TESTBIOTECH Background 27 - 7 -2020

Testbiotech comment on EFSA's assessment of genetically engineered maize MZIR098 for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-DE-2017-142) by Syngenta

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Introduction

Maize MZIR098 was produced by Syngenta to express the following proteins:

- synthetic Bt toxin eCry3.1Ab (fusion of modified Cry3A (mCry3A) gene and a synthetic Cry1Ab),
- synthetic Bt toxin mCry3A (plant codon optimised Cry3A),
- PAT conferring resistance to the active herbicide ingredient, glufosinate ammonium.

Implementing Regulation 503/2013 was applied in the EFSA risk assessment (EFSA, 2020a).

1. Literature review

The review carried out by the showed the absence of any peer-reviewed data on maize MZIR098. This underlines the necessity for the very closest scrutiny of the EFSA risk assessment.

2. Molecular characterisation

In regard to the expression of the additionally inserted genes, Implementing Regulation 503/2013 requests *"protein expression data, including the raw data, obtained from field trials and related to the conditions in which the crop is grown"*.

Environmental stress can indeed cause unexpected patterns of expression in the newly introduced DNA (see, for example, Trtikova et al., 2015). There is plenty of evidence that drought or heat can significantly impact the content of Bt in the plant tissue (Adamczyk & Meredith, 2004; Adamczyk et al., 2009; Chen et al., 2005; Dong & Li, 2006; Luo et al., 2008; Then & Lorch, 2008; Trtikova et al., 2015). Therefore, to assess gene expression, the plants should have been grown under conditions of severe drought, with and without irrigation, as well as compared to more moderately severe climate conditions. All relevant bioclimatic regions should have been taken into account.

Experiments under controlled and defined conditions should have been performed to gather sufficiently reliable data on gene expression and functional genetic stability. This would have to include exposure of the plants to all biotic or abiotic stressors which are relevant but which might have been absent in the field trials. The generation of these data should have taken all relevant patterns of herbicide application and the application of the complementary herbicides as well as various genetic backgrounds into account.

However, the data provided do not represent the conditions in which the plants were grown: (i) no extreme weather conditions which could be expected due to climate change were taken into account; (ii) the field trials did not take current agricultural management practices into account to the necessary extent (see below); (iii) the field sites were only in the US; no other field trials in other GE maize producing countries (like Argentina or Brazil) were used to produce relevant data.

Therefore, the range of data provided for assessing genome x environment interactions is very limited and not representative of the conditions under which these crops will be grown. In addition, gene expression data was only provided for one variety. These poor data sets do not allow sufficiently reliable conclusions to be drawn on the expression of the additional gene constructs.

The need for more data is further underlined by the differences in MZIR098 gene expression compared to expression data from similar constructs of other events. Furthermore, the expression of mCry3A follows a strange pattern with pollen reaching extremely high concentrations. This alone should have highlighted the need for a much more detailed investigation. For example, the event should have been tested not just in one variety, but also in other genetic backgrounds in order to determine whether the pattern of gene expression is impacted.

It also has to be taken into account that the process of genetic engineering led to open reading frames (ORFs) that may give rise to biologically active molecules. One ORF is discussed in more detail because it might generate allergenic proteins. However, all potentially active molecules emerging from the genetic changes should undergo detailed assessment. This includes gene products besides proteins, such as dsRNA. Since detailed assessment is missing, uncertainties remain in regard to the risks of biologically active substances arising from the method of genetic engineering and the newly introduced gene constructs.

The data provided do not allow reliable conclusions to be drawn on gene expression and functional stability.

3. Comparative analysis (for compositional analysis and agronomic traits and GE phenotype)
In regard to the compositional analysis and agronomic traits and the characteristics of the GE phenotype, Implementing Regulation 503/2013 requests the assessment of whether the expected agricultural practices influence the outcome of the studied endpoints. According to the Regulation, this is especially relevant for herbicide resistant plants. Furthermore, the different sites selected for the field trials need to reflect the different meteorological and agronomic conditions under which the crop is to be grown.

Field trials for the compositional and agronomic assessment of maize MZIR098 were conducted in the US only at 8 (9) sites, but not in other relevant maize growing areas, such Brazil, Argentina, Paraguay or Uruguay. Data from only one year (2013) were used to generate data on the relevant meteorological conditions under which the plants may be grown. Due to ongoing climate change, the weather conditions at the same sites can be vastly different from year to year, and therefore data from just one year cannot be regarded as conclusive. Furthermore, data from the US cannot represent all relevant environmental impact factors from regions, such as Argentina or Brazil. Additional data from other sites and for more than one year would have been needed to draw conclusions on the impact of different meteorological and agronomic conditions on the measured endpoints and fulfill the requirements of Implementing Regulation 503/2013.

However, EFSA failed to request further studies, e.g. field trials lasting for more than one season and field sites in other maize growing regions. Furthermore, no data were generated representing more extreme environmental conditions, such as those caused by climate change.

In addition, experiments under controlled and defined conditions should have been performed to gather sufficiently reliable data on gene expression and functional genetic stability. This would have to include exposure of the plants to all relevant biotic or abiotic stressors which might have been absent in the field trials. The generation of these data should have taken all relevant patterns of herbicide application and the application of the complementary herbicides into account. Various genetic backgrounds should also have been tested to assess their impact on plant composition and phenotypical characteristics of the event.

However, no such data were made available. Therefore, no conclusion can be drawn on comparative analysis.

Furthermore, as the complementary herbicide, glufosinate, was not used in high doses as may be expected in the case of increasing weed resistance. Therefore, EFSA should have requested the applicant to submit more recent data from the field trials, also taking into account the highest dosage of glufosinate that can be tolerated by the plants, including repeated spraying. In response to comments made by Member States (2020b), EFSA simply stated that "*for the experimental treatments to be comparable between different locations, the application rate should not differ too strongly between them*." This statement is inadequate. To fulfill the requirements of Implementing Regulation 503/2013, additional data should have been requested to compare not only the treated and the non-treated plants, but also data allowing comparison within the group of treated plants. These data are necessary 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 Regulation. However, no such data were made available.

In addition, there were several significant findings on differences in composition, which should have been investigated in more detail and under the full range of expected agricultural and bioclimatic conditions, including various genetic backgrounds. These investigations should also include so-called 'omics' (transcriptomics, proteomics, metabolomics).

Further, experts from member states pointed to the fact that an analysis for many important maize constituents was missing, e.g. lutein, zeaxanthin, phytosterols, tocopherols or tocotrienols (EFSA, 2020b).

In summary, much more data would be needed to conclude on the comparative analysis and develop a sufficiently defined hypothesis on risk assessment in regard to the phenotypical characteristics and the compositional analysis of the maize.

4. Toxicology

Implementing Regulation 503/2013 requests:

> *"Toxicological assessment shall be performed in order to:*
> *(a) demonstrate that the intended effect(s) of the genetic modification has no adverse effects on human and animal health;*
> *(b) demonstrate that unintended effect(s) of the genetic modification(s) identified or assumed to have occurred based on the preceding comparative molecular, compositional or phenotypic analyses, have no adverse effects on human and animal health;"*

> *"In accordance with the requirements of Articles 4 and 16 of Regulation (EC) No 1829/2003, the applicant shall ensure that the final risk characterisation clearly demonstrates that:*
> *(a) the genetically modified food and feed has no adverse effects on human and animal health;"*

In this regard, the mixed toxicity assessment is most relevant: it is known that maize produces protease inhibitors, which can delay the degradation of the Bt toxins and enhance their toxicity in a synergistic way (MacIntosh et al., 1990). This may also be the case if the maize is mixed into a diet along with other plants such as soybeans, which produce an even higher amount and wider range of protease inhibitors (Pardo-López et al., 2009).

In addition, diets will typically contain residues from spraying with the complementary herbicide, which may also act in a synergistic way and enhance toxicity of the Bt proteins (since specific experimental data are missing, for general overview see Then, 2010).

It is evident that Bt toxins can survive digestion to a much higher degree than has been assumed by EFSA: Chowdhury et al. (2003) as well as Walsh et al. (2011) have found that Cry1A proteins can frequently and successfully still be found in the colon of pigs at the end of digestion when they were fed with Bt maize. This generally shows that Bt toxins are not degraded quickly in the gut and can persist in larger amounts until digestion is completed; there is therefore enough time for interaction between various food compounds.

Further, due to the various modes of action already described in literature (Hilbeck & Otto, 2015; Vachon et al., 2012) in particular in combination with additive or synergistic effects that cause enhanced toxicity, Bt toxins can be much lower in their selectivity than assumed (Then, 2010). Therefore, a much broader range of organisms might be affected. This observation may also be relevant for food and feed if exposed to the mixed toxicity of maize MZIR098.

In general, it is known that not all modes of action of the insecticidal proteins produced in the plants depend on the specific mechanisms occurring only in the target insect species. Only very few Bt toxins (especially Cry1Ab, for overview see, Then, 2010) were investigated in more detail in regard to their exact mode of action, and there is no data on the Bt toxins produced in the maize. On the other hand, several publications exist showing the effects of Bt toxins in mammals: some Cry toxins are known to bind to epithelial cells in the intestine of mice (Vázquez-Padrón et al., 1999, Vásquez-Padrón et al., 2000). As far as potential effects on health are concerned, Thomas and Ellar (1983), Shimada et al. (2003) Huffmann et al. (2004), Ito et al. (2004), Mesnage et al. (2013) and Bondzio et al. (2013) show that Cry proteins could potentially have an impact on the health of mammals. Two recent publications (de Souza Freire et al., 2014; Mezzomo et al., 2014) confirm hematoxicity 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.

Therefore, it should be acknowledged that, in regard to toxicology or potential combinatorial effects, the negative impacts of Bt toxins on human and animal health cannot be excluded a priori. Bt toxins have several modes of action and are altered in their biological quality; therefore, they are not identical to their natural templates (Hilbeck & Otto, 2015). It should not be overlooked that the mode of action of mCry3A as well as eCry3.1Ab was changed to become more effective in pest

insects, therefore data from the naturally occurring Bt toxins are not sufficient. However, as shown in the outcome of the literature review conducted by the applicant (see above), there is a general lack of peer reviewed data on toxicology in regard to the newly synthesized Bt toxins produced by MZIR098.

In this context, there are very general gaps in risk assessment: if new toxins (insecticides) are introduced into the food chain, pesticide regulation requests a defined range of data to assess toxicity, long-term persistence and effects on complex endpoints, such as the immune system and the reproductive system. However, it appears that no such data were made available in the case of the newly synthesized Bt toxins. These toxins were never assessed in accordance with EU pesticide regulation. Instead, in this case, the experts on the GMO Panel have taken it upon themselves to act as pesticide experts. This strongly goes against the GMO and pesticide regulation currently established in the EU.

Some of the few data provided by the applicant seem to indicate that the toxins produced in the plants are comparable to other variants of Cry3 toxins. However, as shown by the comments of the experts from member states (EFSA 2020b), these findings can be disputed since specific data on toxicity are missing and the testing methods are deficient.

Further, according to member states experts (EFSA 2020b), the analysis of the available bioassay data indicates that synergistic effects between eCry3.1Ab and mCry3A cannot be excluded: *"as the given LC50 value for a combination of eCry3.1Ab and mCry3A in a ratio 1.89:1 was estimated to be 0,61 µg/g diet and is substantially lower than estimates for each of the single toxins (e.g. 3.96 µg mCry3A /ml diet in TK0025294 and 9,5 µg eCry3.1Ab/g diet in TK0057497)."* However, no specific experimental data on mixed toxicity were provided. No experimental data were provided on the synergistic and additive effects as described above, which can cause higher toxicity and lower selectivity.

In consequence, specific experimental data are indispensable for any conclusion to be drawn on risks to, e.g. the immune system, inner organs and the intestinal microbiome.

To some extent these questions could be answered by conducting animal feeding studies. They would allow the examination of the whole plant material, including, for example, protease inhibitors and residues from spraying. Therefore, we disagree with EFSA that subchronic feeding studies would not be needed. However, we do agree with comments requesting more data that would allow more specific hypotheses, e.g.: (i) substantial delay in the degradation of the Bt toxins in the plant material which might enhance adjuvant effects; (ii) lowered selectivity of the Bt toxins if combined with residues from spraying with glufosinate; (iii) significant changes in the intestinal microbiome due to exposure to the plant material. Many significant findings were reported from the 90-day feeding study, but without additional data it is difficult to interpret them correctly.

Overall, the data made available do not allow sufficiently reliable conclusions to be drawn on the safety of food and feed products derived from the maize.

In this regard, it also has to be considered that the concentration of the insecticidal proteins will be enriched in processed products such as gluten meal; the concentrations can also reach much higher concentrations compared to the kernels.

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. We conclude that the health risk assessment performed by EFSA is not sufficient to fulfill this requirement.

5. Allergenicity

Implementing Regulation 503/2013 requests:

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

There are several studies indicating that immune responses, such as adjuvanticity in mammals, can be triggered by Bt toxins and need to be considered: in this context, it is also a matter of concern that Bt toxins can cause non-allergic immune responses, such as adjuvant effects (Finnamore et al., 2008; González-González et al., 2015; Ibarra-Moreno et al., 2014; Jarillo-Luna et al., 2008; Guerrero et al., 2004; Guerrero et al., 2007; Legorreta-Herrera et al., 2010; Moreno-Fierros et al., 2000; Moreno-Fierros et al., 2013; Rubio-Infante et al., 2018; Rubio-Infante et al., 2016; Vázquez-Padrón et al., 1999) which might contribute to chronic disease or enhance immune responses. It is widely acknowledged that more data are needed on adjuvant and other potential immune effects caused by Bt proteins (see, for example, Rubio-Infante, 2016; Santos-Vigil et al., 2018).

The synergistic effects described by MacIntosh et al. (1990) and other authors such as Pardo-López et al., 2009, causing higher toxicity of the Bt toxins are also relevant for risk assessment in regard to the immune system: 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 will lead to the intestinal immune system being exposed to Bt toxins for an extended period of time and might therefore trigger or enhance chronic inflammation, including allergies.

In this regard, it has to be further considered that the concentration of the insecticidal proteins will be enriched in processed products such as gluten meal and germ, and that they can reach much higher concentrations compared to the kernels.

In its risk assessment, EFSA did not consider that under real conditions and, contrary to what is suggested by the findings of in-vitro studies, Bt toxins will not be degraded quickly in the gut but are likely to occur in substantial concentrations in the large intestine and faeces (Chowdhury et al., 2003; Walsh et al., 2011). In addition, if mixed into a diet with soybeans, the immune system responses caused by the allergens in the soybeans might be enhanced by the adjuvant effects of the Bt toxins.

In general, it has to be taken into account that so far only very few Bt toxins produced in genetically engineered plants have been investigated in regard to their potential impact on the immune system. As yet, only two Bt toxins (Cry1Ac and Cry1Ab) have been tested in more detail for their possible effects on the immune system. This is especially relevant for mCry3A which has so far not been

subjected to more detailed analysis regarding potential immunological effects. The same is true for eCry3.1Ab.

However, EFSA did not request the applicant to provide experimental data on the allergenic or immunogenic potential of mCry3A and eCry3.1Ab.

Given the fact that potential effects of Bt toxins on the immune system have been discussed for many years (for overview see, for example, Then & Bauer-Panskus, 2017), and already around 40 GE crop events producing Bt toxins have been approved for the EU market, any further delay in resolving these crucial questions 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 maize MZIR098, the risk assessment is inconclusive and market authorisation cannot be granted.

6. Environmental risk assessment

The appearance of teosinte in Spain and France (see Testbiotech, 2016; Trtikova et al., 2017) should have been considered in more depth. As Pascher et al, (2016) show, the volunteer potential of maize is higher than currently assumed. The hypothesis that hybrid offspring from maize MZIR098 and teosinte will show a higher fitness compared to conventional maize is plausible; this is because the Bt toxins may be present in the offspring and teosinte shows higher survival rates compared 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 and the authorisation have to be regarded as not valid.

Further, it is surprising that EFSA did not assess recent findings of Diaz et al (2019) highlighting uncertainties regarding the origin and genetic makeup of teosinte in Spain.

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. Without experimental data on next generation effects (Bauer-Panskus et al., 2020), no conclusions can be drawn on environmental risks of spillage of viable kernels. Consequently, environmental risk assessment carried out by EFSA is not acceptable.

Furthermore, the delay in degradation of the Bt toxins due to protease inhibitors produced in maize, raises questions on environmental exposure via manure or sewage.

7. Others

<u>Monitoring:</u>

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 were 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.

Methods for tracking and tracing the specific maize in comparison to other GE maize with similar gene constructs have to be made available.

8. Conclusions and recommendations

The EFSA risk assessment cannot be accepted.

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