was transferref to the feces (Paul et al. 2010) while 34% of the Cry1Ab protein levels in feed could be detected in liquid manure (Gruber et al. 2011). As Gruber et al. (2011) demonstrated Cry1Ab is relatively stable in liquid manure (decrease of 49% in 24 weeks). The bioactivity of Cry proteins in wastewater or manure is unknown as no bioassays have been carried out so far. Based on the above finding it is likely that all three Bt proteins present in the GMO (Cry1Ab, Cry1F, Vip3A) will contribute to an environmental exposure for which effects cannot be assesses as both quantitative data on the exposure and data on the hazard for soil and water organisms are lacking."*

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.

In addition to geneflow to teosinte, also the environmental exposure to Bt toxins via manure deserves more attention.

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. Monitoring

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.

Insufficiency of the monitoring plan was also observed by experts from Member States (EFSA 2020b). For example it is requested:

> "*The monitoring plan has to be elaborated in more detail in order to meet the following requirements:*
> *- Provision of a fully specified list of monitoring parameters.*
> *- Application of standardised sampling methodologies (…)*
> *- Elaboration of a sampling concept.*
> *- In case of monitoring data being collected by external persons or institutions other than the applicant, binding agreements/contracts with third parties are requested which clearly determine what data are provided and how these data are made available.*
> *- Elaboration of the methods of data analysis including the statistical methods.*

- Application of the concept of adverse effects and environmental damages: Adverse environmental effects can only be determined if they are related to certain relevant subjects of protection."

7. Conclusions

The opinion as published shows how EFSA surrenders in the face of the underlying complexity of risk related issues, scientific findings, uncertainties and open questions. 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.

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 indicating that environmental stress factors, herbicide applications rates, genetic backgrounds, unintended effects 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) or glufosinate are known to impact the composition of the microbiome.

6. 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.

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