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Testbiotech comment on EFSA´s opinion on the assessment of genetically modified maize Bt11 x MIR162 x 1507 x GA21 and three subcombinations independently of their origin, for food and feed uses under Regulation (EC) No 1829/2003 (application EFSA-GMO-DE-2010-86) by company Syngenta.

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Introduction

The GMO Panel (EFSA, 2018) assessed Maize Bt11 x MIR162 x 1507 x GA21, which is derived from crossing four genetically engineered maize events. The maize produces six additional proteins:

- Bt 11 produces the insecticidal protein Cry1Ab and is resistant to glufosinate,
- MIR162 produces the insecticidal protein Vip3Aa20 and the phosphomannose isomerase (PMI) protein (used as a selectable marker),
- 1507 produces the insecticidal protein Cr1F and is resistant to the herbicide glufosinate,
- GA 21 is resistant to glyphosate.

Consequently, the stacked maize produces three insecticidal toxins (Cry1F, Vip3Aa20 and Cry1Ab) that target *lepidoptera* insects. Further, it has been engineered to be resistant to applications of glyphosate and glufosinate, with the resistance to glufosinate 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 (2018a) also declared three subcombinations of the plants to be safe even though they had not been assessed and without requesting experimental data: Bt11 x MIR162 x1507, MIR162 x 1507 x GA21 and MIR162 x 1507. In a minority opinion, a member of the EFSA GMO panel voiced reservations about the assessment of the subcombinations without any specific data. Amongst others, the minority opinion mentions risks for the immune system which should be thoroughly assessed.

1. Molecular characterisation

Updated molecular analysis showed that the DNA sequence present in the stacked maize contained one nucleotide change in the insert sequence compared to the corrected original 1507 maize sequence. From EFSA's point of view this did not raise safety concerns. Testbiotech, however, is of the opinion that a further more detailed assessment should have been performed to assess the overall stability of the DNA construct. As Ben Ali et al. (2014) and Castan et al. (2014) show, mutations can be found in stacked events that do not occur in the parental plants. Therefore, EFSA should have discussed this issue in more detail.

The process of genetic engineering involved several deletions and insertions in the parental maize plants. Several open reading frames were identified in the parental plants. EFSA did not assess these open reading frames in their entirety. No detailed investigations were carried out in regard to gene products apart from newly produced proteins; and unintended proteins were only partially considered in the assessment of the parental plants.

Gene products, such as miRNA from additional open reading frames, were not assessed. Thus, substantial uncertainties remain about biologically active substances arising from the method of genetic engineering and the newly introduced gene constructs. In response to comments from experts from Member States raised in this context (EFSA 2018b), EFSA stated that the findings of Zhang et al (2012), which show cross kingdom activity of plant miRNA in mammals, would not yet have been confirmed. This is not correct, there are several studies with similar results (Yang et al., 2015; Liang et al., 2015; Hirschi et al, 2015; Beatty et al., 2014). In order to enable further risk assessment, the full DNA sequence inserted into the plants should be made available, including all open reading frames.

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 and at one single site. This is not in accordance with current guidelines or EU regulations. Nevertheless, EFSA accepted these deficiencies by stating that the old guidelines from 2006 and 2007 should be applied in this case. However, also in regard to these older guidelines, the necessary standards were not met. For example, EFSA should have requested that the parental plants were grown in direct comparison at the same site (EFSA, 2007). Furthermore, the complementary herbicide should have been sprayed onto the plants. It is also not acceptable that Syngenta did not provide any expression data on forage even though this was requested by EFSA.

Despite the small set of data, findings indicate that gene expression is changed in the plants due to the process of stacking: the Bt content (Cry1Ab) in the leaves was shown to be up to 4 times higher compared to the existing data from parental plants. Therefore, EFSA should have concluded that there were sufficient indications of interactions that could affect the integrity of the events and the levels of the newly expressed proteins in this stacked maize, which would require further investigation.

Instead, EFSA concluded the opposite, by stating that a higher expression of the Cry1Ab might not necessarily be considered to be a risk per se (EFSA 2018b). With this statement, EFSA is confusing two different steps within risk assessment: first there has to be a full investigation into whether and to which extent gene expression is influenced by the stacking. If there are indications that the integrity of the events and the levels of the newly expressed proteins are influenced by the process of stacking, these differences have to be assessed and investigated in detail regardless of whether these effects pose a risk per se.

Therefore, the differences in gene expression as observed need to be assessed in more detail: for example, it is unclear to which extent specific or more extreme environmental conditions (also in other maize producing countries besides the US) can 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 in order to gather reliable data on gene expression and functional genetic stability. This is also relevant in regard to assessing potential effects on the immune system triggered by the Cry proteins (see below).

Further, the method used to determine the amount of Bt toxins (ELISA) is known to be dependent on the specific protocols used. The data are not sufficiently reliable without further evaluation by independent labs. For example, Shu et al. (2018) highlight difficulties in measuring the correct concentration of Bt toxins produced by the genetically engineered plants (see also Székács et al., 2011). Without fully evaluated test methods to measure the expression and the concentration of the Bt toxins, risk assessment suffers from substantial methodological gaps. A similar problem emerges in regard to the Vip3Aa20 produced in the plants.

Furthermore, EFSA and the applicant omitted to assess the stacked maize after 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 glufosinate. The applications of the complementary herbicides will not only lead to a burden of residues in the harvest, but are likely to influence the expression of the transgenes or other genome activities in the plants. 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 genetic stability, changes in gene expression and unintended gene products; also including the use of 'omics' techniques and taking into account specific patterns of herbicide applications.

In awareness of the changes in the gene expression of the Cry1Ab protein, and in light of the deficiencies in the data provided by Syngenta, this should have prompted EFSA to request a full set of new data for the stacked event and its subcombinations. Instead, EFSA used the old guidelines as a general excuse not to perform risk assessment required by EU Regulations.

2. Comparative analysis (for compositional analysis and agronomic traits and GM phenotype)
Field trials for compositional and agronomic assessment of Maize Bt11 x MIR162 x 1507 x GA21 were conducted in the US for only one year. They were not carried out in any other maize growing region, such as Brazil or Argentina.

Contrary to the standards normally applied, the data on agronomic and phenotypic characteristics were derived from field trials without

- the parental plants being grown in comparison,
- the application of the complementary herbicide
- reference lines being grown in parallel.

Further, the small set of data (only 10 out of 23 criteria were assessed) is far below the usual standard. It is remarkable that four out of the ten parameters investigated were found to be different (‘% emerged plants’, ‘heat units to 50% silking’ and ‘heat units to 50% pollen shed’). There is no reason to assume that these effects are not caused by the process of stacking. Therefore, EFSA should have concluded that there are sufficient indications of interactions that affect the integrity of the events, as well as the agronomic and phenotypic characteristics of this stacked maize, which require further investigation.

But EFSA failed to require further studies and data such as:

- field trials that lasted more than one season;
- experiments under controlled conditions representing more extreme environmental conditions, such as those caused by climate change;

- growing the parental plants and other reference varieties in parallel;
- growing the plants with and without the application of the complementary herbicides in parallel.

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.

In awareness of the inadequacies in the design of the field trials, as well as the low number of characteristics investigated and the relatively high number of significant findings, EFSA should have requested a full set of new data for the stacked maize and its subcombinations; supplemented by additional investigations listed above.

For the compositional analysis, a set of field trials was performed in six locations only in the USA and only for one season. The design of these field trials is not acceptable:

- While in this case the complementary herbicides were applied onto the plants, no data were generated without the application of the herbicides. Thus, comparison of plant composition cannot be made as requested under current guidelines.
- The reference lines were not grown at the same sites.
- No parental plants were grown for comparison.

24 out of 50 parameters were found to be significantly different. Data from ILSI and OECD were used to assess these differences; these data are known to be neither sufficiently reliable nor informative.

Despite the large amount of data that was used, the significant differences in regard to the lower level of carotenoids still fell outside the range. This finding was confirmed by further investigations. It is likely that this effect is caused by stacking. Therefore, EFSA should have concluded that there are sufficient indications of interactions that affect the integrity of the events and plant composition of the stacked maize, which require further investigation.

In awareness of the inadequate design of the field trials and the highly significant findings, EFSA should have been prompted to request a full set of new data for the stacked event and its subcombinations; supplemented by additional investigations (see below).

Instead, EFSA took a very reductionist approach, only considering the specific risk of changes in the carotene content. This approach is nothing like the meaningful risk assessment requested in EU regulation. If significant differences in plant composition are found that are likely to be caused by genetic engineering or the process of stacking, they cannot just be set aside because they do not seem to pose a risk per se.

If it is the case that findings indicate interactions affecting the integrity of the events and plant composition, phenotypic characteristics and / or gene expression possibly due to the process of genetic engineering or stacking, then more data must be requested, regardless of whether these specific data are foreseen in any guidelines or not.

Regulation 1829/2003 requires the highest scientific standards to be applied in assessing safety. Therefore, EFSA should have requested new data sets of much better quality not bound by details in older guidelines. It is generally acknowledged that EFSA guidelines are just a starting point for risk assessment that allow the screening of relevant data. Further steps in risk assessment are needed wherever relevant findings indicate unintended changes in the plants. Which data are requested in

detail will depend on the specific case, but in any case, these additional requests can go far beyond what is foreseen in the guidelines.

In this case, 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.
- No field trials were conducted that lasted more than one season.
- No data were generated representing more extreme environmental conditions, such as those caused by climate change.
- No field trials were requested where the parental plants and reference varieties were grown at the same sites.
- Plants were not grown with and without the application of the complementary herbicides in parallel.
- 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.

Furthermore, 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.

In any case, the EFSA opinion has to be rejected since it is in contravention of current standards and EU regulations.

Toxicology
No toxicological tests were conducted with maize Bt11 x MIR162 x 1507 x GA21. This is unacceptable since there clear indications that the process of stacking led to unintended changes in gene expression, plant composition and agronomic and phenotypic characteristics; all of which require further investigation.

In this case, the composition of the stacked maize is also different from that of the conventional plants and the parental plants, therefore, risk assessment cannot be based on previous assessment of the parental plants. Instead, this stacked plant should be considered to be a new event that has to undergo full risk assessment, including detailed toxicological investigations. This also applies to the subcombinations.

Further, combinatorial effects between the newly expressed proteins should have been tested, taking into account the residues from spraying. In the context of risk assessment of this stacked event, the residues from spraying with the complementary herbicides must be considered to be a potent co-stressors. 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).

There is no other regulation in the EU to deal with the specific combinatorial effects of Bt toxins, VIP toxins and residues from spraying with the complementary herbicide, so that this cannot be

considered to be outside the remit of the GMO panel. It also has to be integrated in the final decision making of the Commission.

A conclusion cannot be drawn on the safety of the plants as requested by EU Regulation 1829/2003 if these effects are not taken into account. Due to specific agricultural practices in the cultivation of these herbicide resistant plants, there are, for example, specific patterns of herbicide sprayings and subsequent exposure to specific metabolites, as well as the emergence of combinatorial effects that require special attention.

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 system reactions and reproductive toxicity, also taking combinatorial effects with other plants compounds and the Bt toxins into account.

It should also be acknowledged that the mode of action of VIP proteins is still unknown and simply based on preliminary assumptions derived from the outcome of just a few studies. Particular details that render selectivity and toxicity of the VIP proteins are not understood. No conclusion can be drawn upon their (long-term) effects on the food chain without this knowledge.

There are further relevant issues e.g. the potential impact on the intestinal microbiome also needs 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) poultry (Shehata et al., 2013) and rats (Mao et al., 2018). 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 active ingredients in the pesticide 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. Since both the EU pesticide regulation and GMO regulation require a high level of protection for health and the environment 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.

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 application rates 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 has admitted relevant uncertainties in regard to the immunogenic effects of the Cry proteins: EFSA stated that there is *"limited experimental evidence available"*. Therefore, in the light of current uncertainties, experimental data on the allergenic potential should have been requested.

It should not be ignored that the Bt toxins under real conditions will not be degraded quickly in the gut but are likely to occur in substantial concentrations in the large intestine and faeces. Since adjuvant effects are known from the single Bt toxins (see Rubio-Infante & Moreno-Fierros, 2015), it is not unlikely that a mixture of these toxins could lead to enhanced adjuvant effects.

Further, combined feeding with soybeans, which are known to contain a lot of allergens, should also have been assessed. Finally, the marker protein PMI, which shows a substantially higher concentration in the stacked event compared to the parental plants, has similarity to allergenic parvalbumin in frogs and should, therefore, also have been tested in combination with the Bt toxins (see also EFSA 2018b).

The need for more detailed investigations in regard to potential immunogenic effects is also underlined in the minority opinion (Annex II of EFSA, 2018a):

> *"However, compositional data and reliable information on the actual concentrations of the NEPs [newly expressed proteins] are crucial to achieve a sound safety assessment. Indeed, it has been shown that the genetic background of the recipient plant has a major effect on Cry1Ac expression in GM cotton (Adamczyk & Meredith 2004) and maize (Trtikova et al., 2015; Zeljenkova et al., 2014); it may cause an additional variability (not taken into account by the GMO Panel so far) in Bt protein concentrations which might impact on the safety. The risk of increased expression of the newly expressed Bt proteins and of a possible cumulative effect of their combination on the immune system (e.g. resulting in an adjuvant activity) cannot be ruled out although it is difficult to evaluate in the absence of actual experimental data. Indeed, the scope of AP 86 [this application] is for import and processing which suggests a limited exposure for consumers in the EU. Nevertheless, should those subcombinations (or some of them) be produced and commercialised in the future, the resulting risk for human health, particularly in workers, might be higher than that of singles or of the fully assessed Bt11 x MIR162 x 1507 x GA21 maize."*

The minority opinion also addresses this problem from a more general perspective:

> *"Indeed, unintended effects on the immune system have never been identified in any application where Bt proteins were expressed; but at the same time, it should also be noted that they could not be observed by the toxicological studies (i.e. 28-day repeated-dose tox studies and/or 90-day feeding trials) currently recommended and performed for the safety assessment of GM plants at EFSA because they do not include appropriate tests for this purpose."*

The opinion also addresses the immense importance of risk assessment being conducted thoroughly in this context:

> *"Allergic reactions in general and particularly food allergy are dramatically increasing in the EU (and worldwide) and have become a most important public health issue. The reasons are unclear, but most specialists involve the changes in environmental conditions, in cultivated plant species and in food habits. Indeed, environmental conditions are known to play a major role in the occurrence and/or severity of the allergic reaction in addition to the genetic background of predisposed individuals and characteristics of the allergen. They include the route and doses of exposure to the protein in question but also the presence in the food/diet of compounds known to modulate (e.g. increase) the immune response to other unrelated proteins present in the food. The potential role of these 'adjuvants' is therefore emphasised and especially in the case of immunoglobulin E (IgE)-mediated allergy.*
> *It is thus a pity that a high-double uncertainty due to both a lack of knowledge and a lack of data, still remains which clarification would improve the assessment, clarify the role/absence of role of GMOs in the increasing allergenic risk and finally allow a solid protection and prevention of at risk consumers."*

Consequently, the assessment in regard to allergenicity and immunotoxicity cannot be regarded as conclusive. Contrary to what is expressed in the minority opinion (EFSA 2018a), these problems not only concern the subcombinations, but also the overall risk assessment of maize Bt11 x MIR162 x 1507 x GA21.

Others

No validated method was made available to distinguish the single event from any of the stacked events named in the application. Thus, no targeted monitoring or general surveillance can be performed. Therefore, legal requirements for case specific identification and monitoring are not fulfilled and no market authorisation can be given.

According to Regulation (EU) No 503/2013, 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 stacked maize imported into the EU,
ii) the ports and silos where shipments of the stacked maize were unloaded,
iii) the processing plants where the stacked maize was transferred to,
iv) the amount of the stacked maize used on farms for feed, and
v) transport routes of the stacked maize.

The applicant is further requested to explain how the PMM of the stacked maize in mixed GMO commodities imported, processed or used for food/feed is put into practice. Since traders may co-mingle the stacked maize with other imported commercial GM maize that is processed or used for food/feed, the applicant is requested to explain how the monitoring will be designed to distinguish between potential adverse effects caused by the stacked maize and those caused by other GM maize.

The monitoring should be run in regions where viable kernels of the stacked maize are transported, stored, packaged, processed or used for food/feed. In case of losses and spread of the stacked maize, all receiving environments need to be monitored.

Furthermore, environmental exposure through organic waste material, by-products, sewage or faeces containing Bt11 x MIR162 x 1507 x GA21 maize during or after the production process, and during or after human or animal consumption should be part of the monitoring procedure (see also EFSA, 2018 b).

Environmental risk assessment

Any spillage of 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). EFSA acknowledges that potential gene transfer between maize and weedy Zea species, such as teosintes and/or maize-teosinte hybrids, can occur, but is of the opinion that such a scenario is unlikely (EFSA, 2018a).

Much more detailed investigation would be needed to assess the potential introgression of wild teosinte populations with gene constructs inserted in the stacked maize and their effects on fitness of any progenies. For example, in the light of Fang et al. (2018), it has to be assumed that the transgenic plants will render their offspring higher fitness compared to conventional plants. Therefore, EFSA (2018a) is wrong in its statement saying:

> *"Even if cross-pollination would occur, the GMO Panel is of the opinion that environmental effects as a consequence of the spread of genes from occasional feral GM maize plants in Europe will not differ from that of conventional maize varieties"*

It should be acknowledged that Zhang et al. (2018) also describe higher fitness in offspring from transgenic plants that are resistant to glufosinate.

Further, it has to be assumed that traits, such as herbicide resistance and the production of insecticidal toxins, can substantially enhance the weedy characteristics of teosinte.

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 also underestimates 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 opinion of EFSA has to be rejected because it is in contravention of current standards and EU regulations. It seems that EFSA in this case is intentionally acting in contradiction to existing standards and regulations.

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