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Testbiotech comment on EFSA´s opinion on the assessment of genetically modified maize 1507 x 59122 x MON810 x NK603 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2011-92) by company DowDuPont.

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Introduction

The GMO Panel (EFSA, 2017) assessed Maize 1507 x 59122 x MON810 x NK603, which is derived from crossing four genetically engineered maize events:

- 1507 produces the insecticidal protein Cr1F and is resistant to the herbicide glufosinate
- 59122 produces the insecticidal protein Cry34Ab1 and Cry35Ab1 and is resistant to the herbicide glufosinate
- MON810 produces the insecticidal protein Cr1Ab
- NK603 produces two enzymes rendering resistance to the herbicide glyphosate

Consequently, the stacked maize produces four insecticidal toxins (Cry1F and Cry1Ab that target *lepidoptera* insects and 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 the weed killers onto the maize.

EFSA also declared all subcombinations of the plants to be safe without asking for additional experimental data.

1. Molecular characterisation

The process of genetic engineering 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 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.

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.

Furthermore, environmental stress can 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. In comparison with data from the green house, the expression of the newly introduced enzymes shows a high range of variability. It is unclear, to which extent specific or more extreme environmental conditions (also in other maize producing countries besides the US) will influence the overall concentration of the enzymes in the plants. Thus, 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. This is also relevant in regard to assessing potential immune effects triggered by the Cry proteins (see below).

Furthermore, EFSA and the applicant omitted to assess the stacked event in regard to its higher tolerance to spraying with the complementary herbicides. Due to the pairwise production of the relevant enzymes, which also leads to higher expression rates, it can be expected that the plants can and will be exposed to higher and also repeated dosages of these herbicides. These applications of the complementary herbicides 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. This aspect was completely overlooked in the risk assessment as performed.

EFSA should have requested that the applicant submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, also including repeated spraying.

In general, much more detailed investigations should have been performed to investigate unintended gene products and changes in the gene activity, also including using 'Omics' techniques and taking into account specific patterns of herbicide applications.

2. Comparative analysis (for compositional analysis and agronomic traits and GM phenotype)
Field trials for compositional and agronomic assessment of Maize 1507 x 59122 x MON810 x NK603 were conducted in the US only during one year and not at all in other relevant maize growing areas, such Brazil or Argentina.

Many statistically significant differences were found in regard to agronomic parameters and in compositional analysis. Even if changes taken as isolated data might not directly raise safety concerns, the overall number of effects 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 plants, and must be made available and assessed in detail.
- 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 real tolerance to the complementary herbicides. Higher application rates of the herbicides will not only lead to a higher burden of residues in the harvest, but may also influence the composition of the plants and their agronomic characteristics.

EFSA should have requested that the company submits data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, also including repeated spraying.

Further, EFSA, in accordance with its own guidelines from 2006 and 2007 that were applied in this case, should have requested the parental plants to be grown in direct comparison with the stacked events.

In general, much more detailed investigations should have been performed to investigate unintended changes in the plants composition and phenotypical characteristics, also taking into account specific patterns of herbicide applications.

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 1507 x 59122 x MON810 x NK603. 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. There were several significant changes in the composition of the plants and agronomic characteristics. Even if changes taken as isolated data might not directly raise safety concerns, the overall number of effects has to be taken as a starting point for much more detailed investigation, also in regard to 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 the application of the complementary herbicides, 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 herbicide sprayings and subsequent exposure to specific metabolites and the emergence of combinatorial effects that require special attention.

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.

Furthermore, higher 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. Therefore, EFSA should have requested that the company submits data from field trials with the highest dosage of the herbicides 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 this maize plants is shown to vary substantially.

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.

To our knowledge, EFSA (2017) for the first time admitted relevant uncertainties in regard to the immunogenic effects of the Cry proteins: EFSA not only admitted that there was “limited experimental evidence available” but, in addition, started a call for a comprehensive literature review on adjuvanticity and immunogenicity of proteins. This is appreciated. However, in the light of the existing uncertainties, experimental data on potential adverse health effects have to be requested, before a final conclusion on the safety of the plants can be made.

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). 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. But 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. 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. 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 the complementary weed killers 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.

EFSA admits relevant uncertainties in regard to the immunogenic effects of the Cry proteins: EFSA not only admits *"limited experimental evidence available"* but in addition started a a call for a comprehensive literature review on adjuvanticity and immunogenicity of proteins. But in the light of current uncertainties, experimental data on the allergenic potential should have been requested.

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

Others
No experimental data were provided at all for several subcombinations of the stacked maize. There is, therefore, a high level of uncertainty in regard to all levels of risk assessment as mentioned.

Environmental risk assessment
Any spillage from the kernels has to be monitored closely. EFSA is very well aware 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).

However, EFSA (2017) is mostly ignoring the impact of this potential gene flow. Without any detailed consideration about potential hazards and exposure, EFSA states:

> *"Wild relatives of maize outside cultivation are not known/reported in Europe (…). Therefore, potential vertical gene transfer is restricted to maize and weedy Zea species, such as teosintes, and/or maize-teosinte hybrids, occurring in cultivated areas (…)."*

Since in the EU, teosinte is considered to be a weed that already shows invasive characteristics within the fields, it has to be assumed that traits such as herbicide resistance and the production of insecticidal toxins can substantially enhance their weedy characteristics. Further, tesosinte is known to overwinter and persist in the fields to a much higher degree than maize. This can cause self-sustaining transgenic populations to persist in the maize growing areas. In addition, via teosinte, the transgenes can also be passed to other fields cultivated with conventional maize, where they could persist and spread further.

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. Combinatorial effects were ignored as were the consequences of spraying higher dosages of the complementary herbicides. The environmental risk assessment is not acceptable and 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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