TESTBIOTECH Background 25 - 09 - 2023

Assessment of genetically modified maize Bt11 x MIR162 x MIR604 x MON89034 × 5307 x GA21 for food and feed uses, under regulation (EC) No 1829/2003 (application EFSA-GMO-DE-2018-149) by Syngenta

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Introduction

The GMO Panel assessed the stacked maize which is derived from crossing six genetically engineered maize events (EFSA, 2023a). The maize contains genes conferring resistance to two herbicides and produces six insecticidal proteins.

- Bt11 - expressing the Cry1Ab protein for protection against certain lepidopteran pests and the phosphinothricin acetyl transferase (PAT) protein for tolerance to glufosinate-ammonium-containing herbicides;
- MIR162 - expressing the Vip3Aa20 protein against certain lepidopteran pests and the phosphomannose isomerase (PMI) protein used as a selectable marker;
- MIR604 - expressing a modified Cry3A (mCry3A) protein against certain coleopteran pests and the PMI protein used as a selectable marker;
- MON 89034 - expressing Cry1A.105 and Cry2Ab2 proteins for protection against certain lepidopteran pests;
- 5307 - expressing the eCry3.1Ab protein against certain coleopteran pests and the PMI protein used as a selectable marker;
- GA21 - expressing the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (mEPSPS) protein for tolerance to glyphosate-containing herbicides.

Consequently, the stacked maize produces six insecticidal toxins (Cry1Ab, VIP3Aa20, mCry3A, Cry1A.105, Cry2Ab2, eCry3.1Ab) targeting lepidopteran and coleopteran insects). Further, the maize is resistant to two groups of complementary of herbicides (glyphosate and glufosinate).

1. Systematic literature review

A systematic review as laid down in Regulation (EU) No 503/2013 was only partially provided by the applicant. Unpublished studies were made available, but these did not give a comprehensive overview of all the studies published for each of the parental events or their previous combinations. The applicant made no mention of patent applications filed on unexpected findings relating to maize MIR162 (EP 3632202) in regard to decreased male fertility in several maize inbred lines homozygous for event MIR162 (Testbiotech, 2022; EFSA, 2023b).

2. Molecular characterisation

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

In this context, previous research has shown that expression of Cry1A.105, Cry2Ab2 and EPSPS proteins in GM maize can induce changes in the overall proteome of the respective GM maize line, with impacts on associated endogenous metabolic pathways (Agapito-Tenfen et al., 2014) as well as on levels of (regulatory) micro RNAs in maize lines expressing Cry1A.105, Cry2Ab2 and EPSPS proteins (Agapito-Tenfen et al., 2018; see also comments made by experts from member states, 2023c). However, EFSA did not request any data on changes in metabolic pathways or unintended gene products.

Gene expression under stress conditions

Environmental stress can cause unexpected patterns of expression in the newly introduced DNA (see, for example, Trtikova et al., 2015). More specifically, Fang et al. (2018) showed that stress responses can result in unexpected changes in plant metabolism if they inherit additional EPSPS enzymes. Furthermore, it should also have been noted that the vip3 gene appears to induce unexpected metabolic changes in maize. These effects are influenced by environmental conditions (patent EP 3632202).

Expression of the additional enzymes was, nevertheless, only measured under field conditions in three locations in the US for one year (2015), with no exceptional weather conditions being reported. It is evident that these weather conditions are not representative of all growing conditions, e. g. in 2023 the temperatures were much higher due to ongoing climate change. Therefore, new data on gene expression need to be made available. Data should also cover a much broader range of defined biotic and abiotic stressors to demonstrate stability in gene expression under sufficiently realistic conditions.

Consequently, the GE maize plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are insufficient to conclude on the impact that environmental factors may have on gene expression, as laid down in EU Regulation 503/2013.

Data on herbicide application rates and their impact on gene expression

Due to increased weed pressure, it has to be expected that these plants can and will be exposed to high and also repeated dosages of glyphosate alone and / or in combination with the other complementary herbicides. Higher herbicide application rates will not only lead to a higher burden of residues in the harvest, but may also influence the expression of the transgenes or the expression of other genes in the plants. However, gene expression was only measured in plant material that was not exposed to spraying with the complementary herbicide.

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

Without these data, no reliable conclusion can be drawn, as laid down in Implementing Regulation 503/2013 (in particular for herbicide tolerant GE plants) to assess whether anticipated agricultural practices influence the expression of the studied endpoints (see also Miyazaki et al., 2019).

Consequently, the GE maize plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are insufficient to conclude on the impact herbicide applications have on gene expression, as laid down in EU Regulation 503/2013.

Impact of genetic backgrounds on gene expression
It is known that the genomic background of the varieties can influence both the expression of the inserted genes and plant metabolism (see, for example, Lohn et al., 2020; Trtikova et al., 2015). However, it appears that the data on gene expression were confined to a single variety. Therefore, EFSA should have also requested additional data from different transgenic maize varieties, e. g. those cultivated in South America.

EFSA did not consider any of these issues. Consequently, the GE maize plants tested in the field trials do not sufficiently represent the products intended for import. As such, the data presented by the applicant are insufficient to conclude on the impact the genetic backgrounds may have on gene expression, as laid down in EU Regulation 503/2013.

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.*"

The data presented by Syngenta do not meet the requirements of Implementing Regulation 503/2013: (1) the field trials were not conducted in all relevant regions where the GE maize will be cultivated, and no defined extreme weather conditions were taken into account; (2) the field trials did not take all relevant agricultural management practices into account; (3) not all relevant genetic backgrounds were taken into account.

Data on environmental factors and stress conditions - and their impact on plant composition and phenotype

Field trials to assess plant composition as well as agronomic and phenotypic characteristics of the GE maize, were only conducted in the US for one year (2015) at 9 sites, with no exceptional weather conditions being reported (but one site was excluded from statistical analysis because of heavy rainfall). It is evident that these weather conditions are not representative of all growing conditions, for example, in 2023 when temperatures were much higher due to ongoing climate change. Therefore, new data have to be made available on gene expression, covering a much broader range of defined biotic and abiotic stressors to demonstrate stability in gene expression under sufficiently realistic conditions.

In order to assess changes in plant composition and phenotypic characteristics, the plants should have been grown in various environmental conditions and exposed to well-defined environmental stress conditions. This requirement is especially relevant in this case, as it is known that the additional epsps gene may have pleiotropic effects, thus affecting seed dormancy, growth and stress responses in the plants (see, for example, Fang et al., 2018; Wang et al., 2014; Yang et al., 2017; Beres et al., 2018, Beres, 2019).

Furthermore, it should also should have been noted that the vip3 gene seems to induce unexpected metabolic changes in maize. These effects are influenced by environmental conditions (patent EP 3632202).

It should not be overlooked that, for example, Brazil is among the most important countries for maize imports into the EU: Brazil is a major producer of genetically engineered maize and is one of the largest exporters of maize into the EU (Commission Committee for the Common Organisation of Agricultural Markets, 2023). However, no field trials were conducted outside the US.

No experiments were requested to show to which extent specific environmental conditions influence plant composition and agronomic characteristics. Hence, no data were made available, as laid down in Implementing regulation 503/2013, to assess whether the expected environmental conditions where the plants are likely to be cultivated will influence the expression of the studied endpoints.

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

The mode of action of the active ingredients in the complementary herbicides make it plausible that complementary herbicide applications will cause stress responses in the plants, and thus impact gene expression and plant composition. These effects may vary with the amount of herbicide sprayed onto the crop and the various active ingredients which can be used.

From the available information, it seems that the complementary herbicides were only applied once during cultivation of the plants, which does not appear to be representative of the practical conditions in which these plants are likely to be grown (Miyazaki et al., 2019). Nevertheless, EFSA is of the opinion that the design of the field trials is in accordance with the expected agricultural practices. EFSA should, however, have provided a much more detailed reasoning in order to justify this opinion. Current EFSA practices make it impossible to access the original data from the

companies within the period of consultation. Therefore, the opinion has to provide all the data necessary to allow other experts to conclude on whether the provisions of GMO regulation are fulfilled. In light of the information available, we assume that the application and the data provided do not sufficiently represent the agricultural practices, e. g. single herbicide use, higher dosages and repeated spraying.

EFSA should have requested the applicant to submit data from field trials on all the relevant active ingredients used in agricultural practice, including all dosages and combinations of the complementary herbicides, which might be used in agricultural practice in GE maize producing countries. Without these data, no reliable conclusions can be drawn, as laid down in Implementing Regulation 503/2013 (in particular, for herbicide tolerant GE plants) to assess whether anticipated agricultural practices influence the expression of the studied endpoints (see also Miyazaki et al., 2019).

The data presented are insufficient to conclude on the impact herbicide applications may have on gene expression, plant composition or biological characteristics of the plants, as laid down in EU Regulation 503/2013.

Impact of genetic backgrounds on plant composition as well as on agronomic and phenotypic characteristics

It is known that the genomic background of the varieties can influence both the expression of the inserted genes and plant metabolism (see, for example, Lohn et al., 2020; Trtikova et al., 2015). However, it appears that the data on gene expression were confined to a single variety. Therefore, EFSA should have also requested additional data from transgenic maize varieties that are, for example, cultivated in South America.

EFSA did not take these issues into consideration. Consequently, the GE maize plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are, therefore, insufficient to conclude on the impact that the genetic backgrounds may have on gene expression, as laid down in EU Regulation 503/2013.

Compositional analysis data show the need for further investigations

66 constituents were subjected to statistical analysis (9 in forage and 57 in grain).

- Six-event stacked maize not treated with the intended herbicides - statistically significant differences in the comparison with the non-GE comparator were identified for 19 endpoints (all in grain). Neutral detergent fibre (NDF), stearic acid (C18:0), ferulic acid and p-coumaric acid were considered to show highly significant differences.
- Six-event stacked maize treated with the intended herbicides - statistically significant differences to the non-GE comparator were identified for 20 endpoints (all in grain). Again, NDF, stearic acid, (C18:0), ferulic acid and p-coumaric acid were considered to show highly significant differences.

Given the above reasoning on the impact of environmental factors, herbicide applications and genetic backgrounds as well as the large number of significant findings, EFSA should have requested more data: data on agronomic and phenotypic endpoints should be generated from a wider

range of clearly defined stress factors, including all relevant agricultural practices and genetic backgrounds. This requirement is especially relevant here, as it is known that the additional epsps genes may have pleiotropic effects, which also affect seed dormancy, growth and stress responses of the plants (see, for example, Fang et al., 2018; Wang et al., 2014; Yang et al., 2017; Beres et al., 2018, Beres, 2019).

Furthermore, it should also should have been noted that the vip3 gene seems to induce unexpected metabolic changes in maize, with effects being influenced by environmental conditions (patent EP 3632202).

A more detailed analysis would have been necessary to investigate changes in plant composition and phenotype, and also to investigate potential unintended changes in metabolic pathways and the emergence of unintended biologically active gene products.

The material derived from the plants should have been assessed by using 'Omics' techniques to investigate changes in the gene activity of the transgene and the plant genome, and also to investigate changes in metabolic pathways and the emergence of unintended biologically active gene products (see Benevenuto et al., 2022). Such in-depth investigations should not depend on findings indicating potential adverse effects, they should always be necessary to draw sufficiently robust conclusions to inform the next steps in risk assessment.

However, instead of assessing the overall pattern of changes in plant components, their causes and possible impacts in more detail, EFSA only assessed the observed changes in isolation in regard to evidence of potential harm. This approach turns the comparative approach into a trivial concept of assessing bits and pieces, and it ignores questions concerning the overall safety of the whole food and feed.

The need for more detailed investigation is also underlined in comments made by experts from member states. As German experts note:
"Results of the comparative nutritional analysis showed that all measured parameters corresponding to lignin biosynthesis pathways were elevated. This includes phenylalanine (outcome type 3 = ot 3) and tyrosine (ot 3) of the shikimate-pathway, which are precursors for p-coumaric acid (ot 6) and ferulic acid (ot 7) of the phenylpropanoid pathway, which are precursors for lignin biosynthesis. Lignin was not included within nutrient composition analysis but correspond to ADF (ot 4) and NDF (ot 7 and ot 6 in CHT and IHT, respectively). The impact of this systemic alteration in Bt11 × MIR162× MIR604 × MON89034 × 5317 × GA21 should be further investigated in terms of relevance for food-feed and environmental safety due to the following reasons:
1. The phenylpropanoid pathway is connected with polyamine pathway (Bassard et al. 2010) and polyamines were found to be elevated in genetically modified maize (Mesnage et al. 2016). As polyamines and related secondary plant products could be harmful for human and animal consumption, potential accumulation of such compounds should be further investigated.
2. The phenylpropanoid pathway corresponds to other stress-response pathways and it was frequently reported that stress response was altered in various genetically modified crops (Mesnage et al. 2016; Agapito-Tenfen et al. 2013; Arruda et al. 2013; Liu et al. 2012). Hence alterations in stress-response should be analysed for Bt11 × MIR162 × MIR604 × MON89034 × 5317 × GA21 (e.g. using Omics-analyses) and the following consequences should be considered:

a) As stress response is important for herbicide detoxification processes it should be analysed, if herbicide metabolism has been influenced. Therefore herbicide residues should be analysed.
b) As stress response characteristics can influence outcrossing and distribution behavior potential alterations should be further investigated in terms of environmental safety aspects (cf. II 1.3.5).”

Data from analysis of agronomic characteristics show the need for further investigation
The statistical analysis was applied to only10 endpoints, with the following results: 6 statistically significant differences with the non-GE comparator were identified for the six-event stacked maize (untreated and treated with the intended herbicides).

No data were made available on ear count which is requested as part of the minimum data set that has to be provided by the applicant. Nevertheless, EFSA accepted the incomplete data.

Conclusion on the comparative assessment of plant composition as well as on phenotypic and agronomic characteristics
The data provided by the applicant and accepted by EFSA are insufficient to conclude on the impact that environmental factors may have on herbicide applications and genetic backgrounds, or on gene expression, plant metabolism, plant composition, or on agronomic and phenotypic characteristics.

To gather reliable data on compositional analysis and agronomic characteristics, the plants should have been subjected to a much broader range of defined environmental conditions and stressors. Furthermore, EFSA should have requested the applicant to submit data from field trials which reflect current agricultural practices and all relevant genetic backgrounds. These data should also have been used to gain more in-depth knowledge regarding the causes of several highly significant differences in plant composition

However, only samples from field sites located in the US were used to generate the data, and the impact of environmental factors and agricultural practices were not assessed in detail. The EFSA opinion does not show that herbicide applications in the field trials represent all the relevant agricultural practices. Only one transgenic variety was grown in the field trials.

Consequently, the data presented by the applicant and accepted by EFSA are insufficient to conclude on the impact that environmental factors, herbicide applications or different genetic backgrounds may have on plant composition and agronomic characteristics.

Based on the available data, no final conclusions can be drawn on the safety of the plants. Therefore, the data neither fulfill the requirements of Implementing Regulation 503/2013 nor Regulation 1829/2003. This is also underlined in several statements made by experts from member states (EFSA, 2023c).

In summary, the GE maize plants tested in the field trials do not sufficiently represent the products intended for import.

Due to the complete absence of any independent data on this maize (see literature review, EFSA 2023a), we also strongly recommend establishing a system with independent controls to repeat the trials and double check the data on plant composition and agronomic characteristics.

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;"

Overall assessment

No requests were made to test the whole stacked plant (feeding study), despite the fact that there were many significant changes, especially in the composition of the plants. Even if changes taken as isolated data might not directly raise safety concerns, the overall number of effects should have been considered a starting point for much more detailed investigation of their potential health impacts.

Furthermore, there was no request to investigate the mixed toxicity of the six stacked maize, even though it produces six insecticidal proteins and contains residues from spraying with two herbicides. Without empirical data, potential (chronic) effects on health cannot be excluded, e. g. direct or indirect effects on the immune system that may trigger inflammatory processes. No feeding study with the six-stacked maize was conducted.

Effects of residues from spraying with complementary herbicide specific to GE plants and their mixed toxicity

The residues from spraying were considered to be outside the remit of the GMO Panel. However, without detailed assessment of these residues, no conclusion can be drawn on the safety of the imported products: due to specific agricultural management practices in the cultivation of the herbicide-resistant plants, special attention must be paid to, e. g. specific patterns of spraying, exposure, occurrence of specific metabolites and the emergence of combinatorial effects.

EU pesticide regulation and GMO regulation both 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 a prerequisite for granting authorisation.

EU legal provisions, such as Regulation 1829/2003 (and 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 resulting from combinatorial exposure of various potential stressors need to be tested for mixed toxicity (EFSA, 2019b).

Glufosinate and glyphosate have both been shown to impact or disturb the microbiome, which can have substantial impact on the long-term toxicity (mixed toxicity) of whole food and feed derived from the stacked event. Dong et al. (2020) show that glufosinate can severely impact the microbiome. This is especially relevant in regard to combinatorial (accumulated) effects caused by

the residues from spraying with glyphosate, which is known to cause shifts in the microbial composition and associated microbiomes of plants and animals. Glyphosate has been shown to cause shifts not only in soil organisms (van Bruggen et al., 2018, 2021, Chávez-Ortiz et al., 2022) and rhizosphere microbiome (Cesco et al., 2021), but also in the composition of the intestinal flora of humans (Mesnage et al., 2021a), cattle (Reuter et al., 2007), poultry (Shehata et al., 2013; Ruuskanen et al., 2020), amphibians (Boccioni et al., 2021), earthworms (Owagboriaye et al., 2021) and rodents (Hu et al., 2021; Liu et al., 2022; Mao et al., 2018; Mesnage et al., 2021b, 2021c; 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 this GE maize, are not unlikely to trigger significant changes in intestinal bacteria (see also Testbiotech, 2021).

In general, the microbiome can be seen as a common network of life, encompassing 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 benefits both the host and microorganisms. They act at the interphase and communicate between the organisms and their wider environment and are, at the same time, also part of the closer environment of an organism. Microbiomes are considered to be vital for the health of higher organisms, i. e. human, animal and plants.

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.*"

A 2019 study commissioned by EFSA on adjuvanticity / immunogenicity assessment of proteins included 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*."

These findings are highly relevant in the risk assessment of the GE maize, which inherits combinations of herbicide resistance to glyphosate and glufosinate. The residues may cause gut microbiome perturbation, depending on exposure and combinatorial effects, such as those from Bt toxins. It has to be considered a plausible hypothesis that the effects on the microbiome can trigger effects on the immune system, food uptake and body weight. Both this hypothesis and mixed toxicity need to be tested before any conclusion can be drawn on the health safety of food and feed.

As no such data can be derived from pesticide risk assessment, experimental data on mixed toxicity of the stacked maize have to be requested from the applicant. Furthermore, the combinatorial effects with Bt toxins have to be taken into account, especially in regard to adverse effects on the immune system that are mediated via the microbiome.

In general, antibiotic effects and other adverse health effects might occur from exposure to a diet containing these plants, as they were not assessed under pesticide regulation. These adverse effects on health might be triggered by the residues from spraying with the complementary herbicide (see also van Bruggen et al., 2018). Further attention should be paid to the specific toxicity of the metabolites of the active ingredients in the pesticide, which might occur specifically in the stacked event.

As yet, no attempts have been made to integrate the microbiome into the risk assessment of food and feed derived from the GE maize. This is in clear contradiction 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).

Combinatorial effects with 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), Bondzio et al. (2013) and Bautista-Jacobo et al. (2023) show that Cry proteins could potentially have an impact on the health of mammals. Further studies (de Souza Freire et al., 2014; Mezzomo et al., 2014) confirm hematotoxicity of several Cry toxins, including those used in genetically engineered plants, such as Cry 1Ab and Cry1Ac. Recent research also indicates that Cry1 toxins perturbate intestinal cells in Drosophila and may do so in mammals. Jneid et al. (2023) tested the intestines of Drosophila for effects of Cry1 toxins. Although fruit flies should not be sensitive to the toxins, it was in fact shown that ingestion of the toxins can trigger the death of intestinal cells. As a result, there is a disruption in the formation of new cells: instead of 'normal' intestinal cells, hormone-forming (enteroendocrine) cells grew in greater numbers. These cells are involved in the regulation of many physiological functions, such as feeding behaviour, metabolism and immune response, in both humans and animals. According to the paper, the functionality and hormonal activity of the intestine could also be disturbed by Cry1A toxins in other species, as the described mechanism for the formation of new intestinal cells is the same in all animal species.

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). Relevant findings show that the selectivity

and efficacy of Bt toxins produced in GE plants can be influenced by many co-factors or co-stressors (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 (PIs) which show synergistic effects with Bt toxins, thus strongly enhancing their toxicity. It is likely that PIs 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 studies 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 (PIs), 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 PIs, 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 toxins into account is not reliable and systematically underestimates the risks.

Furthermore, 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 see Rubio-Infante et al., 2016). Since Cry1Ac is also used as adjuvant in vaccines, its risks in regard to food consumption need to be addressed and carefully examined, as these can be promoted by synergistic effects.

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 responses. For example, a study testing maize 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 or shown in 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 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, thus leaving enough time for interaction between various food compounds.

These issues are especially relevant to the stacked events, as 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 PIs. In this context, potential impacts on the microbiome in combination with the residues from spraying with glyphosate and glufosinate also have to be taken into account.

Conclusion on toxicity assessment

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 resulting from combinatorial exposure of various potential stressors need specification, and their assessment needs to be prioritised. We conclude that the current EFSA health risk assessment for the stacked maize is unacceptable. We propose testing these plants following the whole mixture approach, considering them to be "*insufficiently chemically defined to apply a component-based approach*" (EFSA, 2019).

Despite all these open questions regarding potential health impacts, we are not aware of a single whole food and feed feeding study performed with whole food and feed derived from the stacked maize. This observation is supported by the literature review carried out by the company, which did not yield any peer reviewed publication. In this context, it is necessary to consider that the outcome of the feeding studies with the parental plants raised several questions concerning the results, methodology and reliability (see, for example, comments made by experts from member states, EFSA, 2023c).

In conclusion, the EFSA opinion on the application for authorisation of the stacked maize (EFSA, 2023a) cannot be said to fulfil the requirements for assessment of potential synergistic or antagonistic effects resulting from the combination of the transformation events in regard to toxicology.

5. 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. Maize volunteers can be found in the EU on a regular basis, as reported by Palaudelmàs et al. (2009) in Spain or Pascher (2016) in Austria. Further, in awareness of the biological characteristics of the GE maize and the findings of Fang et al. (2018), the stacked maize needs to be examined in detail regarding next generation effects, volunteer potential (persistence) and gene flow. Under these circumstances, even a rare single outcrossing that goes unnoticed can have a huge long-term impact on the agroecosystems.

Testbiotech is aware of an EFSA (2022) opinion regarding the teosinte situation in France and Spain. Here, EFSA comes to the conclusion:
"The new evidence retrieved confirms that where maize and EU teosinte plants co-occur and flower synchronously, maize alleles (transgenic or not), can move into teosinte populations at rates that depend on different factors. Hence, the possible introgression of transgenes from maize MON810, Bt11, 1507 and GA21 into EU teosinte may only provide a selective advantage to GM teosinte hybrid progeny under high infestation of target pests and/or when glufosinate-ammonium- and/or glyphosate-based herbicides are applied. However, this fitness advantage will not allow GM teosinte hybrid progeny to overcome other biological and abiotic factors limiting their persistence and invasiveness. Therefore, EFSA considers that the growth habits of EU teosinte plants and teosinte hybrid progeny are such that the acquisition of insect resistance and/or herbicide tolerance is unlikely to change their relative persistence and invasive characteristics under EU conditions."

This opinion not sufficiently backed by science: the characteristics of potential hybrids and next generations have to be investigated and cannot be predicted simply from the data of the original event. It is well known that there can be next generation effects, and also interference from the genetic background that cannot be predicted from the assessment of the original event (Bauer-Panskus et al., 2020). This issue is relevant for gene flow from maize to teosinte, and from teosinte to maize.

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 cannot be regarded as valid.

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

Consequently, the EFSA environmental risk assessment is not acceptable.

6. Others

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 from 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 the consumption of GM food or feed. 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. If there are losses or spread of viable material (such as kernels), all receiving environments need to be monitored. Furthermore, environmental exposure through organic waste material, by-products, sewage or faeces containing GE products during or after the production process, and during or after human or animal consumption should be part of the monitoring procedure (see also comments made by experts from member states, EFSA, 2023c).

General aspects

A Testbiotech report published in 2021 (Testbiotech, 2021) shows how the European Food Safety Authority (EFSA) intentionally sets aside crucial issues. This careless approach exemplifies the overall decrease in general food safety standards that has been ongoing since the introduction of GE plants. At the same time, the number of events authorised for import has steadily increased.

The Testbiotech report published in 2021 provides evidence that the genetic engineering of food plants has layers of complexity that go far beyond what can be assessed by current standards of risk assessment. The safety of the plants is claimed on the basis of approval processes that only consider risks that are easiest to assess. It shows that, since the introduction of the first transgenic plants into the food chain, uncertainties in regard to safety have been steadily increasing, while risks may have accumulated unnoticed. Overall, the safety of food products has been decreasing while, at the same

time, EFSA and the Commission were unable to present sufficiently robust criteria and methods to significantly improve health safety. Instead, specific areas of risk assessment have been intentionally ignored from the beginning.

In light of these findings, the Commission should try to avoid ‘rubber stamping’ all applications for the import of GE plants, and thus reduce the overall number of products entering the market. They must also ensure that all these products undergo much more thorough risk assessment.

In addition, the Commission should recognise the problem of there being no data from independent risk research for most genetically engineered plants with pending marketing applications, including in the present case. In the case of this six-stacked maize, there is no possibility of comparing or assessing the data from the applicant’s findings with that of scientists not involved in the development of the transgenic plants. Therefore, independent risk research, as laid down in Directive 2001/18, is completely absent.

In response, the Commission and member states should start an initiative to introduce comprehensive, systematic and independent research that follows the perspective of the precautionary principle, and the protection of health and the environment. It is time to end this kind of leap into the dark and organise research that serves the interests of the public as well as the protection of health and the environment.

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