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Testbiotech comment on 'Scientific Opinion on application EFSA-GMO-BE-2013-118 for authorisation of genetically modified maize MON 87427 x MON 89034 x 1507 x MON 88017 x 59122 and subcombinations independently of their origin, for food and feed uses, import and processing submitted under Regulation (EC) No 1829/2003 by Monsanto Company'

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Introduction

The GMO Panel assessed the five-event stacked maize MON 87427 x MON 89034 x 1507 x MON 88017 x 59122 which is derived from crossing five genetically engineered maize events. (EFSA, 2017). The maize contains genes for glyphosate and glufosinate resistance and produces several proteins which confer resistance to specific lepidopteran pests.

- MON 87427 expressing CP4 EPSPS protein for tolerance to glyphosate-containing herbicides;
- MON 89034 expressing Cry1A.105 and Cry2Ab2 insecticidal proteins;
- 1507 expressing the Cry1F insecticidal protein and phosphinothricin acetyl transferase (PAT) protein for tolerance to glufosinate-containing herbicides;
- MON 88017 expressing the Cry3Bb1 and CP4 EPSPS protein for tolerance to glyphosate-containing herbicides; and
- 59122 expressing the Cry34Ab1 and Cry35Ab1 insecticidal proteins and the PAT protein for tolerance to glufosinate-containing herbicides.

Consequently, the stacked maize produces six insecticidal toxins (Cry1A.105, Cry2Ab2 and Cry1F that target *lepidoptera* insects and Cry3Bb1, Cry34Ab1 and Cry35Ab1 that target *coleoptera)*. Further, the resistance to each of the complementary herbicides is based on a pair of enzymes. The pairwise enzymes are likely to confer high tolerance to the spraying of these weed killers onto the maize.

No experimental data were provided for 14 maize subcombinations.

1. Molecular characterisation

Testbiotech had earlier observed that the process of genetic engineering involved several deletions and insertions in the 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 simply assumed that the proteins that might emerge from these DNA sequences would raise no safety issues; no detailed investigations were carried out in this regard.

Furthermore, other gene products, such as miRNA from additional open reading frames, were not

assessed. Thus, uncertainties remain about other biologically active substances arising from the method of genetic engineering and the newly introduced gene constructs.

There are additional reasons for more detailed assessment of the stacked: Ben Ali et al. (2014) and Castan et al. (2014) show that mutations can be found in stacked events that do not occur in the parental plants. Therefore, EFSA should have requested more detailed sequence information from the applicant.

Environmental stress can also cause unexpected patterns of expression in the newly introduced DNA (see, for example, Trtikova et al., 2015). However, the expression of the additional enzymes was only measured under field conditions in the US for one year. It is unclear, to which extent specific environmental conditions will influence the overall concentration of the enzymes in the plants. The plants should have been subjected to a much broader range of defined environmental conditions and stressors to gather reliable data on gene expression and functional genetic stability.

The levels of Cry1A.105 in maize MON 87427 x MON 89034 x 1507 x MON 88017 x 59122 are higher for grain and pollen than in the parental line MON 89034. The same observations were made regarding SmartStax maize MON 89034 x 1507 x MON 88017 x 59122 (see Stillwell and Silvanovich 2007; Testbiotech, 2011). It is surprising that these effects were not investigated further.

Much more surprising, EFSA and the applicant omitted to assess the stacked event in regard to its intended purpose. The reason for crossing MON 87427 with MON 88017 (or the previously authorized SmartStax maize MON 89034 x 1507 x MON 88017 x 59122) was to increase the content of EPSPS enzymes that confer resistance to glyphosate. In consequence, it has to be expected that these plants can and will be exposed to higher and also repeated dosages of glyphosate. Higher applications of glyphosate will not only lead to a higher burden of residues in the harvest, but may also influence the expression of the transgenes or other genome activities in the plants. The same aspect is relevant in regard to the resistance to glufosinate which is also based on two enzyms. This aspect, which is the most relevant in regard to this specific stacked event, was completely ignored in the risk assessment as performed.

EFSA should have requested that Monsanto submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, also including repeated spraying. The material derived from those plants should have been assessed by using Omics techniques to investigate changes in the gene activity of the transgene, as well as the natural genome of the plants.

2. Comparative analysis (for compositional analysis and agronomic traits and GM phenotype)
Field trials for compositional and agronomic assessment of maize MON 87427 x MON 89034 x 1507 x MON 88017 x 59122 were conducted in the US only during one year (2010) and not in other relevant maize production areas, such Brazil or Argentina.

Regarding agronomic parameters, the following statistically significant differences were found:

- between the five-event stack maize <u>not treated with the intended herbicides</u> and the non-GM comparator differences were identified for: early stand count, ear height, plant height, stalk lodged plants, grain moisture and test weight;
- between the five-event stack maize <u>treated with the intended herbicides</u> and the non-GM comparator differences were in: days to 50% pollen shed, days to 50% silking, ear height, plant height, stalk lodged plants, grain moisture and test weight.

All these endpoints fell under equivalence category I.

Compositional data revealed many statistically significant differences:

- Statistically significant differences between the five-event stack maize (not treated) and the non-GM comparator were identified for 47 endpoints. All the endpoints fell under equivalence category I or II.
- Statistically significant differences between the five-event stack maize (treated) and the non-GM comparator were identified for 50 endpoints.

Here, endpoints fell under equivalence category I except for thiamine levels which fell under category III.

Several experts from EU Member States (EFSA, 2017b) took note of the very high number of significant differences (~ 75% of the analytes evaluated) and came to the conclusion that it is highly likely that the genetic modification resulted in unintended effects. However, requests for further tests (toxicological data, sprayings under practical conditions, exposure to a wider range of environmental conditions) were not followed by EFSA.

It has to be assumed that this event is essentially different from its comparator in regard to many compositions and biological characteristics. Even if changes taken as isolated data might not directly raise safety concerns, the overall number of effects and their strong significance has to be taken as a starting point for much more detailed investigations. It is not acceptable that EFSA failed to require further studies e.g.

- No data from Omics (proteomics, transcriptomics, metabolomics) were used to assist the compositional analysis and the assessment of the phenotypical changes.
- More powerful statistical analysis, such as multidimensional analysis, was not applied to the data.
- No field trials were conducted that lasted more than one season. Thus, based on current data, site-specific effects can hardly be assessed.
- Further, no data were generated representing more extreme environmental conditions, such as those caused by climate change. Although no application has been filed for cultivation, data on the interaction between the plants and the environment have to be considered as one of the starting points in risk assessment of the plant, and must be made available and assessed in detail. However, EFSA (2017a) stated that: “Considering the scope of application EFSA-GMO-BE-2013-118, interactions with the biotic and abiotic environment are not considered to be relevant issues.”
- In addition, more varieties carrying the transgenes should have been included in the field trials to see how the gene constructs interact with the genetic background of the plants.

As mentioned, EFSA and the applicant omitted to assess the stacked event in regard to its intended purpose. The reasoning for crossing MON 87427 with MON 89034 x 1507 x MON 88017 x 59122 was to increase the content of EPSPS enzymes that confer resistance to glyphosate. In consequence, it has to be expected that these plants can and will be exposed to higher and also repeated dosages of glyphosate. The specific pattern of applications of the complementary herbicides will not only lead to a higher burden of residues in the harvest, but may also influence the composition of the plants and agronomic characteristics. This aspect was ignored by the risk assessment as performed.

EFSA should have requested that Monsanto submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, also including repeated spraying. The material derived from those plants should have been assessed by using Omics techniques to

investigate changes in the plants composition or agronomic characteristics

Based on the available data, no final conclusions can be drawn on the safety of the plants.

Toxicology
No toxicological tests were conducted with maize MON 87427 x MON 89034 x 1507 x MON 88017 x 59122. This is unacceptable for several seasons:

1. The stacked maize differs from the parental lines with regard to the overall amount of toxin produced which is greater than in the parental lines.

2. Despite many significant changes in the composition of the plants and agronomic characteristics, no testing of the whole plant (feeding study) was requested. It has to be assumed that this event is essentially different from its comparator in regard to many compositions and biological characteristics. Even if changes taken as isolated data might not directly raise safety concerns, the overall number of effects and their strong significance has to be taken as a starting point for much more detailed investigation of their potential health impacts.

3. Beyond that, the residues from spraying were considered to be outside the remit of the GMO panel. However, without detailed assessment of these residues, no conclusion can be drawn on the safety of the imported products: Due to the specific agricultural practices that go along with the cultivation of these herbicide resistant plants, there are, for example, specific patterns of applications, exposure, occurrence of specific metabolites and emergence of combinatorial effects that require special attention.

The assessment of herbicide and metabolite data was also requested by Member State experts (EFSA, 2017b): *"The amount of residues of the herbicide treatment should be assessed including amounts of herbicide metabolites present in the produced material. For this analysis the notifier should take into consideration that CP4 EPSPS transgenes are expressed at a higher level in GM maize MON87427 x MON89034 x 1507 x MON88017 x 59122 which might affect the maximum level of glyphosate herbicides that could be used in the crop."*

Also the publication by Kleter et al. (2011) shows, using herbicides to spray genetically engineered herbicide-resistant plants does indeed lead to patterns of residues and exposure that need to be assessed in detail.

More detailed assessment is also in accordance with pesticide regulation, which requires specific risk assessment of imported plants if the usage of pesticides is different in the exporting countries compared to the one in the EU. In this regard, it should be taken into account that EFSA (2015a) explicitly stated that no conclusion can be derived on the safety of residues from spraying with glyphosate occurring in genetically engineered plants resistant to this herbicide.

Further, there is a common understanding that commercially traded formulations of glyphosate, such as Roundup, can be more toxic than glyphosate itself. Therefore, the EU has already taken measures to remove problematic additives known as POE tallowmine from the market. Problematic additives are still allowed in those countries where the genetically engineered plants are cultivated. The EU Commission has confirmed the respective gaps in risk assessment:

> *"A significant amount of food and feed is imported into the EU from third countries. This includes food and feed produced from glyphosate-tolerant crops. Uses of glyphosate-based plant protection products in third countries are evaluated by the competent authorities in*

those countries against the locally prevailing regulatory framework, but not against the criteria of Regulation (EC) No. 1107/2009. (…).” (www.testbiotech.org/node/1637)

The European Food Safety Authority (EFSA) agrees that further investigations and data are needed (EFSA, 2015b).

In any 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. In addition, cumulative effects have to be investigated if a plant contains or produces other compounds with potential toxicity.

The reason for crossing MON 87427 with MON89034 x 1507 x MON88017 x 59122 was to increase the content of EPSPS enzymes that confer resistance to glyphosate. In consequence, it has to be expected that these plants can and will be exposed to higher and also repeated dosages of glyphosate. These applications of glyphosate will not only lead to a higher burden of residues in the harvest, but may also influence the composition of the plants and agronomic characteristics. EFSA should have requested that Monsanto submit data from field trials with the highest dosage of glyphosate that can be tolerated by the plants, also including repeated spraying. The material derived from those plants should have been assessed in regard to organ toxicity, immune reactions and reproductive toxicity, also taking combinatorial effects with other plants components and the Bt toxins into account.

In the context of risk assessment of this stacked event, the residues from spraying with the complementary residues must also be considered to be a potent co-stressor. The impact on cells and organisms exposed to several stressors in parallel can be of great importance for the efficacy of Bt toxins. As, for example, Kramarz et al. (2007 and 2009) show, parallel exposure to chemical toxins can lead to Bt toxins having an effect on organisms that are not normally susceptible. In addition, Bøhn et al. (2016) show additive effects of several Cry toxins. Cry toxins interact with Roundup / glyphosate when co-exposed to *Daphnia magna*. These cumulative effects also have to be assessed in regard to food and feed usages (see also Bøhn, 2018).

In regard to immunogenicity (non-IgE-mediated immune adverse reactions), it is generally acknowledged that Bt toxins are immunogenic (Rubio-Infante & Moreno-Fierros, 2016; Adel-Patient et.al., 2011; Andreassen et.al., 2015a,b; Andreassen et.al., 2016; see also Then & Bauer-Panskus, 2017). These observed effects are likely to be dose-dependent. Stacked events have a much higher concentration of Bt toxins than other plants, such as the single plants which were tested in feeding studies. Further, the concentration of Bt toxins in the plants varies substantially (see Testbiotech 2011).

Moreover, it is evident that Bt toxins can survive digestion to a much higher degree than has been assumed by EFSA: Chowdhury et al., (2003) as well as Walsh et al. (2011) have found that Cry1A proteins can frequently and successfully still be found in the colon of pigs at the end of digestion when they were fed with Bt maize. Thus, the Cry1A proteins can show much higher stability, at least in monogastric species, than predicted by current in vitro digestion experiments. Thus, Bt toxins are not degraded quickly in the gut and can persist in larger amounts until digestion is completed and there is enough time for interaction between various food compounds. Consequently, there is substantiated concern that especially the stacked event can trigger immune reactions and show adverse health effects.

There are further relevant issues: For example, the potential impact on the intestinal microbiome also has to be considered. Such effects might be caused by the residues from spraying since glyphosate has been shown to have negative effects on the composition of the intestinal flora of cattle (Reuter et al., 2007) and poultry (Shehata et al., 2013). Further, Bremmer and Leist (1997) examined the possible conversion of NAG to glufosinate in rats. Up to 10% deacetylation occurred at a low dose of 3 mg/kg bw as shown by the occurrence of glufosinate in the faeces. The authors concluded, however, that most of the conversion was caused by bacteria in the colon and rectum, although toxicity findings indicate partial bioavailability (Bremmer & Leist, 1997, see also EFSA 2017b). In general, antibiotic effects and other adverse health effects might occur from exposure to a diet containing these plants that were not assessed under pesticide regulation. These adverse effects on health might be triggered by the residues from spraying with the complementary herbicide (see also van Bruggen et al., 2017). Further attention should be paid to the specific toxicity of the metabolites of the pesticide active ingredients that might occur specifically in the stacked event. For example, glufosinate is classified in the EU as showing reproductive toxicity.[1] But there were no detailed investigations into the metabolites arising from spraying glufosinate onto these plants; these metabolites might also differ from those of the parental plants.

In any 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. In addition, cumulative effects have to be investigated if a plant contains or produces other compounds of potential toxicity.

In addition, cumulative effects have to be investigated if a plant contains or produces other compounds of potential toxicity. It should be acknowledged, that no new methodology is needed to assess the health risks emerging from the combinatorial application of the herbicides and their potential interaction with the other plant constituents. Suitable methodology to assess combinatorial effects that emerge from *simultaneous exposure* to a *fixed combination* of potential stressors via a *defined route of exposure* (as it is the case with food and feed products derived from genetically engineered plants that are made resistant to several herbicides) is available and widely used. For example, chronic feeding or multigenerational studies are a well-established method to generate the relevant data.

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

Allergenicity

No data were presented to show that plant composition is unchanged in regard to allergenic potential.

There might be various reasons why the allergenic potential in the stacked event is increased: Higher applications of glyphosate will not only cause a higher burden of residues in the harvest, but may also change the composition of the plants in regard to naturally occurring allergens. Higher concentration of Bt toxins might trigger adjuvant effects in regard to other components in the diet. No data were presented to assess such potential effects.

Consequently, the assessment in regard to allergenicity cannot be regarded as conclusive.

[1] http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN

Others
For 14 subcombinations of the five-event stacked maize, no experimental data were provided at all. There is, therefore, a high level of uncertainty in regard to all levels of risk assessment as mentioned above.

Environmental risk assessment
Any spillage from the kernels has to be monitored closely. EFSA completely overlooked that populations of teosinte are abundant in Spain and France; these have to be considered to be wild relatives that enable gene flow and potential spread of the transgenes throughout the fields and the environment (Trtikova et al., 2017).

In this regard, the opinion of EFSA (2017a) is extensively flawed since the authority refers to completely outdated literature on the occurrence of wild relatives in Europe: "*Populations of sexually compatible indigenous wild relatives of maize are not known in Europe (Eastham and Sweet, 2002; OECD, 2003), therefore vertical gene transfer is not considered to be an environmental issue in the EU.*" However, since 2009, teosinte, a wild relative of maize, is known to occur in Spain. There are further reports from France about its occurrence that might encompass further regions in the EU (Trtikova et al., 2017).

Thus, 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.

Further, as shown by Pascher (2016), EFSA is also underestimating the risks posed by occurrence of volunteers from maize plants.

Consequently, environmental risk assessment carried out by EFSA is not acceptable.

Conclusions and recommendations
The EFSA risk assessment should not be accepted. EFSA did not request any empirical data regarding toxicity and impact on the immune system, and did not name the knowledge gaps or uncertainties. Combinatorial effects were ignored as well as the consequences of spraying higher dosages of the complementary herbicides . The environmental risk assessment is based on wrong assumptions. The monitoring plan has to be rejected because no evaluated method was made available that would allow case specific identification. Further, no system is foreseen to perform case specific monitoring of spillage and potential health effects.

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